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ԲԱՆԲԵՐ ՍՏՈՄԱՏՈԼՈԳԻԱՅԻ ԵՎ ԴԻՄԱԾՆՈՏԱՅԻՆ ՎԻՐԱԲՈՒԺՈՒԹՅԱՆ
Գիտագործնական հանդես

Գլխավոր խմբագիր - Հակոբյան Գ.Վ.
Գլխավոր խմբագրի տեղակալ-
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RESEARCH ARTICLE

CRESTAL BONE LOSS IN PATIENTS WITH DIFFERENT SURFACE MODIFIED DENTAL IMPLANTS: INSIGHTS FROM COMPREHENSIVE REGISTRY DATA-BASED COHORT STUDY

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ABSTRACT

Background: Several surface modifications have been performed on implant surfaces to improve osseointegration. The clinical results of these implants over a long term, however, have not yet been thoroughly assessed. Accordingly, the present registry data-based cohort study aims to examine the crestal bone loss (CBL) associated with sandblasted acid-etched and anodized dental implants.

Materials and Methods : Case records of a total of 162 patients who underwent implant surgery (311 implants) using three different i mplant systems implants with sandblasted acid-etched [Group 1: SLA ® (93 implants), Group 2: SLActive ® (99 implants)] and anodized topographies [Group 3: TiUnite ® (119 implants)], followed by conventional loading between January 2022-June 2023 were enrolled. Digital X-rays at baseline, 3 months and 1-year intervals were collected from case records and crestal bone loss was measured. A one-way ANOVA was conducted for statistical analysis, followed by Tukey's HSD post hoc test for further comparisons.

Results: Implants in group 3 exhibited significantly higher CBL than those in groups 1 and 2 at 3 months and 1-year ($p = 0.000$). At 3 months and 1-year, the prevalence of CBL ≥2 mm was higher in group 3 implants (1.7%, 10.6%) followed by group 1 (0%, 2.2%) and group 2 (0%,0%).

Conclusion: During a one-year follow-up period, anodized implants showed a higher incidence of CBL relative to sandblasted acid-etched implants.

Keywords: Anodization, Bone loss, Dental implants, Sandblasting, Surface topography

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INTRODUCTION

In recent years, both fully and partially edentulous patients tends to opt for dental implant placement as part of their oral rehabilitation. Literature evidence supports high success and survival rates of different implant systems.[1–5] Nevertheless, crestal bone loss from pathological processes like peri-implantitis or physiological remodelling can occur in osseointegrated implants.[6–10]

Crestal tissues undergo constant changes following implant placement and restoration.[11] A frequent observation is that the soft tissues around the implant gradually recede, accompanied by the resorption of the underlying crestal bone.[12] Thus, it has been suggested that for the sustained success of implant-supported restorations, tight soft tissue seal and high peri-implant crestal bone level are crucial. Various geometrical implant designs, and surface modifications have been developed to preserve high crestal bone levels thereby to enhance the osseointegration influence.[13–15]

The primary approaches for modifying the surface of implants are sandblasting, acid treatments, and different oxidation processes. Sandblasted, large grit, acid-etched (SLA) implant surfaces are created by modifying the implant's macrostructure through sandblasting with coarse particles, and then creating micro-irregularities on the surface by etching with an acid. This surface treatment creates irregularities, which in turn favours adhesion of bone cells leading to bone deposition.[16–19] An advancement over SLA surface is SLActive, where, the implant surface is cleaned using nitrogen protection, hydroxylated, and stored in saline solution. This process makes the surface hydrophilic. Currently, ultraviolet (UV) photo functionalization is a prominent implant surface modification technique that enhances osteoblast adhesion. This process transforms the implant's hydrophobic surface into a superhydrophilic one, thereby promoting better osseointegration. Increased hydrophilicity improves the initial attachment of osteoblast cells to the implant surface, facilitating more effective integration with the surrounding bone tissue.[20] Another electrochemical method is anodization, which creates a thick titanium dioxide layer thereby making the surface osteoconductive.[21]

Surface modifications alter the roughness, surface texture, wettability features of the implant surface. Several experimental studies have demonstrated that highly rough surface dental implants typically experience greater and more noticeable crestal bone loss.[22–24] However, there are no research involving humans to assess the impact of surface modifications. In studies evaluating a single type of implant, crestal bone level changes at 5 years vary from 0.15 mm for SLA implants,[25] to 1.07 mm for titanium dioxide sandblasted surface implants,[26–28] and 1.5 mm for anodized implants.[29–31] Comparable crestal bone loss was observed in the various groups after five years in studies that examined two different surface modified implants: turned and sandblasted implants,[32] or turned and anodized implants.[33–38] Notably, insertion of the final prosthesis was the starting point in these investigations.

Literature evidence reveals there are no follow-up studies assessing the crestal bone level changes starting from implant placement. Even though structured, extended-duration prospective studies are necessary for accurately identifying risk factors in conditions with complex and multiple causes, they are challenging to carry out and frequently involve small sample sizes and ideal research conditions, which reduces their external validity. Therefore, the objective of this registry data-based cohort study was to examine the crestal bone loss (CBL) associated with sandblasted acid-etched and anodized dental implants.

MATERIALS AND METHODS

This registry data-based cohort study was conducted in Department of Periodontology, Saveetha Dental College, India. The present study included case records of patients who underwent implant surgery with internal-hex varying platform root analog bone level implants, featuring sandblasted acid-etched and anodized surfaces. These implants were conventionally loaded and restored with cement-retained, porcelain-fused-to-metal prosthetic crowns between January 2022 and June 2023. The ethical clearance was obtained from the Institutional Ethical ReviewCommittee (IHEC/SDC/FACULTY/PERIO/1913).

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Case records of periodontally healthy individuals without systemic diseases and patients with controlled systemic diseases (ASA I and ASA II) of both gender within the age group of 25-60 years were included; patients with bone augmentation procedures during implant placement were excluded.

Case records of 162 patients (311 implants) from January 2022-June 2023, meeting the above inclusion and exclusion criteria were involved in the study. Based on sandblasted acid-etched and anodized topographies, they were categorized into: Group 1 consisted of 93 implants (SLA®, Straumann, Basel, Switzerland) placed in 47 patients. Group 2 included 99 implants (SLActive®, Straumann, Basel, Switzerland) placed in 54 patients. Group 3 included 119 implants (TiUnite®, Nobel Biocare, Gothenburg, Sweden) placed in 61 patients. Table 1 describes details of implants utilized in this study.

Table 1. Implants utilized

Implant Dimension	Group 1 (n)	Group 2 (n)	Group 3 (n)	Total (n)
Maxillary Anteriors				
3.3 * 10	12	10		22
3.3 * 12	12	16		28
3.5 * 10			17	17
3.5 * 11.5			12	12
Maxillary Posteriors				
4.1 * 10	15	14		29
4.8 * 10	11	12		23
4.3 * 10			18	18
4.3 * 11.5			15	15
Mandibular Anteriors				
3.3 * 10	9	9		18
3.3 * 12	6	7		13
3.5 * 10			12	12
3.5 * 11.5			8	8
Mandibular Posteriors				
4.1 * 10	8	12		20
4.1 * 12	11	10		21
4.8 * 10	9	9		18
4.3 * 10			17	17
4.3 * 11.5			14	14
5 * 10			6	6
Total (n)	93	99	119	311

Data collection

Digital periapical radiographs at baseline (T0 - on the day of implant surgery), 3rd month (T1 - during stage 2 uncovery procedure) and 1st year (T2 - after cementation of the prosthesis) were collected from the case records to assess CBL. At mesial and distal aspects of each radiograph, distance from platform of implant to coronal level of bone was measured and then average was taken as primary outcome variable. If the crest of the bone was coronal to the implant's platform, a positive value was assigned; conversely, when it was apical to the implant's

platform, a negative value was assigned. Prior to the start of the implant surgeries, the following patient-related factors were assessed as secondary variables from the case records that might have an impact on the primary outcome: age, gender, smoking, systemic disease, oral hygiene by Silness and Loe plaque index, and local inflammation by Loe and Silness gingival index.

Statistical Analysis

Study data was analysed using SPSS Software, Version 23.0; IBM Corp., Armonk, NY, USA. Shapiro-Wilk test of normality was used to assess the normal distribution of the data. The results followed normal distribution accordingly parametric tests were chosen. One-way ANOVA was used to compare age, plaque index, gingival index, CBL between the three groups. Frequency distribution was calculated and the prevalence for the various CBL levels was computed (0-1.9 mm and ≥ 2mm) and expressed in mean percentages. Tukey's HSD post hoc test was done for pairwise comparison. Chi-square test was used to compare gender, smoking and systemic disease distribution. A p-value of less than 0.05 was deemed statistically significant.

RESULTS

The sample population conprised of 311 implants (162 patients) evaluated at baseline. The 1-year data from 303 implants (156 patients) is presented in this study. The patient characteristics of the sample, categorised by implant type, are shown in Table 2.

Table 2. Patient characteristics at baseline based on the type of implants

	SLA	SLActive	TiUnite	p value
Age	55.2±11.3	52.7±13.5	57.2±15.4	0.879
Gender	Male n=76 Female n=86	Male n=74 Female n=88	Male n=77 Female n=85	0.612
Smoking	Yes n=31 No n=131	Yes n=28 No n=134	Yes n=27 No n=135	0.423
Systemic disease	Yes n=64 No n=98	Yes n=59 No n=103	Yes n=61 No n=101	0.674
Plaque Index	0.57±0.03	0.61±0.06	0.62±0.06	0.864
Gingival Index	0.56±0.05	0.51±0.08	0.58±0.01	0.943

The three implant types that were assessed in this study had an equal distribution of these patient characteristics. No significant difference was noted at baseline between the groups in terms of age, gender, smoking, systemic disease, plaque index and gingival index (p>0.05).

Crestal bone loss (CBL)

During evaluation at 1 year, number of implants lost in group 1, group 2 and group 3 were 1 (1.07%), 1 (1.01%) and 6 (5.04%) respectively. Table 3 depicts the mean CBL among the three groups. At baseline, the crestal bone level in group 1, 2 and 3 implants were 0.35±0.05, 0.34±0.04 and 0.36±0.05 mm, respectively and there was no significant difference between three groups (p = 0.409). At 3 months, the CBL in group 3 implants was notably higher compared to groups 1 and 2 (-0.86±0.11 vs. -0.53±0.04 and -0.44±0.48 mm, respectively; p = 0.000). Similarly at 1 year, group 3 implants exhibited significantly greater CBL than those in groups 1 and 2 (-1.96±0.92 vs.-0.85±0.06 and -0.73±0.05 mm, respectively; p = 0.000). Furthermore, pairwise comparisons revealed significant differences between group 1 and group 2, group 1 and group 3, and group 2 and group 3 at both the 3-month and 1-year follow-ups (p = 0.00).

Table 3. Comparison of crestal bone loss (CBL) between the groups

Type of implants	Baseline (T0)	3 months (T1)	1 year (T2)
SLA	n=93 Mean±SD: 0.35±0.05	n=93 Mean±SD: -0.53±0.04	n=92 Mean±SD: -0.85±0.06
SLActive	n=99 Mean±SD: 0.34±0.04	n=99 Mean±SD: -0.44±0.08	n=98 Mean±SD: -0.73±0.05
TiUnite	n=119 Mean±SD: 0.36±0.05	n=119 Mean±SD: -0.86±0.11	n=113 Mean±SD: -1.96±0.92
ANOVA	p = 0.409	p = 0.000*	p = 0.000*
Tukey's HSD post hoc	SLA vs SLActive Mean Difference: -0.004 p = 0.825	SLA vs SLActive Mean Difference: 0.091 p = 0.000*	SLA vs SLActive Mean Difference: 0.122 p = 0.000*
	SLA vs TiUnite Mean Difference: -0.009 p = 0.379	SLA vs TiUnite Mean Difference: -0.335 p = 0.000*	SLA vs TiUnite Mean Difference: -0.708 p = 0.000*
	SLActive vs TiUnite Mean Difference: -0.005 p = 0.747	SLActive vs TiUnite Mean Difference: -0.426 p = 0.000*	SLActive vs TiUnite Mean Difference: -0.831 p = 0.000*

**Statistically significant*

Table 4 shows the frequency distribution of the implants at three months and one year based on two pre-established CBL thresholds (0-1.9 mm and ≥2 mm). At three months, group 3 implants (1.7%) had a prevalence of CBL ≥2 mm, compared to 0% and 0% for group 1 and group 2 implants, respectively. Similarly, group 3 implants (10.6%) had a prevalence of CBL ≥2 mm at 1 year, whereas group 1 and group 2 implants had corresponding percentages of 2.2% and 0%, respectively.

Table 4. Prevalence of described CBL thresholds at 3 months and 1 year

Type of implants	CBL at 3 months		CBL at 1 year	
	0-1.9 mm	≥ 2 mm	0-1.9 mm	≥ 2 mm
	n (%)	n (%)	n (%)	n (%)
Group 1	93/93 (100%)	0/93 (0%)	90/92 (97.8%)	2/92 (2.2%)
Group 2	99/99 (100%)	0/99 (0%)	98/98 (100%)	0/98 (0%)
Group 3	117/119 (98.3%)	2/119 (1.7%)	101/113 (89.4%)	12/113 (10.6%)

DISCUSSION

In this study evaluating 311 implants, higher CBL was observed in relation to anodized relative to sandblasted acid-etched implants at 3 months and 1 year. Throughout the entire study, anodized surface implants showed higher CBL than sandblasted acid-etched implants. The acquired data are analogous to those observed in longitudinal case reports involving the referenced implant surfaces, despite the lack of similar comparative studies in the literature. Nicolau P et al., reported that at the end of 1 year, CBL was around 1.07 mm in SLActive implants.[39] Following a 20-month observation period, the average CBL values for the SLActive and SLA implants were 0.17 and 0.24 mm respectively, reported by Sener-Yamaner ID et al.[40] Similarly, another study demonstrated that the total mean bone level change at 12 months was 0.77±0.93 mm in SLActive implants.[41] Heberer S et al.,[42] observed 0.9 mm and 0.5 mm of bone loss around SLA and SLActive implants at 1 year follow-up in a split-mouth randomized controlled clinical trial. These findings are similar to the CBL observed in the present study (0.85±0.06 mm in SLA and 0.73±0.05 mm in SLActive).

Anodized implants had a significantly higher CBL (1.92 mm) than turned (1.46 mm) and hybrid implants (1.02 mm) according to a study comparing anodized with turned and hybrid implants. Also, six anodized implants were failed after four to five years.[43] A recent animal study examined the impact of microgeometry of the implant on osseointegration and crestal bone formation. Three implant groups were compared using histomorphometric analysis: (1) commercially available anodized implant; (2) customized replica of implant "1" with hydrophilic sandblasted acid-etched surface; and (3) commercially available implant with surface identical to implant "2" but different implant geometry. In this study, a standardized dehiscence model was created in pigs and observed up to 2 to 8 weeks of healing. The study's finding showed that, in comparison to group 1 implants, crestal bone level was high around groups 3 and 2 implants and was found to be significantly higher after 8 weeks.[44] Glauser R et al., reported crestal bone loss of 1.54±0.99 mm around anodized surface implants,[29] which is similar to the range reported in the present study (1.96±0.92 mm). However, when the standard deviation values are high, these mean bone loss values might be obscuring the clinical relevance. The results of this research indicate that anodized implants behaved more inconsistently in terms of CBL, with the anodized implants' standard deviation measuring 0.92 mm, compared to 0.06 mm and 0.05 mm for SLA and SLActive implants, respectively.

All the implants considered in the present study have moderately rough surfaces. Higher CBL in moderately rough surface implants has been reported in meta-analysis,[45] systematic review,[46] randomized studies,[47,48] and in various retrospective and prospective studies.[49–51] A frequency distribution analysis would be beneficial instead of focusing only on comparing mean values, which conceals the severity of the problem and makes it difficult to identify those who are most severely affected. In light of this, we analyzed the frequency distribution of CBL thresholds across various time intervals.

When comparing anodized implants to sandblasted acid-etched implants, the frequency of implants with bone loss ≥ 2 mm at a one-year follow-

up was nearly five times higher (10.6% vs. 2.2%). Similarly, Gallego L et al., reported the prevalence of CBL ≥ 2 mm at 2 years follow-up was 8.98% in the anodised implants.[52] According to the current clinical study's findings, anodized implants are likely to have a higher failure rate (5.04%). These findings are consistent with those of Ferrantino L et al., who reported 8.1% failure rate for anodized surface implants.[53]

There are a few limitations associated with this study, which include: a) its design, which makes it impossible to establish cause-and-effect relationship; b) the radiological analysis method, which might pose a barrier to the validity of the results obtained because periapical radiographs are known to underestimate actual bone loss; and c) omission of an analysis of the crown-to-implant ratio. Nonetheless, the study's findings are reliable because the patient population was fairly homogeneous consisting of periodontally healthy individuals without systemic diseases and patients with controlled systemic diseases. Also, all implants utilized were commercially available with textured collar design, internal connection and the connection was platform switched, they were placed using standardized surgical and prosthetical protocols and all procedures were performed in the same institution. In addition, throughout all of the time periods, CBL measurements were obtained from a single reference point. Furthermore, the outcomes are consistent with previous research assessing variations in crestal bone loss based on implant microgeometry.

CONCLUSION

This registry data-based cohort study reports a greater CBL around implants with anodized implants relative to sandblasted acid-etched implants with 1 year follow-up period. Therefore, having detected that CBL, it is crucial to set up extended duration follow-ups with this patient group to determine whether the early indication of increased CBL in anodized surface implants progresses, potentially culminating in implant failures.

DECLARATIONS

Conflicts of interest and financial disclosures

The authors declare no conflict of interest and there was no external source of funding

Ethical Approval

Ethics approval and consent to participate: The ethical clearance was obtained from the Institutional Ethical Review Committee (IHEC/SDC/FACULTY/PERIO/1913).

Informed Consent

All participants signed an informed consent form.

Author contributions

Conceptualization AR, MDB, GC; methodology, AR; LF; GM; and VR; software, AR; and VR; formal analysis, AR; investigation, AR; data curation, AR; writing—original draft preparation, GM, VR; MMM; writing—review and editing, GC; LF and GM; supervision, GC; LF and GM. All authors have read and agreed to the published version of the manuscript.

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RESEARCH ARTICLE

PLATINUM –COATED TITANIUM NANOTUBES -ENHANCING THE CORROSION RESISTANCE AND BIOMINERALIZATION

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ABSTRACT

Background: Titanium has long been the preferred material for oral rehabilitation due to its excellent mechanical properties and biocompatibility. However, despite its widespread clinical success, implant failures remain a significant concern, primarily due to peri-implant diseases. Studies indicate that over 56% of implants experience complications over time, highlighting the need for improved materials or treatment strategies. While surface modifications to enhance osseointegration by creating bioactive surfaces have shown promise, there remains a need to improve the antimicrobial properties of implant materials. This study explores a novel approach of coating platinum onto the titanium nanotube arrays leveraging its inherent antimicrobial properties, to enhance both biological performance and corrosion resistance.

Materials and Methods: Titanium surfaces were anodized to fabricate platinum-doped TNA on titanium alloys, aimed at promoting bioactivity and improving osseointegration. Platinum, known for its antimicrobial and antioxidant properties, was sputtered onto the nanotubes to further support healing and reduce microbial colonization. Surface morphology was analyzed using scanning electron microscopy (SEM), and electrochemical tests were conducted to assess corrosion resistance. Biomineralization potential was evaluated by immersing the samples in Dulbecco's Modified Eagle Medium (DMEM) with fetal bovine serum (FBS) for seven days. ATR-IR spectroscopy was used to confirm the formation of biomimetic structures.

Results:SEM images revealed uniformly aligned platinum-doped nanotubes with partial coverage by platinum nanospheres. Corrosion resistance tests demonstrated enhanced stability of the platinum-coated TNA. Immersion studies showed a flower-like biomimetic morphology resembling water lettuce, confirmed by ATR-IR spectroscopy to be formed by proteins and calcium phosphate molecules.

Conclusion: In conclusion, platinum-coated titanium nanotube arrays (TNA) enhance antimicrobial properties and corrosion resistance, improving implant performance. This innovative approach offers potential for reduced microbial colonization and better osseointegration, providing a promising solution to reduce implant failures and improve long-term outcomes in oral rehabilitation. Further clinical research is needed.

Keywords: Titanium Nanotube, Platinum, Corrosion Resistance,Periimplant Diseases

INTRODUCTION

Titanium has become the cornerstone material for oral rehabilitation due to its remarkable biocompatibility, mechanical strength, and corrosion resistance. Its widespread application in dental and

orthopedic implants has been driven by its ability to integrate with bone tissue effectively, a process termed osseointegration. Despite these advantages, the long-term success of titanium implants is often compromised by peri-implant diseases, which include peri-implant mucositis and peri-implantitis. These conditions, characterized by inflammation and bone loss, remain a significant challenge in implantology.[1]Addressing this issue necessitates innovative approaches to enhance both the bioactivity and antimicrobial properties of implant surfaces.

Surface modification techniques, such as anodization techniques have been extensively studied to improve osseointegration Anodized titanium nanotube arrays (TNA) have garnered considerable interest due to their unique ability to mimic natural bone structure and provide a favorable environment for cell attachment and proliferation.[2] These nanostructures not only promote osteogenesis but also offer opportunities for incorporating antibacterial agents, addressing a critical limitation of conventional titanium implants.

Recent advancements have focused on incorporating antimicrobial elements such as silver, zinc, and copper into titanium nanotube arrays (TNA) to prevent bacterial colonization.[3,4] However, these materials often present challenges, including cytotoxicity and limited long-term efficacy.[5]Unlike silver-based coatings, which raise concerns over bacterial resistance, platinum (Pt) offers a unique antibacterial mechanism that does not rely on ion release. Platinum, a noble metal known for its biocompatibility, antimicrobial properties, and antioxidant activity, has emerged as a promising candidate for implant surface modification. Its catalytic activity promotes the generation of reactive oxygen species (ROS), such as superoxide radicals and hydrogen peroxide, which induce oxidative stress in bacterial cells, damaging essential biomolecules and leading to cell death.Furthermore, Pt-coated surfaces modify surface charge and wettability, reducing bacterial adhesion and biofilm formation.[6] These attributes make Pt an ideal choice for next-generation implant coatings, ensuring long-lasting antibacterial efficacy without concerns over ion release or cytotoxicity.

The present study investigates a novel approach of doping TNA with platinum to develop a multifunctional implant surface. By combining the osteogenic potential of TNA with the antimicrobial efficacy of platinum, this strategy aims to address the dual challenges of bacterial infections and peri-implant diseases. The incorporation of platinum through sputtering not only enhances antimicrobial properties but also improves corrosion resistance, a critical factor for the longevity of titanium implants in the oral environment.

To assess the effectiveness of this approach, platinum-doped TNA surfaces were analyzed using scanning electron microscopy (SEM) to examine surface morphology and subjected to electrochemical testing to evaluate corrosion resistance. Biomineralization studies were conducted by immersing the samples in Dulbecco's Modified Eagle Medium (DMEM) with fetal bovine serum (FBS), providing insights into their bioactivity. Additionally, ATR-IR spectroscopy was used to confirm the formation of biomimetic structures.This study builds upon previous research demonstrating the potential of nanostructured titanium surfaces for enhanced osseointegration, while addressing the critical need for antimicrobial functionality.[7] By harnessing the unique properties of platinum, this surface modification presents a promising strategy to reduce implant failures and enhance the clinical success of titanium-based oral rehabilitation. Further investigations could lead to the development of next-generation implant materials with improved biological integration, mechanical performance, and long-term durability.

MATERIALS AND METHODS

Sample Preparation

A 2 mm thick commercially pure titanium (Cp-Ti) sheet was obtained from M/s Uniforce Engineers, Chennai, India. The sheet was mechanically cut into 15 mm × 20 mm × 2 mm samples. To prepare the surface for electrochemical anodization, the samples were ground using silicon carbide paper with varying grit sizes. Ultrasonic cleaning was then performed using acetone and double-distilled water to remove surface contaminants. Following a previously reported method, the cleaned samples were etched using Kroll's reagent (a mixture of 7.5 mL water, 1.5 mL concentrated sulfuric acid, and 1.5 mL hydrofluoric acid) for 10 seconds. The etched samples were then rinsed thoroughly with double-distilled water and left to air-dry at room temperature before further processing.[8]

Electrochemical anodization was performed at room temperature using an electrolyte solution of 0.5 M sodium fluoride and 1 M orthophosphoric acid. A cleaned Cp-Ti sample functioned as the anode, while platinum served as the cathode in a two-electrode electrochemical setup. The process was conducted

under a constant voltage of 30 V for 90 minutes to facilitate nanotube formation.Following anodization, the sample was rinsed thoroughly with double-distilled water and air-dried at room temperature. To improve nanotube stability, the samples were sintered at 400°C for 3 hours, enabling the formation of the anatase phase. Finally, a thin platinum layer was sputtered onto the TNA surface for 45 seconds at 20 mA using a JEC-3000FC Auto Fine Coater.[9]

The surface topography of the platinum-sputtered TNA (Pt-TNA) samples was analyzed using a scanning electron microscope (SEM, JOEL-JSM-IT800), equipped with energy-dispersive X-ray spectroscopy (EDS, Oxford Instruments) for elemental composition analysis.[10] Additionally, the functional groups present on the Pt-TNA surfaces were identified using Attenuated Total Reflectance-Infrared Spectroscopy (ATR-IR, Spectrum Two).Electrochemical corrosion behavior was assessed using electrochemical impedance spectroscopy (EIS) and potentiodynamic polarization analyses, conducted on a CHI 730E electrochemical workstation. A three-electrode electrochemical setup was used, consisting of a saturated calomel electrode (SCE) as the reference electrode, a graphite rod as the counter electrode, and the sample as the working electrode. Corrosion studies were performed in simulated body fluid (SBF) solution, following our previous methodology . To stabilize the open circuit potential (OCP), the sample was immersed in SBF for 1 hour before testing.

For EIS analysis, a 10 mV sinusoidal voltage was applied within a frequency range of 0.01 Hz to 100 kHz, with results represented using Nyquist and Bode plots. Data fitting and equivalent circuit modeling were conducted using ZSimpwin software (Princeton Applied Research, USA). Potentiodynamic polarization studies were performed over a ±250 mV potential range, recorded against SCE at a scan rate of 1 mV/s. The corrosion potential (Ecorr) and corrosion current density (icorr) were derived from the polarization curves, with the Stern-Geary equation used to calculate the corrosion current density.[11]

RESULTS

The surface topography and elemental constituents of TNA and Pt-TNA are provided in Figure 1. The anodized surface exhibits consistently aligned vertical nanotubes. At higher magnification, the nanotubes appear to fuse together, forming a uniform structure. The resulting The EDX profile of TNA (Figure 1b) confirms the presence of Ti, O, and P. The incorporation of phosphate ions in the nanotubes is beneficial for the bone mineralization process. In contrast, Pt-TNTA (Figure 1c) displays a uniform distribution of platinum deposits on the TNA surface. The rim of the nanotubes remains visible, with partial coverage of sputtered Pt (Figure 1d). Despite Pt deposition, the nanotubular structures retain their integrity, indicating that the sputtering process does not significantly alter their morphology.[2]

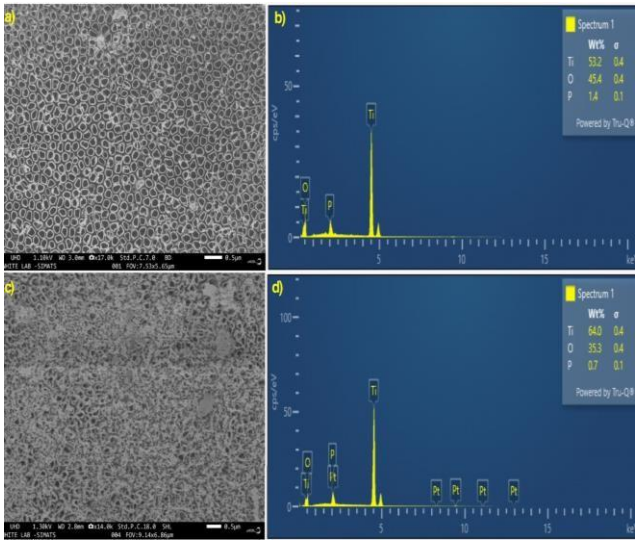


Figure 1. Surface Morphology and Elemental Analysis of Titanium Nanotubes and Platinum Sputtered Titanium Nanotubes

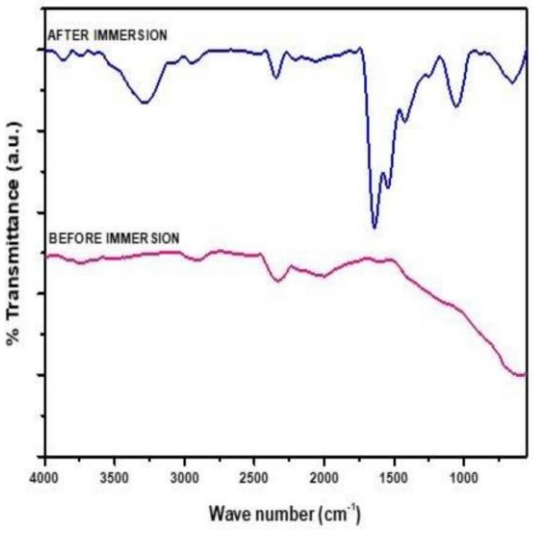


Figure 2. FTIR Analysis of Surface Modifications Before and After Immersion

The FTIR spectrum presented in the image compares the material before and after immersion in a specific solution. The x-axis represents the wavenumber (cm^{-1}), indicating the vibrational frequencies of molecular bonds, while the y-axis represents the percentage transmittance, showing how much

infrared light passes through the sample. Before immersion, the pink curve appears relatively featureless, suggesting a minimal presence of functional groups absorbing IR radiation. However, after immersion, the blue curve exhibits significant peaks, particularly in the range of 1600 cm^{-1} to 1000 cm^{-1}.[13] These peaks likely correspond to phosphate (PO_4^{3-}) or carbonate (CO_3^{2-}) groups, which are commonly associated with biomineralization. The observed spectral changes indicate surface modifications or the deposition of biomolecules and minerals after immersion. This suggests that the material undergoes chemical transformations, potentially forming a bioactive or mineralized layer, which could be beneficial for biomedical applications such as bone integration or bio-coatings.

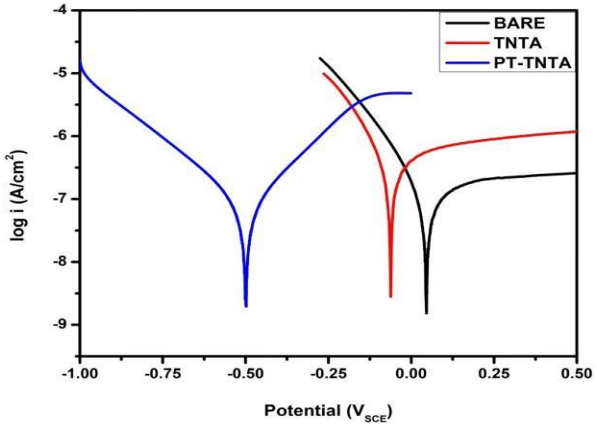


Figure 3. Polarization curves for Bare Titanium, Titanium Nanotube and Platinum Nanotube

The polarization curves in the image illustrate the electrochemical behavior and corrosion resistance of three different surfaces: bare (black), TNA (red), and Pt-TNA (blue). The bare surface exhibits the most negative corrosion potential (E_corr) and the highest corrosion current density (i_corr), indicating greater susceptibility to corrosion. In contrast, the TNA and Pt-TNTA curves shift towards more positive potentials, demonstrating enhanced corrosion resistance due to the formation of a protective nanotubular oxide layer. The Pt-TNA surface shows the lowest i_corr, signifying the best corrosion protection among the three. The shift in the anodic branch of the Pt-TNA curve further suggests improved electrochemical stability, likely due to the catalytic and passivating effects of platinum. Overall, the results indicate that TNA enhances corrosion resistance compared to bare titanium, while Pt-TNA provides the highest level of protection, making it a promising material for biomedical and electrochemical applications.[14]

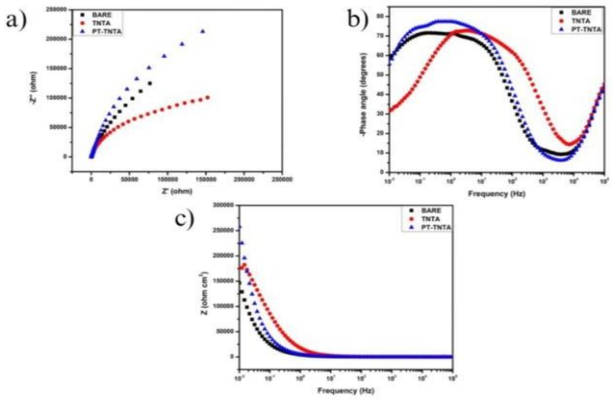


Figure 4. Electrochemical impedance spectroscopy (EIS) analysis of BARE, TNTA, and PT-TNTA samples: (a) Nyquist plot, (b) Bode phase angle plot, and (c) Bode magnitude plot.

Figure 4 presents three electrochemical impedance spectroscopy (EIS) plots—(a) Nyquist, (b) Bode phase, and (c) Bode magnitude—comparing the electrochemical behavior of BARE (black squares), TNA (red circles), and PT-TNA (blue triangles) samples. The Nyquist plot (a) depicts the real (Z′) and imaginary (Z″) impedance components, showing the highest impedance for PT-TNA, followed by BARE and TNA, indicating increased charge transfer resistance and improved corrosion resistance. The Bode phase plot (b) illustrates the phase angle versus frequency, with PT-TNA exhibiting the highest peak phase angle, suggesting enhanced capacitive behavior. The Bode magnitude plot (c) demonstrates impedance variation across frequencies, where PT-TNA maintains the highest impedance at low frequencies, indicating superior barrier properties. Overall, the PT-TNA modification enhances electrochemical performance, likely improving corrosion resistance and surface stability.[15]

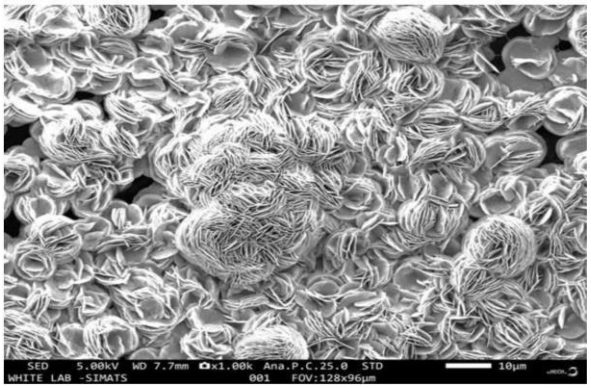


Figure 5. SEM Image of Pt-TNTA Surface Morphology After 7 Days of Immersion in DMEM and FBS Solution

The Figure 5 illustrates a SEM micrograph of Pt-TNTA after immersion for 7 days in DMEM and FBS solution, captured at 1000× magnification with a 10 μm scale bar. The image reveals nanoplatelet-like apatite structures that have merged to form a distinct flower-like morphology. This hierarchical architecture suggests the progressive precipitation of calcium phosphate compounds, along with amino acids and proteins, resulting in structurally ordered apatite formation. The incorporation of phosphate within the nanotubes facilitated strong conjugation with calcium ions from the DMEM and FBS solution, leading to the development of biologically active apatite complexes integrated with amino acids and proteins. This surface transformation indicates enhanced bioactivity, potentially beneficial for biomedical applications.[16]

DISCUSSION

The proposed platinum-doped titanium nanotube array (TNA) structure offers adaptive features crucial for enhancing cell interactions with implant materials. Its vertically aligned nanotube topography provides a significantly larger surface area compared to flat titanium surfaces, a feature that has been shown to address limitations of current clinical implants.[17] This enhanced surface area creates additional sites for cell interaction, particularly at the extracellular matrix level, and may mimic the pore size of natural bone, which is essential for bone cell response.[18,19] Moreover, the bioactive layer formed by inward growth TiO_2 nanotubes demonstrates improved adherence to the titanium metal, addressing challenges associated with weak interfacial bonding observed in existing ceramic coatings.[20,21]
The increased surface energy of these nanostructures promotes interactions with proteins like vitronectin and fibronectin, which are critical for specific cell adhesion and improved osseointegration.[22,23]
Additionally, the platinum coating not only enhances the biomineralization potential but also provides antimicrobial and antioxidant properties, further supporting the healing process and reducing microbial colonization. Yu et al. [24] demonstrated that anatase TNA optimally supports cell adhesion, proliferation, and differentiation while reducing inflammatory responses.[25] In addition to the above TNA-modified implant surface, we have sputtered it with a noble metal such as platinum.
Hence, this study presents compelling evidence in favor of using platinum as a monometallic coating for implants, in contrast to conventional bimetallic or alloy-based coatings.

Platinum, as a noble metal, possesses unique chemical and electrochemical properties that contribute to its superior biocompatibility, corrosion resistance, and antibacterial potential. One of the key advantages of platinum is its exceptional chemical inertness, which prevents undesirable interactions with biological tissues and bodily fluids, ensuring long-term stability and reducing the risk of adverse immune responses. This property is shared among noble metals such as gold and silver, but platinum's superior corrosion resistance further enhances its suitability for biomedical applications.[26] Unlike other metals, platinum does not readily oxidize or degrade, even in aggressive physiological environments, thereby maintaining the structural integrity of the implant over extended periods.[27,28]

Electrochemically, a monometallic platinum coating eliminates the presence of a galvanic pair, thereby preventing the preferential oxidation of a more reactive metal. While bimetallic coatings can facilitate controlled ion release, they also introduce the risk of accelerated degradation of the less noble component due to galvanic coupling.[29,30]

This effect is particularly pronounced in silver-based systems, where the presence of platinum enhances silver ion release, improving antibacterial efficacy but potentially shortening implant longevity.[31] By utilizing pure platinum, these degradation risks are avoided while maintaining its inherent antibacterial properties and biocompatibility. Additionally, concerns regarding unintended inflammatory reactions or unpredictable biological responses from alloying elements are minimized.

Although bimetallic nanoparticles and coatings have garnered interest for their synergistic antibacterial effects, the potential for bacterial resistance to monometallic nanoparticles—especially silver—remains a concern. While Ag-Pt and Au-Pt bimetallic systems have demonstrated improved antimicrobial performance, monometallic platinum coatings offer a stable, long-term alternative without the complications of silver ion release or the risk of bacterial resistance development.[32]

Furthermore, platinum coatings eliminate uncertainties related to compositional variations and structural inconsistencies, which can influence the biological response of bimetallic systems.[33,34] While the present study demonstrates the potential of platinum-coated titanium nanotube arrays (TNA) as surface modification in improving antimicrobial

properties and corrosion resistance, limitations include the need for long-term clinical validation and potential cost implications. Future research should explore the biological interactions of platinum coatings over extended periods and optimize deposition techniques for uniform coverage. Additionally, in vivo studies are essential to confirm the long-term stability, osseointegration efficiency, and resistance to peri-implant diseases in real-world applications.

CONCLUSION

In conclusion, platinum-coated titanium nanotube arrays (TNA) surface modification shows significant promise for enhancing both antimicrobial properties and corrosion resistance, addressing key challenges in oral implantology. The incorporation of platinum improves the biological performance of titanium implants, offering potential for reduced microbial colonization and enhanced osseointegration. The findings suggest that platinum-coated TNA could provide a viable solution to reduce implant failures and improve long-term outcomes in oral rehabilitation, warranting further research and clinical exploration.

Declarations

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Conflict of Interest

The author declares that he has no conflict

Ethical Approval

Not Applicable

Informed Consent

Not Applicable

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RESEARCH ARTICLE

INFLUENCE OF TEMPERATURE AND SINTERING DURATION ON HYDROXYAPATITE'S STRUCTURAL AND MORPHOLOGICAL CHARACTERIES EXTRACTED FROM OVINE BONE

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ABSTRACT

Bone grafts are widely used in dentistry worldwide. Still, none of the products possess all the desirable qualities for a bone substitute material, including low immunogenicity, low patient morbidity, affordability, and angiogenic potential. This study evaluated the effect of temperature and sintering time on the hydroxyapatite derived from ovine bone. The ovine bone samples were categorized based on their temperature and sintering time into six groups: 120°C for 4 hours, 120°C for 8 hours, 240°C for 1.5 hours, 240°C for 3 hours, 360°C for 1.5 hours, and 360°C for 3 hours. Physiochemical properties using scanning electron microscopy, X-ray diffractometry, and Microculture Tetrazolium Assay were compared to commercially available bone grafts of BioOss and Osseograft, serving as a benchmark for the sintered ovine bone samples and highlighting their potential superiority. BioOss showed increased proliferation initially, but a decline was observed after 72 hours. In contrast, the sample sintered at 360°C for 3 hours exhibited increased cell aggregation after 72 hours, potentially enhancing osteogenic differentiation. In addition, the samples sintered at 360°C for 3 hours displayed the most distinctive and interconnected pore structure, suggesting higher sintering and consolidation effects. The unique and intriguing qualities of the sintered ovine bone samples, such as their distinctive pore structure and potential for strengthening osteogenic differentiation, could open new and promising avenues for bone graft materials in dentistry and orthopedics, offering optimism for their future use. The ovine bone showed physicochemical properties like BioOss with increased sintering time and temperature. Processing ovine bone with solvents like toluene and ethylene di amine and subjecting it to a temperature of 360° C for 3 hours showed similar results to commercially available xenograft-BioOss.

Keywords: Hydroxyapatite, Osteoblasts, Bio-Oss, Differentiation

INTRODUCTION

Bone grafts are a critical component in modern medical and dental treatments, facilitating the repair and regeneration of bone tissue. These grafts can be sourced from the patient's body or a donor or synthetically created, each type offering unique benefits and challenges. They are pivotal in treating fractures, bone defects, conditions like osteoporosis, and dental implant procedures. The ability to effectively replace or augment bone tissue not only aids in physical recovery but also enhances patients' overall quality of life, underscoring the significance of advancements in bone grafting techniques and materials.[1] Autogenous bone grafting has been considered the "gold standard." Autografts offer osteogenic, osteoconductive, and osteoinductive qualities. However, several disadvantages exist to using autologous bone transplants, including their restricted availability, donor site morbidity, and longer recovery times.[2,3] Owing to the rising need to correct cranial bony abnormalities and the improvements in dental implantology, the use of bone grafts and replacements in dentistry has expanded significantly in recent years.[4] These skeletal or bone abnormalities can result from oral cancer, trauma, periodontal conditions, surgical excision, cranioplasty, infection, or congenital deformities.[5] In dentistry, the most common indicator of insufficient bone following tooth loss is the rapid resorption of alveolar bone due to the absence of intraosseous stimulation typically provided by periodontal ligament fibers. For the successful placement of dental implants, adequate alveolar bone dimensions are crucial, requiring a minimum height of 10 mm and a diameter of 3 mm to 4 mm.[6,7] According to estimates, bone grafts will be used in as many as 50% of dental implant surgeries.[8] The development of innovative bone grafting materials, such as alternatives for bone grafts, has gained momentum despite the need for evidence-based studies on safety and indications. Consequently, in conjunction with the global aging population and the continuous, discernible rise in the need for bone graft materials, these issues make it imperative to conduct further comprehensive research into creating novel materials with ideal properties for diverse bone grafting techniques.[9,10] Ovine bone grafts, sourced from sheep, are increasingly employed in medical and dental sectors due to their advantageous properties and availability. These grafts are a viable alternative to human bone, providing a natural scaffold for bone regeneration.[11]

Ovine bone closely resembles human bone structurally and compositionally, making it an adequate substitute. Using ovine bone grafts can reduce the risks associated with human donor grafts, such as disease transmission and immune rejection. Additionally, they are readily available and can be processed to ensure biocompatibility and safety. The osteoconductive properties of ovine bone grafts promote new bone tissue formation, aiding in healing fractures, bone defects, and dental implant integration.[12] With ongoing research, the application of ovine bone grafts is expanding and promising, with improved bone repair and regeneration outcomes. Their efficacy and safety profile make them an asset in bone grafting. The temperature and sintering time are critical parameters in the processing of hydroxyapatite derived from ovine bone. These factors significantly influence the material's crystallinity, phase composition, mechanical properties, and bioactivity. Proper control of temperature ensures the stability and purity of hydroxyapatite. At the same time, optimal sintering time affects the densification and grain growth, ultimately impacting the performance of the bone graft in clinical applications. Understanding and optimizing these parameters can improve bone repair and regeneration outcomes, making the grafts more effective and reliable. Thus, the present study assessed the impact of temperature and sintering time on hydroxyapatite derived from ovine bone.

MATERIALS AND METHODS

The present in-vitro study was conducted at Saveetha Dental College and Hospitals, Chennai, India. The Scientific Review Board (SRB) approved the study on 26 August 2022. (SRB/SDC/PROSTHO-20/22/TH-073).

Ovine Bone Processing

Samples from a local slaughterhouse were obtained from the cortical hind limb-femur goat bones. They were cut into smaller pieces, and pretreatment with hydrothermal and wet and dry processing was done.[13] A portion of the organic phase was eliminated by pretreatment, which involved cutting broad portions of ovine bone from the middle of the femur bone and physically removing any attached soft tissue. The bone marrow and residual soft tissues were emptied by simmering the slices for 12 hours at 100° C, then

changing the water. This cycle was carried out for 96 hours. The samples were then cleaned and stored at 4° C for 12 hours and placed in a hot air oven at 100° C for 12 hours. This cycle was carried out for 48 hours. The samples were cleared of all visible soft tissue and debris (Figure 1 a).

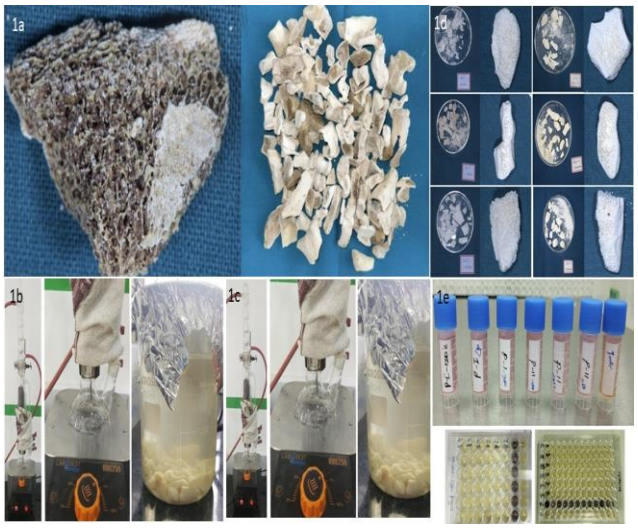


Figure1.1a) Presence of residual organic components-fats, proteins and debris post boiling the samples at 100° C for 12 hours followed by a water change every 12 hours for 96 hours; **1b)** Chemical processing using hot extraction method. Soxhlet extractor along with Toluene to aid in removal of organic contents; **1c)** Soxhlet extractor along with Ethylene di amine to aid in removal of organic contents; **1d)** Samples sintered at 120° C for 4 hours; Samples sintered at 120° C for 8 hours; Samples sintered at 240° C for 1.5 hours; Samples sintered at 240° C for 3 hours; Samples sintered at 360° C for 1.5 hours; Samples sintered at 360° C for 3 hours; **1e)** Samples prepared for MTT analysis.

The chemical processes for removing lipids, fat, and protein were carried out using the hot extraction method. 150 ml of toluene and the soxhlet extractor were used as an organic solvent. This process was carried out for 48 hours. The samples were soaked for 12 hours and then washed using ethanol to remove traces of the organic solvent.[13,14] The ethanol wash samples were heated in a hot air oven at 100° C for 12 hours (Figures 1b and c). For a thorough removal of organic components, 150 ml of ethylene di amine was used along with the soxhlet extractor as an organic solvent. This process was carried out for 48 hours. The samples were soaked for 12 hours and then washed using ethanol to remove traces of the organic solvent. The samples were then boiled at 100° C of distilled water for 12 hours. The samples were then washed twice in ethanol to remove any traces of organic components and the chemicals used for processing.

Sintering Time and Temperature

After being processed with chemical solvents, the ovine bone samples were categorized based on their temperature and sintering time into 6 groups: 120°C for 4 hours, 120°C for 8 hours, 240°C for 1.5 hours, 240°C for 3 hours, 360°C for 1.5 hours, and 360°C for 3 hours.[13,14] High and low sintering times and temperatures were applied in independent samples (Figure 1d).

The experimental group included these groups, whereas commercially available bone grafts- BioOss and Osseograft were grouped as the Control category. The sintering process is essential in material science, particularly for bone samples, as it influences the material's microstructure, mechanical properties, and biological behavior. Temperature and Sintering Time Ranges used in this study: 120°C for 4 hours and 8 hours, 240°C for 1.5 hours and 3 hours, 360°C for 1.5 hours and 3 hours. The reasons for choosing these ranges are thermal stability and phase changes, comparative analysis, and preservation of bone integrity.[13]

Thermal Stability and Phase Changes

120°C: This temperature is relatively low and is often used to remove organic components and water content without significantly affecting the mineral structure. Prolonged durations (4 and 8 hours) ensure thorough dehydration and initial decomposition of organic residues.

240°C: At this intermediate temperature, more organic material decomposes, and initial sintering processes begin, leading to the formation of a more cohesive structure. The two durations (1.5 and 3 hours) help study the impact of time on the intermediate phase of sintering.

360°C: Higher temperatures lead to significant sintering, densification, and possible bone mineral content phase changes (such as hydroxyapatite transformation). The chosen times (1.5 and 3 hours) allow for examining these changes and their effect on the mechanical properties of the bone samples.[13,14]

Comparative Analysis

Using different temperatures and times helps us understand the sintering kinetics and the role of thermal treatment in modifying the bone's structure. It allows for comparing low, intermediate, and high-temperature effects, comprehensively analyzing the sintering behavior, and optimizing parameters for desired properties.[13]

Preservation of Bone Integrity

Lower temperatures (120°C) preserve the bone's mineral structure while removing organic matter. Higher temperatures (240°C and 360°C) are applied to achieve significant sintering and modification of the bone structure while ensuring that the bone does not degrade excessively.[13,14]

Physicochemical characterization

After chemical processing and sintering, the samples were crushed and assessed for their physicochemical characterization. The experimental and control group samples were subject to physicochemical studies. The analysis included Scanning Electron microscopes (SEM) to evaluate their physical properties and X-ray diffraction (XRD) to analyze their mineral phases and crystallinity.[13]

Scanning electron microscopy

Particle size, structure, and surface topography were evaluated using SEM. The samples' modest size increased their electrical conductivity, and their stable placement on the specimen stage ensured their stability. The granules were air-dried, severely point-dried, and cleaned with acetone and distilled water to remove the moisture. The material was imaged utilizing a high-energy electron beam in an SEM. This interaction created secondary electrons, backscattering of electrons, and distinctive X-rays. Several detectors gathered these impulses to make pictures. The shorter wavelength of the electron beam allows for higher resolution and a greater depth of focus in SEM micrographs, which gives the material a three-dimensional aspect. Five scales were used to view the samples' topography: 1µm, 5µm, 10µm, 50µm, and 100µm. The investigation used a JSM-IT800 NANO SEM (JEOL Benelux, Netherlands). Using SEM pictures, the average particle size was calculated using Image J software.[13]

X-ray diffractometry

XRD was used to ascertain the materials' crystalline phases and crystallinity. The ground samples were placed on the analysis holder and subjected to a 2θ range of 10° to 80° using a D8 Advance (Bruker AXS GmbH, Karlsruhe, Germany). A cathode ray tube produced the X-rays, filtered out to generate monochromatic radiation directed toward the sample. This resulted in diffracted rays, which were detected, analyzed, and tallied. The sample contained enough material to fill an empty sample container. The X-ray's wavelength corresponded to the bond lengths between the atoms in the crystal, resulting in a pattern specific to the arrangement of atoms in the sample. The results of each material were compared to standards to determine their phase clarity and crystal structure, and the origin was used to plot the generated graphs.13

Compatibility analysis

Samples of the ovine bone were crushed and made into fine powder for the included Microculture Tetrazolium Assay (MTT) assay. The MTT assay was carried out to assess cell viability and proliferation(Figure1e).Eagle minimum essential medium F12 (1:1) containing 15% (vol/vol) heat-inactivated fetal bovine serum, two mM L-glutamine, 50 IU/mL penicillin, and 50 mg/mL streptomycin was used to cultivate MG-63 cells. It was cultured in T-25 cm2 culture flasks at 37°C with 95% air and 5% CO2 until it reached confluence, or 70% to 80%. Following two weeks, the cells were separated using a trypsin solution and multiplied at a density of 2.5 x 10^5 cells per well in 6-well plates. Each well received two milliliters of complete Dulbecco's Modified Eagle Medium F-12 (DMEM F-12) medium after the cells had been attached for 24 hours.

MTT assay

Each six-well plate was filled with one milliliter of complete culture media. 0.5 mg/mL of MTT was added to each well's bottom. The plate was subsequently incubated for four hours at 37°C. After incubation, the culture media was removed from the insert and the well, and 100µl of Dimethyl Sulfoxide (DMSO) solution was added to each well to dissolve the produced formazan crystals. The cell types were gently shaken for two minutes to ensure an even mixing of the solvent and the blue reaction product. Finally, 100µl of the colored DMSO solution was

transferred from each insert and well to a new 96-well plate to measure cell viability. Absorbance at 450 nm was measured using a microplate reader (Figure 1e). Percent cell viability was calculated using the following equation: Cell viability (%) = 450od of (sample)/A 450 od of (control) x 100

RESULTS

This study investigated the effect of sintering time and temperature on hydroxyapatite derived from ovine bone. The sintering parameters included six groups: 120°C for 4 hours, 120°C for 8 hours, 240°C for 1.5 hours, 240°C for 3 hours, 360°C for 1.5 hours, and 360°C for 3 hours. The synthesized HA samples were evaluated and compared with commercially available bone graft materials, BioOss and Osseograft, through scanning electron microscopy (SEM), X-ray diffraction (XRD) analysis, and 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay.

Scanning Electron Microscopy

The Scanning electron microscopy (SEM) analysis of BioOss depicts a well-interlaced matrix with dense inter-articles and certain bioactive surface coatings on the surface of the bone graft. More interconnected pore structures need to be identified in the SEM analysis of commercially available bone graft-Osseograft. The samples sintered at 120° C for 4 hours and 120° C for 8 hours showed poorly connected pore structure and matrix and a decreased porosity level compared to BioOss. Similar results were also obtained for samples sintered at 240° C and 360° C for a shorter enjoyable time of 1.5 hours. The samples sintered at 240° C and 360° C for 3 hours showed a well-defined pore structure and a dense structure with a well-defined surface morphology compared to BioOss. SEM analysis revealed a well-defined porous structure in all the sintering groups, indicative of successful synthesis of HA derived from ovine bone. Notably, the samples sintered at 360°C for 3 hours displayed the most distinctive and interconnected pore structure, implying a higher degree of sintering and consolidation. The samples sintered at 360° C showed a relatively smooth surface with a high level of inter-particle bonding and increased consolidated structure of Hydroxyapatite (Figure 2 a -e).

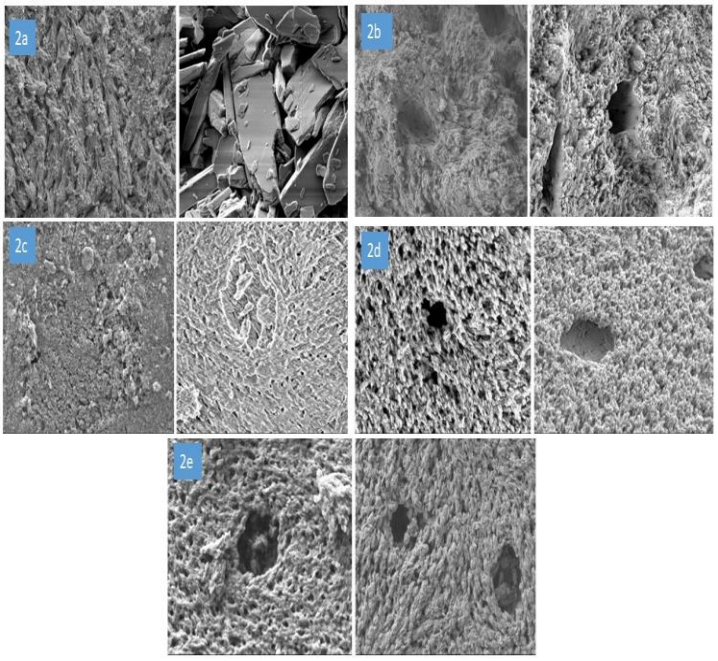


Figure 2. 2a) SEM analysis of commercially available xenograft- BioOss; Osseograft; **2b)** samples post Soxhlet extraction with Toluene; Ethylene di amine; **2c)** Samples samples sintered at 120° C for 4 hours; sintered at 120° C for 8 hours; **2d)** Samples sintered at 240° C for 1.5 hours; sintered at 240° C for 3 hours; **2e)** samples sintered at 360° C for 1.5 hours; sintered at 360° C for 3 hours**.**

X-ray Diffraction

The XRD patterns reveal the crystalline structures of hydroxyapatite [Ca4.854(H0.126(PO4)3)(OH)0.834] (01-074-9776) and calcium oxide phosphate [Ca5(PO4)3O0.5] (04-023-8826), respectively. The calcium oxide phosphate phase was predominantly observed, with peaks at 25.8° (002), 31.8° (211), 32.2° (112), 32.9° (300), 46.7° (222), and 49.5° (213). In addition, peaks in the region around 34.2° (202), 40° (221), 42.5° (302), and 43.6° (113) indicate the presence of the hydroxyapatite crystalline phase, which corresponds to the hexagonal crystal structure, as confirmed by the hkl indices. The XRD analysis suggests the primary presence of calcium oxide phosphate, with secondary contributions from the hydroxyapatite crystalline phase. From the XRD data, it was observed that hydroxyapatite (PDF 01-074-9776 Ca4.854(H0.126(PO4)3) (OH)0.834) and Calcium oxide phosphate (PDF 04-023-8826 CA5(PO4)3O0.5) crystalline phases were observed with the slight attribution of silica. In terms of the

graph, the High-intensity peak was observed first at around 31.7 deg. The two theta analyses observed a shift towards the right concerning increasing sintering temperature from 31.7 to 32.1 degrees. The maximum right shift was noted from 31.7 to 32.1 degrees at the highest sintering temperature at 360° C for 3 hours. The graph for BioOss also revealed three highly distinctive peaks suggestive of the presence of Hydroxyapatite in its crystalline form. The peaks ranged from 30 to 36 degrees, almost like ovine bone samples sintered at 360° C for 3 hours. However, the graph for Osseograft samples showed no distinctive peaks, indicating the absence of Hydroxyapatite and only the presence of Collagen(Figures 3 A, B, and C).

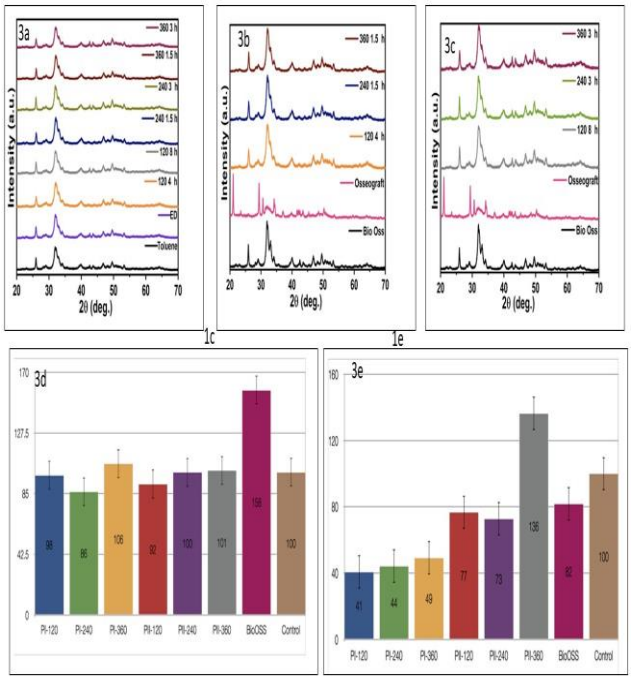


Figure 3. XRD peak data showing hydroxyapatite peaks when comparison made **3a)** between sample treated with Toluene, Ethylene di amine and sintered at 120°C for 4 hours, 120°C for 8 hours, 240°C for 1.5 hours, 240°C for 3hours, 360°C for 1.5 hours, and 360°C for 3 hours; **3b)** between 120°C for 4 hours, 240°C for 1.5 hours, 360°C for 1.5 hours and commercially available bone graft BioOss and Osseograft; **3c)** between 120°C for 8 hours, 240°C for 3 hours, 360°C for 3 hours and commercially available bone grafts BioOss and Osseograft; **3d & e)** Bar graph depicting the percentage of MG 63 cells proliferation for samples sintered at sintered at 120°C for 4 hours, 120°C for 8 hours, 240°C for 1.5 hours, 240°C for 3 hours, 360°C for 1.5 hours, and 360°C for 3 hours and its comparison to that of BioOss after an incubation period of 24 hours and 72 hours respectively.

A comparison with the Osseograft cannot be made as XRD confirmed no presence of Hydroxyapatite, and even the SEM data revealed a lack of interconnected pore structure. Therefore, even with the XRD results, ovine bone samples sintered at a higher temperature for a longer time showed comparable results to those of commercially available xenograft BioOss. When considering the results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, all the samples showed biocompatibility and growth of MG-63 cells at 24 hours. BioOss samples showed an increased proliferation and development of MG-63 cells (158 %) compared to the other samples. However, at 72 hours, there was a decline (82%) of up to 2 folds in the BioOss samples (Figure 4).

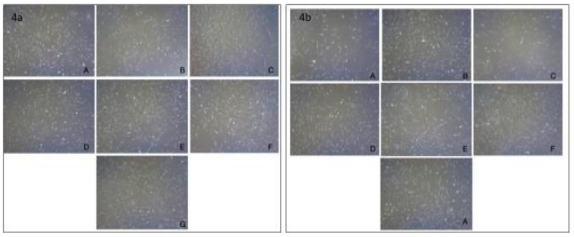


Figure 4a & b. Cell culture of MG-63 cells at 24 hours and 72 hours respectively for (A) commercially available bone graft- BioOss; (B) ovine bone graft sintered at 120° C for 4 hours (C) ovine bone graft sintered at 120° C for 8 hours; (D) ovine bone graft sintered at 240° C for 1.5 hours; (E) ovine bone graft sintered at 240° C for 3 hours; (F) ovine bone graft sintered at 360° C for 1.5 hours; (G) ovine bone graft sintered at 360° C for 3 hours.

The sample sintered at 360 ° C at 3 hours showed increased aggregation of the MG-63 cells of up to 1.2 folds after 72 hours, unlike the 2-fold decrease observed with that of BioOss. This can significantly affect and enhance the osteogenic differentiation potential of the ovine bone graft sintered at 360° C for 3 hours. The samples from other groups showed less than 50% cell viability, indicating toxicity (Table 1, 2 and Figure 3d, e).

Table 1. Sample groups based on Sintering Temperature and Time

GROUPS	SINTERING TEMPERATURE	SINTERING TIME
Group I	120° C	4 hours
Group II	120° C	8 hours
Group III	240° C	1.5 hours
Group IV	240° C	3 hours
Group V	360° C	1.5 hours
Group VI	360° C	3 hours

Table 2. The percentage of MG 63 cell proliferation for samples sintered at 120°C for 4 hours, 120°C for 8 hours, 240°C for 1.5 hours, 240°C for 3 hours, 360°C for 1.5 hours, and 360°C for 3 hours and its comparison to that of BioOss after an incubation period of 24 hours and 72 hours.

Groups	Cell Viability% After Incubation Period of 24 hours	Cell Viability% After the Incubation Period of 72 hours
PI-120	98	41
PI-240	86	44
PI-360	106	49
PII-120	92	77
PII-240	100	73
PII-360	101	136
BioOss	158	82
Control	100	100

DISCUSSION

This study observed the physicochemical properties of ovine bone, processed with solvents toluene and ethylene diamine and subjected to a temperature of 360°C for 3 hours to be like those of the commercially available xenograft BioOss. Improved results were observed with increased sintering time and temperature. Osseograft consists solely of collagen and lacks hydroxyapatite. The temperature and sintering duration affected the color of the ovine bone powder, which can range from grayish black to light yellow to white. Temperature differences caused changes in the organic compounds' vibrational states, which in turn caused changes in the chemical composition and the effective reflective index, which reflected variations in the sample's hue. The occurrence of ashes from the breakdown of the

organic component in the bone was indicated by the gray hue of the preprocessed samples. Therefore, further chemical processing is necessary to remove organic contents. Processing with toluene and ethylene di amine led to the additional removal of organic compounds. This was analyzed using data from SEM where certain impurities were present, and the pore structure was not as well defined as that of BioOss's SEM data.

The MTT analysis conducted using MG-63 cells indicates the impact of the tested substance on the viability and metabolic activity of these cells, which are commonly used as a model for bone-related studies. The MTT assay measures the conversion of MTT into a colored formazan product by mitochondrial enzymes, reflecting the overall cell metabolic activity. A higher intensity indicates increased cell metabolic activity and, thus, higher cell viability. Conversely, a lower intensity suggests reduced metabolic activity and decreased cell viability. However, the attachment of MG63 cells onto the surface of bone grafts could not be verified by optical microscopy. However, the proliferation of cells adjacent to the bone graft was confirmed. MTT assay can provide only a valuable initial assessment of the ovine bone graft's impact on cell viability. However, it should be supplemented with additional experiments and analyses to understand its effects on ovine bone comprehensively. Further investigations, such as gene expression analysis, alkaline phosphatase activity assays, and mineralization assays, can provide a more detailed understanding of the influence of ovine bone graft on bone cell differentiation and mineralization.

Previous literature and studies on the processing protocol regarding bovine bone-derived hydroxyapatite have been reported extensively.[15,16] Hydroxyapatite was also obtained while a three-step hydrothermal process processed bovine bone.[14] Studies on bovine bone have been previously carried out based on various processing methods.[17]

Alkaline hydrothermal hydrolysis was the method used by Barakat et al. to extract HAp from bovine bone.[18]

After heating the extracted HAp to 250 °C, a 1.86 Ca/P ratio nanoflake HAp was formed after five hours. In addition to cow bone, HAp has also been extracted from the bones of camels, horses, and pigs. Calcination of camel bone produced pure crystalline HAp with variable forms in the nanoscale range of 79-97 nm.[19]

Rahavi et al. reported asymmetrically shaped nano-sized HAp by calcining horse and camel bones for two hours at 700 °C.[20]

While in the present study, chemical treatment was carried out using a Soxhlet extractor along with toluene and ethylene di amine with a temperature range below 400° C, previous studies based on bovine bone employed a combination of alkaline heat treatment and calcination to extract Hap.[21]

However, there was limited evidence of obtaining hydroxyapatite and processing it associated with ovine bone. The literature revealed limited data regarding hydroxyapatite derived from fish bone, which was sintered at 800°C, 900°C, 1000°C, 1100°C, and 1200 °C.[22,23]

However, the only drawback observed was decreased mechanical properties, especially its fragility and decreased mechanical properties.[24]

The present study yielded significant insights into the impact of sintering time and temperature on hydroxyapatite derived from ovine bone; it is essential to acknowledge certain limitations. Firstly, the focus was on the sintering parameters without considering other factors, such as the chemical composition and impurities within the starting material. Future research should investigate the combined influence of sintering parameters and material composition for a more comprehensive understanding. Furthermore, although the employed characterization techniques (SEM, XRD, and MTT analysis) provided valuable information on the microstructure, crystallography, and biological aspects of sintered hydroxyapatite, there remains potential for more advanced characterization techniques. Techniques like FTIR or Raman spectroscopy could be incorporated to examine the material's chemical bonding and functional groups. Additionally, while the MTT assay offered insights into cell viability and proliferation, further investigations encompassing comprehensive biocompatibility assessments, including cell adhesion, differentiation, and gene expression analysis, are warranted. In vivo studies employing animal models would also be essential to evaluate the material's performance within a more realistic biological environment.

Further studies should explore advanced processing techniques like additive manufacturing or 3D printing to fabricate intricate structures using sintered hydroxyapatite. The incorporation of bioactive molecules, growth factors, or drug delivery systems could be investigated to enhance the material's regenerative properties. Furthermore, long-term degradation studies and mechanical testing under physiological conditions would provide crucial insights into the material's stability and mechanical integrity over time. The present study contributed to understanding sintering time and temperature effects on hydroxyapatite derived from ovine bone; there are still substantial opportunities for future research to address limitations and expand the scope of knowledge. These endeavors would further refine the understanding and application of hydroxyapatite derived from ovine bone for various biomedical purposes.

CONCLUSION

The influence of temperature and sintering duration on hydroxyapatite characteristics is well-documented. Still, this study offers new insights by focusing on hydroxyapatite derived from ovine bone, which is less extensively studied than other sources like bovine or synthetic hydroxyapatite. One essential contribution is using ovine bone as a novel alternative source, which could provide a more accessible or affordable option depending on regional availability and ethical considerations. Additionally, the study identifies an optimized sintering parameter of 360°C for 3 hours, which produced hydroxyapatite with highly interconnected pore structures, potentially enhancing biocompatibility and osteogenic potential. This specific sintering condition may not have been widely explored in previous studies. Furthermore, by directly comparing the ovine bone-derived hydroxyapatite to commercial bone grafts like BioOss and Osseograft, the study offers practical insights into the potential of ovine bone as a viable alternative for bone grafting in dental and orthopedic applications. The findings suggest that ovine bone-derived hydroxyapatite can achieve superior properties to or even superior to commercial products, particularly in biocompatibility and osteogenic differentiation. Also, the compositional or structural advantages of ovine bone hydroxyapatite over other sources would be valuable information in the future. This study unravels the intricate relationship between sintering time and temperature and their consequential impact on the processing of hydroxyapatite derived from ovine bone. The results unequivocally demonstrate that prolonged sintering time and elevated sintering temperature positively influence the material's properties, forming a remarkably uniform and interconnected porous network like the commercially available xenograft BioOss.

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Conflict of interest

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Author Contributions

Khushali K. Shah: Conceptualization, Methodology, Data Collection, and Manuscript Writing

Deepak Nallaswamy Veeraiyan: Study Supervision, Review, and Final Approval of the Manuscript

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Rajlakshmanan Eashwarmoorthy: Biochemical Analysis, Interpretation of Results, and Technical Guidance

Ravleen Nagi: Literature Review, Data Interpretation, and Manuscript Editing

Junad Khan: Statistical Analysis, Interpretation, and Critical Review of the Manuscript

Suresh Venugopalan: Experimental Design, Validation, and Manuscript Review

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RESEARCH ARTICLE

EFFECTIVENESS OF MODIFIED MASSON TRICHROME STAINING VS MODIFIED VOF STAINING FOR HARD TISSUE PATHOLOGIES

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ABSTRACT

Background: Differentiating between various types of bone—such as osteoid, calcified bone, woven bone, and lamellar bone-can be challenging with standard histological techniques for decalcified sections. Several stains, including Verde Luz-orange G-acid fuchsin (VOF), Von Kossa, solochrome cyanine, Masson's Trichrome, and silver staining (applied before decalcification), have been developed to address this issue. These stains are quick, easy, affordable, and effective at distinguishing between hard and soft tissue components in various specimens.
Aim: This study aimed to compare the efficacy of modified Masson Trichrome staining with modified VOF staining for diagnosing hard tissue pathology.
Materials and Methods: We analyzed 18 formalin-fixed, paraffin-embedded, decalcified bone samples with known histological diagnoses. These samples were subjected to staining using standard techniques, as well as modified version of Masson Trichrome and VOF stains. The evaluation criteria included stain intensity, clarity, translucency, and contrast. These factors are critical for accurately distinguishing between different types of bone tissue and assessing pathological changes.
Results: Our analysis focused on grading the quality of the staining techniques based on background clarity, morphological detail, nuclear feature definition, and overall differentiation. The modified VOF stain consistently showed superior performance in these categories compared to the modified Masson Trichrome stain. This indicates a higher efficacy in revealing detailed histological features necessary for accurate diagnosis.
Conclusion: Our findings suggest that modified VOF staining outperforms modified Masson Trichrome staining for evaluating hard tissue pathologies. This enhanced staining method provides clearer differentiation and better detail, making it a more reliable choice for histological assessment in clinical and research settings.

Keywords: Histological Techniques, Connective Tissue Stains, Masson Trichrome Staining, Osteoid Differentiation and Hard Tissue Pathologies

INTRODUCTION

Throughout the development of a tooth, hard tissues including enamel, dentin and bone, as well as the existence of mature and immature collagen, make histochemical staining identification difficult. Different percentages of mature bone, immature bone, and osteoid make it difficult to distinguish between them and, consequently, to diagnose [1]. Hard tissue lesions of the head and neck exhibit different

degrees of ossification within the fibrous connective tissue stroma. To name a few, benign fibro-osseous lesions include soft tissue osteomas, central and peripheral giant cell granulomas, osteomyelitis, and osteosarcomas. Other examples are fibrous dysplasia, cemento-osseous dysplasia, and ossifying fibroma [23].

Histological examination is crucial for biological research and disease diagnosis. The examination exposes the state of cells and tissues and allows us to deduce the general condition of the individual as well [4]. The hematoxylin and eosin staining (HE) method of tissue staining is one of the most common methods of evaluating the condition of cells and tissues. Routine histological techniques like hematoxylin and eosin (H & E) for decalcified bone sections may not clearly identify osteoid from calcified bone, woven bone, and lamellar bone; additionally, both calcified and soft tissue may resemble the same shade of pink [5,6]. Early calcifications are frequently overlooked, and it can be challenging to tell the difference between the organic matrix of calcified tissue and soft tissue [7]**.** Another difficulty is the misdiagnosis of bone vs cementum-like tissue [8].

Over the years, various connective tissue stains have been developed and utilized to distinguish between the stroma's hard and soft tissue components. Among the stains identifying stromal hard tissues are Von Kossa, Masson's Trichrome (MT), solochrome cyanine, and silver staining before decalcification [9]. The tetrachrome technique makes use of the idea that aging, primary and secondary mineralization, and structural and chemical changes in bone tissue are all interrelated. Changes allow for differential staining even after decalcification and distinguish bone matrix from the initial non-mineralized osteoid.

Differential staining has been accomplished using the tetrachrome approach by carefully mixing phosphotungstic acid with dyes. Another advantage of using the technique is its ease of use, low cost, lack of particular equipment requirements, ability to stain large sections, and ability to be used in conjunction with polarized light to show the collagenous matrix's underlying lamellar and woven structure [7,9]. The MT stain is primarily used to stain collagen, and as the name suggests, three dyes are used to stain different tissues selectively. The porosity of the tissue influences the staining procedure because the smallest dye molecule colors less porous tissue [10].

Sarasquete and Gutiérrez changed the original trichromic hematoxylin-Gutiérrez' Verde Luz-orange G-acid fuchsin (VOF) stain to tetrachromic VOF stains to more readily differentiate diverse hard tissue components. The tetrachromatic Verde Luz-orange G-acid Fuchsin (VOF) stain is a promising approach for identifying the components of hard and soft tissues. It is a dye combination including orange G, acid fuchsin, light green, and methyl blue that selectively stains distinct tissue components [11]. The current study compares the efficacy of modified Masson Trichrome staining against modified VOF staining to aid in the identification of histological characteristics of hard tissue pathologies.

MATERIALS AND METHODS

The archives of the Department of Pathology were searched to identify 18 tissue blocks with known histopathological diagnoses (decalcified bone samples). Two sections from each block were cut using a soft tissue microtome for staining. This study was conducted following the approval of the Institutional Human Ethical Committee under the reference number IHEC/SDC/UG-2178/24/GPATH/111.

For the VOF stain preparation, 260 mg of light green, 140 mg of methyl blue, 500 mg of orange G, and 600 mg of acid fuchsin were dissolved in 100 mL of boiling distilled water. After allowing the solution to cool completely, 1.5 g of phosphotungstic acid and 3 mL of glacial acetic acid were added. Then, 200 mL of pure ethanol was mixed into the solution. The VOF stain was now ready for use.

Harris's Hematoxylin stain was prepared by dissolving 2.5 g of hematoxylin in 25 mL of pure alcohol. Separately, 50 g of potassium alum was dissolved in 500 mL of warm distilled water in a 2-liter flask. The hematoxylin solution was then combined with the potassium alum solution in the flask, and the mixture was brought to a boil quickly. Gradually, 1.25 g of mercuric chloride or 0.5 g of sodium iodate was added to the boiling liquid. The stain was rapidly cooled by immersing the flask in cold water or a sink filled with chipped ice. Finally, 20 mL of glacial acetic acid was added to the cooled stain, making it ready for immediate use.

To prepare the Eosin solution, a 0.5% or 1% solution of Eosin Y was made in distilled water. A crystal of thymol was added to the Eosin solution to prevent fungal growth. Additionally, a small amount of acetic acid (0.5 mL per 1000 mL of stain) was added to enhance the sharpness of the staining.

Xylene is used to deparaffinize the tissue slices for 20 minutes. Xylene is a typical solvent for removing

paraffin wax from tissue slices, which allows the stains to permeate the tissues. After that, the deparaffinized pieces are rehydrated using a succession of alcohol solutions. For about 10 minutes, the slides are immersed in decreasing concentrations of alcohol (e.g., 100% alcohol, 95% alcohol, and 70% alcohol). Rehydration aids in the removal of any residual xylene and prepares the tissue for staining. Following rehydration, the portions are cleaned for 3-5 minutes under running tap water. This procedure eliminates any remaining alcohol and prepares the tissue for the subsequent staining phase. For 5 minutes, the slices are stained with Harris' hematoxylin. Hematoxylin is a natural pigment that attaches to cell nuclei, allowing them to be seen under a microscope. The nuclei are stained blue in this stage. The slides are briefly bathed in acid alcohol to distinguish the stained nuclei from the background. Acid alcohol is a combination of alcohol and hydrochloric acid that aids in the removal of superfluous hematoxylin from tissue, leaving only the stained nuclei. The slides are then briefly immersed in ammonia. Ammonia helps to "blue" the hematoxylin-stained nuclei, increasing contrast and clarity. The slides are then placed in the VOF stain for 5 minutes. The VOF stain is a solution-based mixture of light green, methyl blue, and orange G dyes. This stain gives varied colors to different tissue constituents and aids in the visualization of diverse structures. Following VOF staining, the slides are dehydrated in 80% ethanol and absolute ethanol. The slides are dehydrated by soaking them in increasing concentrations of alcohol to eliminate water from the tissue sections and prepare them for mounting. Instead of employing the VOF stain, another batch of 15 slides can be transferred to eosin for a single dip. Eosin is a red dye that emphasizes cytoplasmic structures and collagen fibers in tissues, allowing for more detailed inspection.

After staining with eosin (if desired), the slides are dehydrated in successive grades of alcohol to eliminate water and excess dye. After that, the slides are cleaned with xylene. Clearing chemicals such as xylene aid in the transparency of tissue slices, allowing them to be mounted. The slides are mounted using DPX, a typical mounting media used to preserve stained tissue slices under a coverslip for microscopic analysis. The commercially available Mason trichrome stain kit (Merk) was used for staining the slides. The slides were washed in increasing grades of alcohol and two observers examined the slides under the microscope and graded the intensity, clarity, translucency, and contrast of the two stains. The grading was tallied, and statistical analysis was performed with SPSS 20 software. To assess interobserver agreement, an independent t Test and Kappa statistics were utilized.

RESULTS

When the MMT stain and VOF stain were compared based on staining parameters such as mean background (p-value = 0.762), mean morphology (p-value = 0.421), mean nuclear characteristics (p-value = 0.220), mean staining (p-value = 0.010) and mean differentiation, the VOF stain was shown to be significantly more effective than the MMT stain (Refer Table 1). Even when comparing hard tissue samples under a microscope, VOF stain enabled a clear visualization and identification of stained components such as mature bone and immature trabecular bone by producing vivid and distinct colors. Mean morphology typically pertains to the average morphological characteristics or features observed in stained cells or tissues. This includes the shape, size, and structural properties of the stained structures, such as cells or organelles (Figure1and 2).

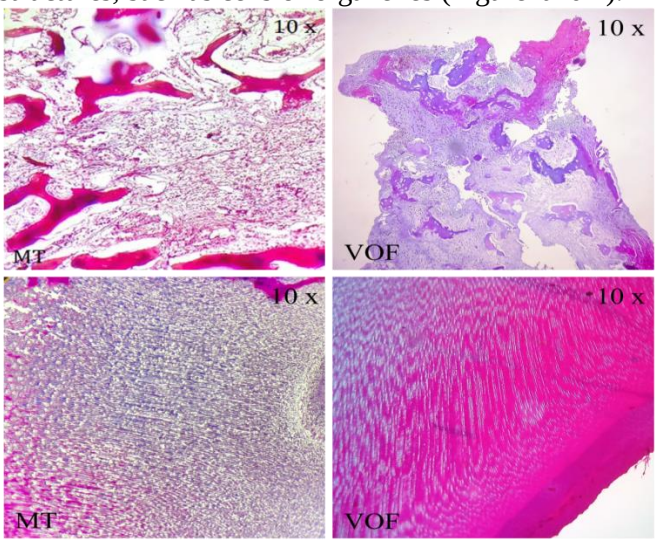


Fig 1. Comparing the features between different stains. The mean background intensity (A), nuclear stain (B), morphology (C), staining characteristics (D), and differentiation (E) of the VOF stain are better than the MMT stain.

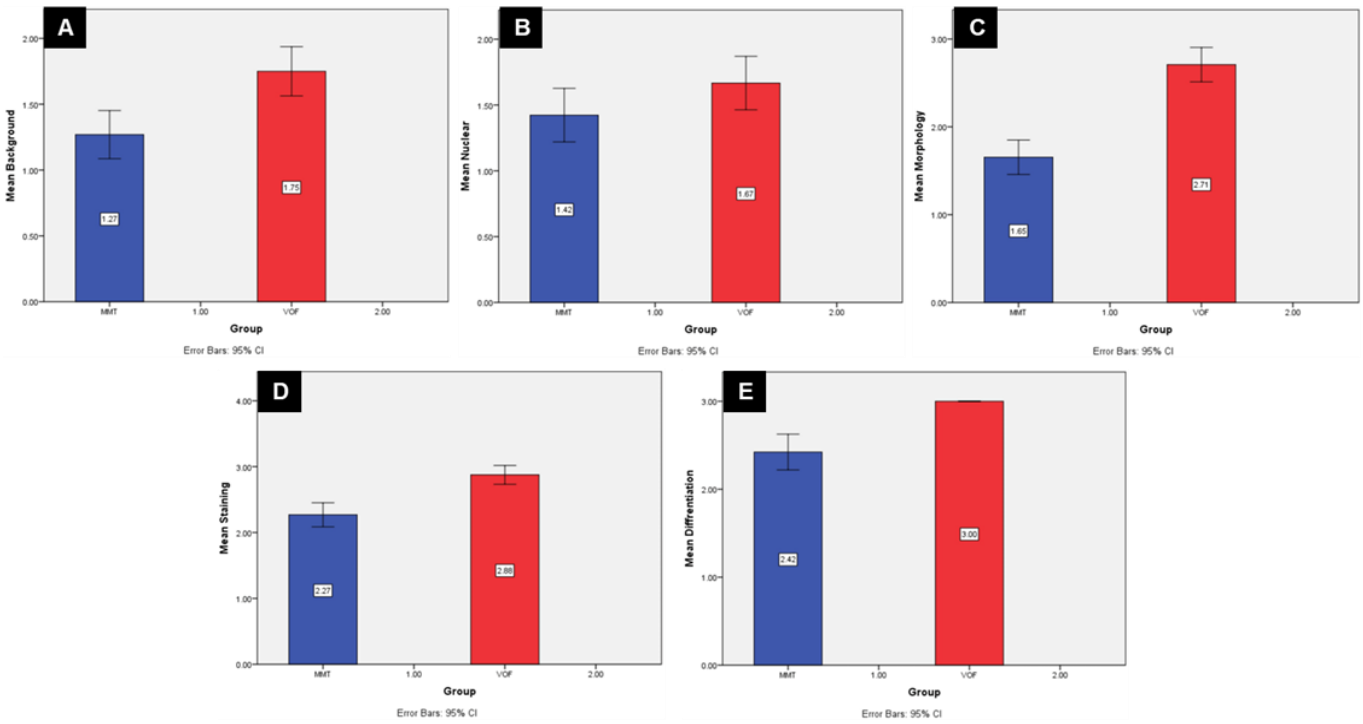


Figure 2. Photomicrograph of a hard tissue sample stained with modified Masson's Trichrome (A). It demonstrates clear and visible differentiation of fine and coarse collagen fibers and their arrangement and it provides contrasting and distinguishable features, allowing examination of tissue architecture, and collagen distribution. Photomicrograph of the hard tissue sample stained with modified VOF staining (B). It provides vivid and distinct colors, allowing for clear visualization and identification of the stained structures. It also provides good contrast between the stained components and the background, making it easier to observe and analyze the targeted structures.

Table1. Comparing the characteristic properties of the stains

Characteristics		**Mean ± Std Deviation**	**p Value**
Background	MMT	1.26 ± 0.45	0.762
	VOF	1.75 ± 0.44	
Morphology	MMT	1.65 ± 0.48	0.421
	VOF	2.70 ± 0.46	
Nuclear characteristics	MMT	1.42 ± 0.50	0.220
	VOF	1.66 ± 0.481	
Staining	MMT	2.26 ± 0.452	0.010
	VOF	2.87 ± 0.33	
Differentiation	MMT	2.42 ± 0.50	0.00
	VOF	3	

DISCUSSION

The study's findings revealed that VOF stain is superior to MMT stain in terms of its characteristic staining qualities and differentiates the stained structures of the sample better than MMT stain. Bone development in various skulls and lesions might be mature or immature. Immature bone has a larger percentage of osteocytes than mature bone. It has two subtypes: woven bone and coarsely bundled bone [12].

Coarsely bundled bone (immature bone) is distinguished from woven bone (immature bone) by the presence of thick collagen bundles, the majority of which are oriented parallel to one another with osteocytes in between. However, with H and E staining, the distinction may not always be evident [12,13]

Mature bone differs from immature bone in several microscopic ways, including 1) a relatively even acidophilic staining of its matrix, 2) a relatively regular arrangement of its lamellae, and 3) the presence of fewer, more evenly distributed osteocytes in flatter lacunae. However, it may be challenging to categorize hard tissues using such a detailed description because the calcified components may manifest at different phases of maturity [14,15]. As a result, bone and other hard tissue components, as well as soft tissues, are eosinophilic in typical H and E-stained sections, making differentiation difficult [16]. For simple identification, a dye combination can stain hard and soft tissue structures differentially. In these cases, trichrome and tetrachrome staining techniques are advantageous.

JM Tater et al employed histochemical staining with hematoxylin and eosin (H&E) and tetrachromatic Verde Luz-orange G-acid fuchsin (VOF) to distinguish between mature and immature (osteoid production) in ossifying fibroma [17]. After H&E staining, bone and osteoid in ossifying fibromas showed varying degrees of pink staining, as well as soft tissue components including collagen. Collagen was dyed blue by VOF, while osteoid and bone were stained purple-red. The blue, purple, and red VOF staining made it simpler to detect structures inside peripheral and central ossifying fibromas. In addition, H&E staining requires many stages, whereas VOF requires only one [18].

Soft tissue stroma in Masson's trichrome commonly merges with calcification colors while being distinct over H and E and staining in the same slightly different shades of blue, green, and red. However, if the bone is sparse, it becomes challenging to identify mature bone in Masson's trichrome because the mineralized bone takes on the same tones of color as the stroma of the surrounding soft tissue [19].

H&E, Masson's Trichrome, and Modified Gallego's Stains were compared in a research by P Singh et al. to distinguish between healthy and diseased hard tissues. The results were 75, 50, and 45% for MG, MT, and H&E, respectively [20]. The results showed that while H&E stain is often used for regular histopathology exams and provides an overall evaluation of tissue form, MMT stain is particularly useful for assessing collagenous connective tissues and fibrosis [21,22]. A multi-step procedure called MMT stain is used to selectively stain distinct tissue components using a variety of dyes. Muscle fibers turn red, nuclei turn black or brown, keratinized tissues turn black or brown, and collagen fibers turn blue and hard tissue diseases such fibro-osseous lesions and others that are challenging to identify, the MG stain may be a viable differential stain. The MG stain helps clinicians identify ambiguous lesions, determine the type and etiology of lesions, and distinguish between aggressive and nonaggressive lesions. This information helps them comprehend the biological activity of the lesions and develop effective treatment plans [23].

Several investigations have demonstrated that Modified VOF staining uses only one step and takes less time than H and E and Masson's trichrome staining, and it is also less expensive [24]. It stains all hard tissue components, including bone, cementum, and dystrophic deposits, and differentiates them from stromal components. The stain's efficiency is further supported by the fact that the smallest calcified deposits pick up the stain brilliantly against the pale blue collagen background, interpreting it as considerably simpler. Degenerating bone and tumor osteoid also have a distinct staining pattern [24,25]. Because VOF stain appears to be reliable in recognizing hard tissue components of stroma, it may be used to determine the nature of calcified deposits in all suspected cases of normal/abnormal calcifications. This would certainly help with pathology treatments.

CONCLUSION

The study compared the efficacy of Modified VOF staining and Modified Masson Trichrome staining in evaluating hard tissue pathologies. The results showed that Modified VOF staining outperformed Modified Masson Trichrome staining across several

key parameters, including background clarity, morphological detail, nuclear feature definition, overall staining quality, and differentiation. Modified VOF staining provided superior contrast and translucency, making it easier to distinguish between different tissue components. This enhanced clarity is particularly beneficial for accurately identifying and diagnosing various hard tissue pathologies.

Furthermore, the Modified VOF stain demonstrated a higher mean staining intensity and better differentiation of tissue types, which are crucial for precise histological analysis. These attributes make Modified VOF staining a more reliable and effective method for examining decalcified bone samples and other hard tissues. Given its advantages in terms of clarity, contrast, and overall staining quality, Modified VOF staining should be considered the preferred technique for histopathological evaluation of hard tissue lesions.

In conclusion, this study highlights the superiority of Modified VOF staining over Modified Masson Trichrome staining for hard tissue pathology. The findings suggest that adopting Modified VOF staining in clinical and research settings could improve the accuracy and reliability of histological evaluations.

DECLARATIONS

Conflicts of interest and financial disclosures

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Ethical Approval

The ethical clearance was obtained from the Institutional Ethical Review Committee

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ORIGINAL ARTICLE

EVALUATING BONE MINERAL DENSITY AND TRABECULAR BONE MICROSTRUCTURE AROUND IMPLANTS BY CBCT

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ABSTRACT

Aim: The purpose of this study is to evaluate Bone Mineral Density (BMD) by Hounsfield Units (HU) and Trabecular Bone microstructure by Fractal Dimension (FD) around dental implants pre-operatively and post operatively by CBCT.

Materials and Methods: Twelve patients with partially edentulous sites of age group between 20 to 50 years with good systemic health were enrolled. Patients are assessed for BMD before (baseline) and after placement of implants (9 months) using analysis by Hounsfield Units (HU) and FractalDimension (FD).

Results: BMD by HU improved from baseline (412.25 ± 105.16) to 9 months (626.58 ± 114). Trabecular Bone Microstructure by FD improved from baseline (0.78 ± 0.06) to 9 months (1.40 ± 0.05). Unpaired t test and paired t test were performed for inter groupand intra group analyses respectively.

Conclusion: Present study was undertaken to evaluate bone changes on implants placed in the suitable edentulous sites. All the implant Osseo integrated without any uneventful healing. The marginal bone loss found was also minimal and found to be statistically non-significant. With the limited sample size it can conclude that fractal analysis holds good for bone architectural analysis.

Key words: Bone Mineral Density, Trabecular Bone Microstructure, Hounsfield Units, Fractal Dimension, Cone Beam Computed Tomography.

INTRODUCTION

Dental implants have become a predictable treatment option for restoring missing teeth. Provides adequate function and esthetics without affecting adjacent hard and/or soft tissue structures. A dental implant is a titanium screw which is placed into bone to replace missing teeth. The implant mimics the root of a tooth in function. It is not only biocompatible, but actually fuses to bone by osseointegration. Osseointegration is a direct structural and functional union between living bone and surface of the load carrying implant.[1] Traditional radiographs like periapical and panoramic

radiography provide adequate information along with neighboring vital structures that must not be violated. However, these radiographic modalities provide a two-dimensional (2D) representation of three-dimensional (3D) structures. Their limited film size, image distortion, magnification, and 2-D view restrictttheir use in some cases. In an effort to overcome this limitation, Cone Beam Computed Tomography (CBCT) has become available for 3D visualization of the craniofacial complex.[2]

CBCT is an essential tool for treatment planning and post-procedure monitoring. By providing highly accurate 3-D images of the patient's anatomy from a single, low-radiation scan, CBCT technology delivers a comprehensive understanding of the patient's jaw and the anatomical structures that provides accurate treatment planning.[3]

Bone Mineral Density (BMD) is the amount of bone tissue in a certain volume of bone. Assessment of jaw BMD may be considered useful in implant planning (Gulsahi et al., 2010).[4] Clinical studies show greater implant survival & osseointegration in mandible than maxilla. This survival is limited by bone quality, i.e. bone density.[5] Bone mineral density is lower in maxillary when compared to mandibular Bone Mineral Density. HU value was used to assess the bone density on the implant site, and the standard value of jaw bone density varies from one individual to other.[6]

Fractal analysis is based on fractal mathematics for describing complex shapes and structural patterns. It indicates a figure's complexity and expressed as "Fractal Dimension" (FD), which measures self-similarity. Trabecular bone has branching pattern that exhibits fractal properties. Therefore, when applied to trabecular bone images on radiographs, this method can be considered as a reflection of trabecular bone micro architecture.[7] In fractal analysis, a box-counting algorithm is mainly used to quantify the trabecular pattern by counting the trabecular bone and bone marrow interface.[8]

MATERIALS AND METHODS

STUDY DESIGN

The objective of this study is to evaluate modalities to measure the peri- implant changes i.e. BMD by HU and Trabecular bone microstructure by FD. The peri- implant changes were assessed using CBCT at baseline and 9 months.

Clinical parameters such as Bone Mineral density, Trabecular Bone Microstructure were assessed at baseline and 9 months. Approval of the study was obtained from the ethical committee of St. Joseph Dental College and an informed consent was taken from all participants before commencement of the study.

STUDY POPULATION

The study population included patients who reported to the department of Periodontics St Joseph dental college during the period April 2017 to April 2018.

INCLUSION CRITERIA

- Patients in the age group of 20 to 45 years.
- Partially edentulous patients.
- Patients showing compliance to maintain good oral hygiene.
- Good systemic health.

EXCLUSION CRITERIA

- Patients with known systemic disease.
- Patients on medication and antibiotic therapy since last 6 months.
- Patients with psychiatric disorders.
- Pregnant or lactating women.
- Smokers.
- Teeth with occlusal interferences and restorations.
- Poor oral hygiene and mobility.

PRE-TREATMENT RECORDS

- Detailed medical and dental history.
- Routine blood investigations.
- Periodontal assessment using clinical parameters.
- Diagnostic casts.
- Periapical, panoramic radiographs and CBCT scan whenever required.
- Clinical photographs (fig1,2).



Figure 1. Pre Operative scaling



Figure 2. Post Operative scaling

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CLINICAL PARAMETRS

- Hounsfield Units
- Fractal Dimension(fig.3,4).

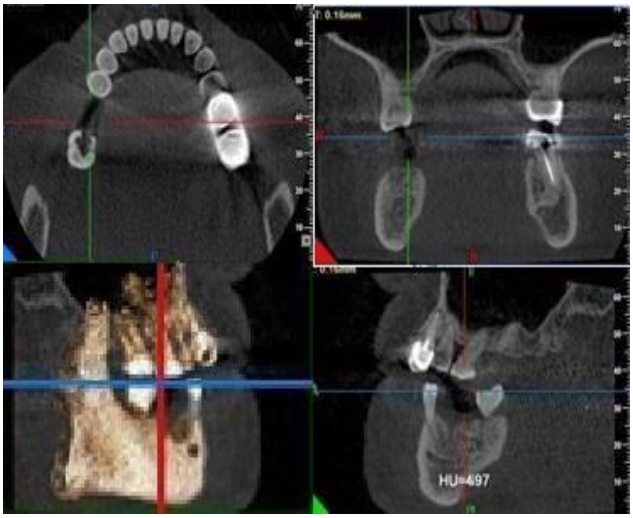


Figure 3. Pre-Operative Hounsfield Units (HU) Value

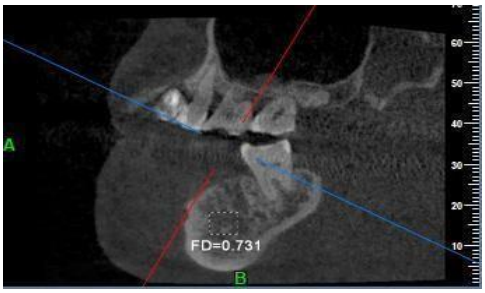


Figure 4. Pre-Operative Fractal Dimension Units(FD

PRESURGICAL PROCEDURE

Prior to commencement written consent was taken. Following initial examination and treatment planning, the selected patients underwent phase 1 therapy. Detailed instructions and plaque control measures were given. Two weeks after phase 1 therapy, patients were subjected to the surgical procedure. Selected patients underwent routine blood investigations and radiographs of the area of interest were taken. On completion of baselineexamination and initial therapy, dental implants were placed at the edentulous sites.

IMPLANT MATERIALS

- Self-tapered threaded implants were used.
- Minimum dimensions - 3.3mm in diameter and 9.5mm long. Maximum dimensions - 4.5mm in diameter and 11mm long.
- Crown placed- metal ceramic.

SURGICAL PROCEDURE

Local anesthesia (1:80000 lignocaine) is administered at the site where implant is tobe placed.The sequenceof implant placement is(fig.5-8)-

1. Initial marking of the implant site with a round bur.
2. Use of a 2-mm twist drill to establish depth and align the implant.
3. Guide pin is placed in the osteotomy site to confirm position and angulation.
4. Pilot drill to increase diameter of the coronal aspect of the osteotomy site.
5. Final drill used is the 3-mm twist drill to finish preparation of the osteotomy site.
6. Countersink drill is used to widen the entrance of the recipient site and allow forthe sub crestal placement of the implant collar and cover screw.
7. Implant is inserted into the prepared osteotomy site with a handpiece.
8. The cover screw is placed and soft tissues are closed with 3-0 suture.



Figure 5. Mid crestal incision given



Figure 6. Full thickness mucoperiosteal flap elevatedand Osteomy site prepared



Figure 7. Implant placement done

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Figure 8. Mucoperiosteal flap approximation doneusing silk sutures

POSTOPERATIVE CARE

All patients were prescribed Amoxicillin 500 mg thrice daily for 5 days and a combination of Ibuprofen 400mg and Paracetamol 325mg thrice daily 3days. They were also instructed to rinse with 10 ml of Chlorhexidine gluconate (0.2%) mouthwash twice daily for two weeks.

Postoperative instructions given are as follows:

1. For first 3 hours after operation, avoid taking hot liquids for the first 24 hours.
2. Do not brush over the surgical site.
3. During the first day, apply ice pack intermittently on face over the operated area.
4. If unusual pain or bleeding from the operated sites seen, asked to report to the hospital immediately.

POSTSURGICAL PROTOCOL

Patients were recalled 24 hours after the surgery to evaluate signs of postoperative complications. After 7 days sutures were removed and the area was irrigated with saline. The patients were advised to use extra-soft brushes for mechanical plaque control to maintain good oral hygiene. The patients were again instructed to rinse with chlorhexidine mouthwash twice daily for another one week. All the 30 patients were kept under a recall program for loading after 9 months.

PROSTHODONTIC PHASE

In both the groups, delayed loading was done. Cover screws were removed and healing abutments were placed after 3 months in the mandibular implants and after 6 months in the maxillary implants. After 15 days of placement of the abutments, abutment level closed tray putty impressions were taken and given for crown fabrication (fig 9-12).



Figure 9. Healing abutments placed



Figure 10. Prosthesis place



Figure 11. 9 Months Post Operative Hounsfield

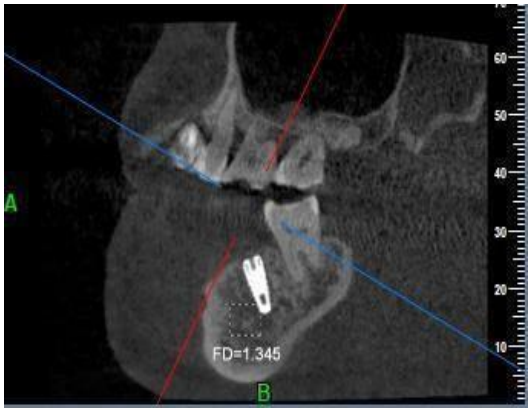


Fig: 12 9 Months Post Operative Fractal Dimension Units (FD)

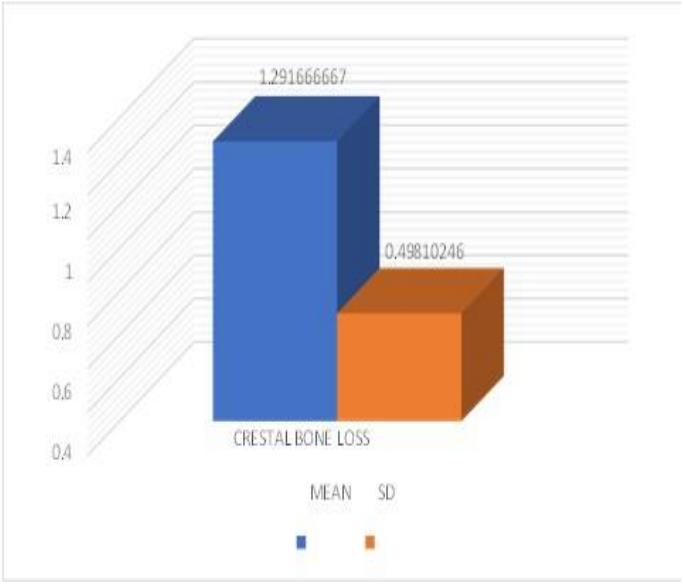
RESULTS

The mean crestal bone loss at base line was 4.5 ± 0.2 and the mean crestal bone loss at 9 months was 5.2 ± 0.4. When the pre operative mean was compared with 9 months post operative mean, it yielded a p value of 0.09, which was not statistically significant (P > 0.05).

Table 1. Mean, standard deviation and test of significance for crestal bone loss at baseline and 9 months post operatively.

	BASELINE		9 MONTHS		BONE LOSS OCCURED		P value
CRESTAL BONE LOSS	MEAN	SD	MEAN	SD	MEAN	SD	
	4.5	0.223	5.2	0.425	0.7	0.202	0.09

Statistical Analysis: Paired t Test. P value was > 0.001 meaning statistically non- significant



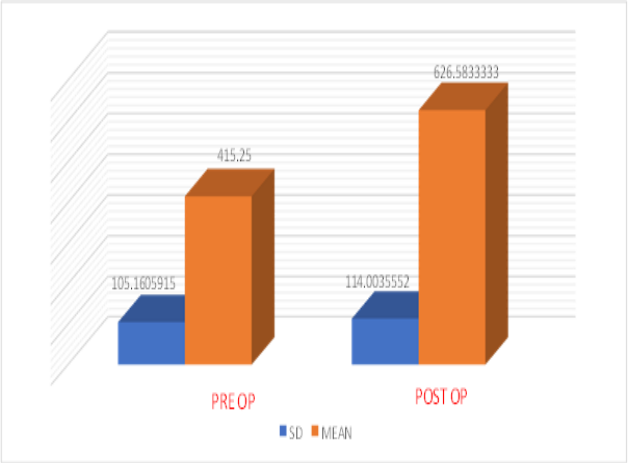
GRAPH 1. Mean crestal bone loss at 9 months post operatively

Table 2. Mean, standard deviation and test of significance for Bone Mineral Density by Hounsfield Units at baseline and 9 months post operatively.

	BASELINE		9 MONTHS		Mean Diff from pre op & 9months		P value
HOUNSFIELD UNITS	MEAN	SD	MEAN	SD	MEAN	SD	
	415.253	105.1606	626.583	114.003	211.33	8.8424	0.004 ** S

Statistical Analysis: Paired t Test. **S: Statistically highly significant.

Inference- The mean bone mineral density (HU) at base line was 412.25 ± 105.16 and the mean bone mineral density (HU) at 9 months was 626.58 ± 114. When the pre operative mean was compared with 9 months post operative mean, it yielded a p value of 0.004, which was statistically significant (P < 0.05).



GRAPH 2. Comparison of mean Bone Mineral Density by Hounsfield Units at baseline and 9 months post operatively

Table 3. Mean, standard deviation and test of significance for Trabecular bone microstructure by Fractal Dimension at baseline and 9 months post operatively.

	BASELINE		9 MONTHS		Mean Diff from pre op & 9months		P value
HOUNSFIELD UNITS	MEAN	SD	MEAN	SD	MEAN	SD	
	415.253	105.1606	626.583	114.003	211.33	8.8424	0.004 ** S

Statistical Analysis: Paired t Test. **S: Statistically highly Significant.

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Inference- The mean Trabecular Bone Microstructure (FD) at base line was 0.78 ± 0.06 and the mean Trabecular Bone Microstructure (FD) at 9 months was 1.40 ± 0.05.When the pre operative mean was compared with 9 months post operative mean, it yielded a p value of 0.001, which was statist ically significant (P < 0.05).

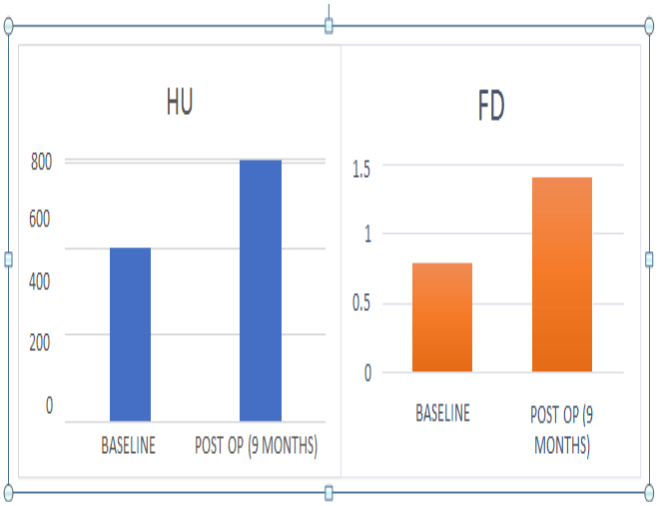


GRAPH 3. Comparison of mean Trabecular bone microstructure by Fractal Dimension at baseline and 9 months post operatively.

Table 4. Mean, standard deviation and test of significance for Bone Mineral Density by Hounsfield Units and Trabecular bone microstructure by Fractal Dimension at baseline and 9 months post operatively.

	BASELINE		9 MONTHS		P value
	Mean	SD	MEAN	SD	
HOUNSFIELD UNITS(HU)	415.253	105.1606	626.583	114.003	0.004 * S
FRACTAL DIMENSION (FD)	0.781917	0.068182	1.40125	0.058518	0.001 ** S

Inference-The mean bone mineral density (HU) at base line was 412.25 ± 105.16 and at 9 months was 626.58 ± 114. When the pre operative mean was compared with 9 months post operative mean, it yielded a P value of 0.004, which was statistically significant (p < 0.05). The mean trabecular bone microstructure (FD) at base line was 0.78 ± 0.06 and at 9 months was 1.40 ± 0.05.When compared it yielded a P value of 0.001, which was statistically significant (P < 0.05). In comparison, Fractal Dimension Analysis showedhigh significance than Hounsfield units.



GRAPH 4: Mean inter group comparison of Bone Mineral Density by HU and Trabecular bone microstructure by FD at baseline and 9 monthspost operatively.

DISCUSSION

The Implant-Tissue interface is an extremely dynamic region of interaction. The process of osseointegration involves an initial interlocking between alveolar bone and the implant body and later, biological fixation through continuous bone apposition andremodelling. This entire process is followed by changes in Bone Mineral Density and Trabecular bone microstructure. Cone Beam Computed Tomography (CBCT) is an essential 3-dimensional tool for measuring the bone mineral density. It is measured in terms of Hounsfield units.

Trabecular bone microstructure which is measured in terms of fractal dimensions is a method for describing complex shapes, structural pattern and also to estimate microstructure of bone tissue and trabecular pattern and is expressed numerically as fractal dimension. The debate on which method (BMD or FD) is accurate in recording the changes has always been a matter of concern for periodontists when placing implants.

Thus, the purpose of this study was to compare and evaluate Bone Mineral Density (BMD) by Hounsfield Units (HU) and Trabecular Bone microstructure by Fractal Dimension (FD) analysis around implant cases pre-operatively and post operatively.

Bone mineral density, most important parameter, can be measured using hounsfield units.

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The units are based on a linear scale defined only by two points: the attenuation of dry air, set at) 1000 HU, and the attenuation of pure water, set at 0 HU.After the 9 months follow-up it was found that bone mineral density (HU) at base line was 412.25 ± 105.16, while at 9 months was 626.58 ± 114. The bone mineral density increased considerably after implant placement which states the physiological changes that have occurred in the bone to be healthy.

The mean bone density was highest in the anterior mandible followed by the anterior maxilla, the posterior mandible, and the posterior maxilla. The mean bone density recorded in the present study was higher than the studies reported previously by Y. Hao et al. 2014[9], this could be because of the physiological differences between the living bone and the cadaver bone. This increase in bone mineral density is due to the reactive bone formation that occurs around the implant for Osseo- integration. It was also stated by Farre-Pages et al 2010 that HU can be used as a diagnostic parameter to predict possible implant stability. The results in the present are in correlation with the studies performed by Kyou Hiasa et al. in 2016, Stoppie et al. 2011, Farre-Pages et al. 2010 and L.J. Fuh[10] 2010.

Fractal Dimensions has some advantages when compared with hounsfield units such as lower radiation dose, shorter acquisition time, and reduced costs. HU should be better understood as “relative” density rather than “true” density. This relative density is determined by CT numbers, which represent the total X-ray attenuation of different body tissues. Thus, the comparative evaluation between bone mineral density (HU) and trabecular bone microstructure was considered. The trabecular bone micro structure (FD) at base line was 0.78 ± 0.06 and at 9 months was1.40 ± 0.05. The trabecular bone micro structure has also improved from baseline to 9 months after implant placement indicating osseointegration. The results are in the accordance studies done by Bollen et al., 2013[11]. Bone quality can improve around a functional osseointegrated dental implant due to the positive bone stimulation, the more bone that is present at an implant site, the better the possibility for implant success. Mechanical injury to bone results in an increase of its volume and density as stated by Yan Hua 2008[12].

This effect, however, as stated by Branemark et al. 1964 and Slotte et al. 2003. Hounsfield units and Fractal dimensions could record physiological changes occurring in the bone around implant. HU represents the relative bone density of the jaw bone in a calibrated gray-level scale. In accordance to the studies done by *Y. HAOet al 2014* stated that the anterior mandible bone density (HU) > anterior maxilla (HU) and posterior mandible (HU) > posterior maxilla.

In accordance to the studies done by Yan Hua 2008 stated thatfractal analysis is one of the most suitable method for texture analysis. Beam hardening and heel effect are the two phenomenon that gives the accurate results of trabecular bone microstructure by fractal analysis. Beam hardening is a phenomenon resulting from the increase of mean energy of the X ray beam when passes through an object. Heel effect is a phenomenon that causes a angular distribution of the X ray beam intensity. Trabecular bone micro texture analysis thus plays a vital role in bone quality assessment by micro architectural composition. However, further clinical trials with larger sample size and longer duration are required to determine the study.

CONCLUSION

In conclusion the present study was undertaken to evaluate bone changes on implants placed in the suitable edentulous sites. All the implants have osseointegrated without any uneventful healing. The oral hygiene measures followed properly showed successful implants without peri-implantitis. The marginal bone loss found was also minimal and found to be statistically non- significant. With the limited sample size it can concluded that fractal analysis holds good for bone architectural analysis. However, the study has certain limitations. Limited number of patients and shorter follow up period may have contributed to the lack of any detectable significance between the groups. Longer follow up with larger sample size is necessary to study the efficiency of the material in attaining a predictable treatment outcome.

DECLARATION

Ethics approval

Obtained from the St.Joseph Dental College and Hospital, CEC/20/2018-19 **Consent to participate:** Obtained from all the study participants

Patient consent for publication

Obtained

Acknowledgments

Not applicable

Conflicts of interest and financial disclosures

The author declares that he has no conflict percent and there was no external source of funding for the research in question

Sources of Funding

This research received no external funding (self-funding).

Availability of data and materials

From the Institution

Clinical trial registry number

Not applicable

Prospero registry number

Not applicable

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ORIGINAL ARTICLE

CLINICO-PATHOLOGICAL COMPARISON OF SALIVARY TNF-α AMONG MENOPAUSE WOMAN WITH AND WITHOUT PERIODONTITIS - A SHORT STUDY

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Abstract

Background: Menopause is associated with significant hormonal changes that can influence systemic and oral health, including an increased risk of inflammatory conditions such as periodontitis. Tumor Necrosis Factor-alpha (TNF-α) is a key pro-inflammatory cytokine involved in periodontal disease progression and has been detected in saliva, serving as a potential biomarker for disease severity.

Objectives: To determine the association between elevated salivary TNF-α levels and periodontitis in menopausal women.

Materials and Methods: An in vivo cross-sectional study was conducted on 40 menopausal women, divided into two groups: Group I (with periodontitis; n=20) and Group II (without periodontitis; n=20). Saliva samples were collected using randomized sampling, and TNF-α levels were quantified using enzyme-linked immunosorbent assay (ELISA). Data were analyzed using SPSS software, with Independent t-tests employed for group comparisons; significance was established at $p < 0.05$.

Results: All participants exhibited detectable salivary TNF-α levels. Group I demonstrated significantly higher TNF-α concentrations with Mean ± SD of 20.48 ± 3.10 compared to Group II with 11.32 ± 1.74, with the difference being statistically significant ($p = 0.001$). These results revealed the association between elevated TNF-α levels in periodontitis in menopausal women, reflecting the combined impact of hormonal changes and local inflammation.

Conclusion: Elevated salivary TNF-α levels in menopausal women with periodontitis suggest that TNF-α could serve as a potential biomarker for periodontal inflammation influenced by estrogen deficiency. The findings contribute to a better understanding of the interplay between systemic hormonal alterations and local inflammatory responses in periodontal disease, warranting further research with larger cohorts and exploration of targeted therapeutic strategies.

Keywords: TNF-α, periodontitis, menopause, salivary biomarkers, inflammation

INTRODUCTION

Menopause is a significant physiological milestone in a woman's life, marked by the cessation of ovarian function and subsequent estrogen depletion. This hormonal change profoundly impacts various body systems, including the oral cavity. One of the lesser-known consequences of menopause is its association

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with oral health alterations, particularly the susceptibility to periodontal diseases.[1] Periodontitis, a chronic inflammatory condition that destroys the supporting structures of teeth, are influenced by both local and systemic factors, including hormonal fluctuations and immune responses. Among the immune-related mediators implicated in periodontitis, tumor necrosis factor-alpha (TNF-α) plays a pivotal role. TNF-α is a pro-inflammatory cytokine that regulates immune responses and contributes to the destruction of periodontal tissues by enhancing the recruitment of inflammatory cells, inducing osteoclastogenesis, and disrupting the balance of bone metabolism. Elevated levels of TNF-α have been detected in various bodily fluids, including saliva, in individuals with periodontitis, highlighting its potential as a biomarker for periodontal inflammation (Figure 1). [2,3]

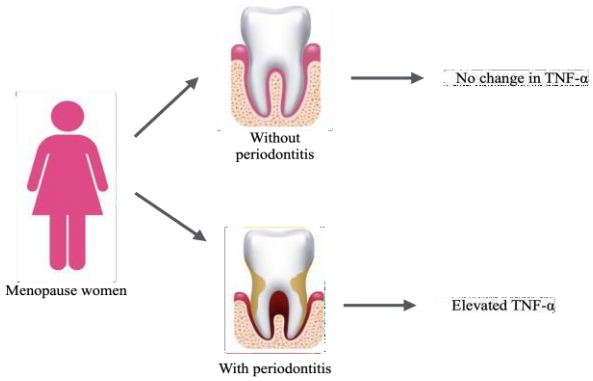


Figure1. Relationship between Menopause, Periodontal health, and Salivary TNF-α levels

Menopausal women are particularly vulnerable to periodontal diseases due to estrogen deficiency, which exacerbates inflammatory responses and disrupts the protective role of estrogen in maintaining bone and soft tissue health.[4] Estrogen receptors are present in periodontal tissues, and their activation modulates inflammatory cytokine expression. A decline in estrogen levels, therefore, creates a pro-inflammatory environment, which may increase the risk and severity of periodontitis in menopausal women. Consequently, investigating the levels of inflammatory mediators such as salivary TNF-α among menopausal women with and without periodontitis can provide insights into the interplay between systemic hormonal changes and oral inflammatory conditions.[5,6]

Several studies have explored the association between menopause, systemic inflammation, and periodontal health. For instance, Chakrabarti et al. (2014) reported elevated levels of TNF-α in postmenopausal women with periodontitis, suggesting that hormonal changes influence the inflammatory milieu of periodontal tissues.[7]

Similarly, Feres et al. (2016) demonstrated that menopausal women exhibited a higher prevalence and severity of periodontitis compared to premenopausal women, which was correlated with increased systemic and local inflammatory markers .[8] Furthermore, salivary biomarkers, including TNF-α, as non-invasive tool for assessing periodontal disease activity in systemic conditions such as menopause.[9]

Despite the growing body of evidence linking menopause to periodontal inflammation, clinicopathological comparisons of inflammatory markers like TNF-α in saliva among menopausal women with and without periodontitis remain underexplored. Saliva offers a convenient and non-invasive medium for assessing systemic and local inflammatory states, making it an attractive option for clinical research.[10] Studies comparing salivary TNF-α levels in menopausal women with varying periodontal statuses can bridge the knowledge gap and aid in understanding the underlying mechanisms linking menopause, inflammation, and periodontal health.[11]

The interaction between menopause-induced hormonal changes, inflammatory mediators such as TNF-α, and periodontal health represents a critical area of research with significant clinical implications. This study aims to investigate the clinicopathological differences in salivary TNF-α levels between menopausal women with and without periodontitis. By examining the relationship between salivary TNF-α levels and periodontal status, this research seeks to elucidate the role of systemic hormonal changes in modulating inflammatory responses in periodontal diseases. Additionally, the findings may contribute to the development of targeted strategies for the prevention and management of periodontal diseases in menopausal women, thereby improving their overall oral and systemic health.

MATERIALS AND METHODS

Study Design

This in vivo cross-sectional study was conducted on saliva samples collected from menopausal women,

both with and without periodontitis. The study was non-invasive and designed to minimize inconvenience to participants. Ethical approval was obtained from the Scientific Review Board (IHEC/SDC/BDS/1977/01). Despite its strengths, the study had a limitation of a small sample size.

Study Setting

A total of 20 samples (n=20) were collected from patients coming to clinics at Saveetha Dental College and Hospitals who were divided into two groups Group I consists of menopause women with periodontitis n=10, Group II consists of menopause women without periodontitis n=10.

Randomized sampling ensured unbiased selection, and validation of samples was performed by an expert pathologist. All participants were from the Dravidian ethnic group of South India. Informed consent was obtained from all participants, ensuring anonymity and compliance with ethical standards.

Selection Criteria for Study Subjects

Participants included menopausal women diagnosed with periodontitis and those without the condition. Patients with systemic comorbidities or terminal illnesses were excluded.

Sample Collection

Unstimulated saliva samples (1 ml) were collected from participants into Eppendorf tubes. Samples were immediately stored at -20°C. Before analysis, the samples were thawed and centrifuged. Collection took place between August 2024 and October 2024.

Principle of the Test

The assay was based on an enzyme-linked immunosorbent assay (ELISA) using a competitive binding technique. Tumor necrosis factor-alpha (TNF-α) in the samples competed with horseradish peroxidase (HRP)-labeled TNF-α for binding to a human monoclonal antibody immobilized in the wells. The bound TNF-α was detected using a biotinylated anti-human TNF-α antibody followed by HRP-conjugated streptavidin. The substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added to develop color, which changed from blue to yellow upon the addition of Stop Solution. The intensity of the yellow color was measured at 450 nm.

Reagent Preparation

Reagents and samples were brought to room temperature (18-25°C) prior to use. Assay Diluent B was prepared as a 5-fold dilution with deionized water, while Assay Diluent A was used for sample dilution, with a suggested dilution range of 2-20 folds for standard preparation, a 50 pg/ml TNF-α standard was serially diluted using Assay Diluent A for serum/plasma samples, with Assay Diluent B serving as the zero standard. The Wash Buffer was prepared by diluting a 20X concentrate with deionized water to yield 400 ml of 1X Wash Buffer. The detection antibody and HRP-streptavidin were briefly spun, mixed gently, and diluted as required for the assay.

Assay Procedure

Reagents and samples were equilibrated to room temperature before use, and all standards and samples were run in duplicate. A volume of 100 µL of each standard and sample was added to the wells and incubated at room temperature for 2.5 hours with gentle shaking. The wells were then washed four times with 1X Wash Buffer, followed by the addition of 100 µL of biotinylated antibody to each well, which was incubated for 1 hour with gentle shaking. After another washing step, 100 µL of prepared Streptavidin-HRP solution was added and incubated for 45 minutes. Subsequently, 100 µL of TMB substrate was added and incubated in the dark for 30 minutes. The reaction was stopped with 50 µL of Stop Solution, and the absorbance was measured at 450 nm.

Statistical Analysis

Statistical analysis was performed using SPSS Version 23.0. Salivary TNF-α levels were calculated with independent t-test to compare the groups and $p<0.05$ was considered significant.

RESULTS

Prevalence of Salivary TNF-α Levels in Group I menopausal women with periodontitis and Group II menopausal women without periodontitis exhibited detectable levels of salivary TNF-α (pg/ml), with Group I showing a higher mean concentration of 20.48 ± 3.10 compared to 11.32 ± 1.74 in Group II (Table 1). There was a notable and statistically significant difference in TNF-α levels between the two groups. Women with periodontitis exhibited

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significantly elevated TNF-α levels compared to those without periodontitis, suggesting a potential link between elevated TNF-α and the presence of periodontitis. A statistically significant difference ($p < 0.05$) was observed between the groups, with higher salivary TNF-α levels detected in menopausal women with periodontitis compared to those without periodontitis (Figure 2).

Table 1. Table showing Mean ± SD of Salivary TNF-α of Menopause women

Group	Salivary TNF-α levels (pg/ml) (Mean ± SD)	P value
Association between Menopause women with Periodontitis	20.48 ± 3.10	P = 0.001*
Association between Menopause women without Periodontitis	11.32 ± 1.74	

** statistically significant*

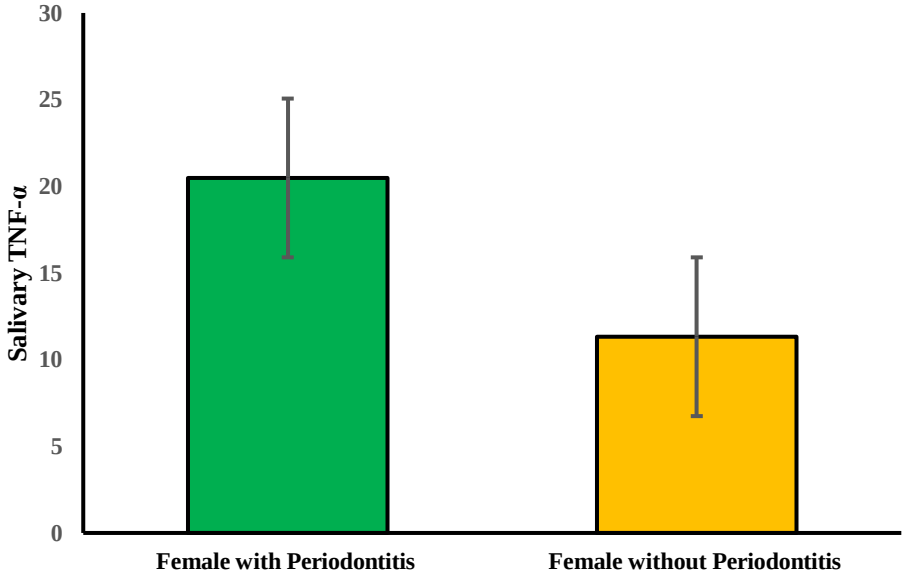


Figure 2. The bar graph illustrates the comparison of salivary TNF-α levels between women with and without periodontitis. The findings reveal that salivary TNF-α levels are higher in females with periodontitis compared to those without periodontitis, with error bars indicating standard deviations.

DISCUSSION

The present study aimed to compare salivary TNF-α levels between menopausal women with and without periodontitis. Results revealed a significantly higher concentration of salivary TNF-α in menopausal women with periodontitis (20.48 ± 3.10 pg/mL) compared to those without periodontitis (11.32 ± 1.74 pg/mL), with a *p*-value < 0.05. These findings are consistent with previous studies that highlight TNF-α as a key pro-inflammatory cytokine involved in periodontal pathogenesis and its exacerbation under systemic conditions such as menopause. Several studies in recent years have corroborated the role of TNF-α in both menopause and periodontal diseases. Sharma et al. (2020) reported elevated TNF-α levels in postmenopausal women with periodontitis, suggesting that estrogen depletion exacerbates periodontal inflammation by increasing pro-inflammatory cytokines.[12] Similarly, Rai et al. (2021) emphasized that reduced estrogen levels during menopause lead to systemic inflammation, manifesting as increased TNF-α in saliva and serum,

which contributes to periodontal tissue destruction.[13]

Further, Gupta et al. (2022) explored salivary biomarkers in menopausal women and observed significantly higher TNF-α levels in those with periodontal disease compared to their healthy counterparts. Their findings align with the current study, suggesting that menopause creates a pro-inflammatory environment that predisposes women to periodontal inflammation.[14]

While López-Marcos et al. (2021) proposed that salivary TNF-α can serve as a non-invasive biomarker for periodontal disease progression, particularly in systemic conditions like menopause, where inflammation have already heightened.[15]

From a pathophysiological perspective, estrogen deficiency in menopausal women disrupts immune homeostasis by promoting osteoclastogenesis and inflammatory cytokine expression, including TNF-α.[16] TNF-α, a potent pro-inflammatory mediator, contributes to periodontal tissue degradation by inducing matrix metalloproteinase (MMP) activity and enhancing the recruitment of inflammatory cells to the periodontium [17]. Taba et al. (2023) emphasized that elevated salivary TNF-α levels reflect ongoing periodontal tissue destruction and can differentiate periodontal disease severity.[18]

The present study also highlights the utility of saliva as a diagnostic fluid for detecting inflammatory markers. Saliva has been widely recognized as a non-invasive alternative to blood in assessing systemic and local inflammation.[19] Recent studies, including those by Al-Rawi et al. (2022), have demonstrated that salivary TNF-α levels correlate well with periodontal status and can be effectively used to monitor inflammatory conditions in menopausal women.[20]

Despite these findings, the small sample size of the present study remains as a limitation. Future studies with larger cohorts are required to confirm the generalizability of these results. Longitudinal studies assessing TNF-α levels before and after periodontal therapy in menopausal women could further clarify its diagnostic and prognostic value. Additionally, exploring other salivary inflammatory markers, such as IL-1β and IL-6, alongside TNF-α, may provide a more comprehensive understanding of the inflammatory burden in menopausal women with periodontitis.

CONCLUSION

The elevated salivary TNF-α levels observed in menopausal women with periodontitis interplay between systemic hormonal changes and local inflammatory responses. These findings align with recent literature and highlight the potential of TNF-α as a biomarker for periodontal disease assessment, particularly in vulnerable populations like menopausal women.

DECLARATIONS

Abbreviations

TNF-α – Tumour Necrosis Factor - Alpha

Conflict of Interest

There is no Conflict of Interest

Acknowledgement

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RESEARCH ARTICLE

EVALUTION OF ANTICANCER POTENTIAL OF PIPERINE IN HUMAN ORAL SQUAMOUS CELL CARCINOMA-AN IN VITRO EXPERIMENTAL STUDY

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is one of the most prevalent and aggressive malignancies, characterized by uncontrolled cell proliferation and high recurrence rates. Despite advancements in therapy, resistance to conventional treatments remains a major challenge. *Piperine,* a bioactive alkaloid from black pepper, has demonstrated anticancer potential in various cancer models through apoptosis induction and cell cycle arrest. However, its specific effects on oral cancer cell lines remain underexplored. This study evaluates the anticancer activity of Piperine in KB (Human oral cancer) cell line, focusing on its impact on cell viability and apoptotic gene expression.

Material and Method: KB oral cancer cells were cultured and treated with Piperine at 25 µM, 50 µM, and 100 µM. Cell viability was assessed using the MTT assay, while morphological changes were observed under an inverted microscope. The expression of key apoptotic genes (Bcl-2, Bax, and p53) was analyzed using quantitative real-time PCR (qRT-PCR). Data were statistically analyzed using one-way ANOVA, and results were expressed as mean ± SEM.

Results: Piperine exhibited a dose-dependent cytotoxic effect, significantly reducing cell viability at higher concentrations (50 µM and 100 µM). Microscopic observations revealed cell shrinkage, detachment, and membrane blebbing, indicating apoptotic cell death. Gene expression analysis showed downregulation of Bcl-2 (anti-apoptotic gene) and upregulation of Bax and p53 (pro-apoptotic genes), confirming Piperine induced apoptosis.

Conclusion: These findings suggest that Piperine exerts anticancer effects on KB oral cancer cells by inducing apoptosis via the mitochondrial pathway. Its ability to modulate Bcl-2, Bax, and p53 expression highlights its potential as a therapeutic agent for OSCC. Further preclinical and clinical studies are warranted to explore its bioavailability and translational applications in oral cancer therapy.

Keywords: Apoptosis, Anti-cancer, Cell line, Oral squamous cell carcinoma, Piperine

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a disease characterized by uncontrolled and abnormal cell growth, which can progress slowly if not managed. When cancer infiltrates a single cell or a group of cells, its progression rate doubles over time[1].

This type of cancer can affect various tissues in and around the mouth, including bones, muscles, and nerves, leading to both benign and malignant growths. It is a significant global health concern due to its high incidence and mortality rates[2]. The complexity of treatments and the economic burden further challenge patient survival. Despite post-surgical treatment, there is an increased risk of local and regional recurrence[3]. Patients diagnosed early have a 90% survival rate, while those with late-stage cancer face a mortality rate of around 60%[4].

Black pepper, a member of the Piperaceae family, produces mature but unripe fruits used as a popular spice. Many spices, particularly pepper, contain compounds with insulin resistance properties and antioxidant potential[5,6]. *Piperine*, a component of black pepper, has been traditionally used as a flavour enhancer and in traditional medicine[7]. Studies have shown that *Piperine* induces cell death by depleting Matrix Metalloproteinase (MMP) and activating caspase-3. It also reduces DNA levels and arrests cell growth in G2/M phase, as demonstrated in cell cycle studies. These findings suggest that *Piperine* effectively induces cell death by reducing MMP levels and Reactive Oxygen Species (ROS) release, subsequently triggering caspase-3 activation and cell cycle arrest[8]. *Piperine* has displayed a wide range of physiological effects, including anti-hypertensive, anti-coagulant, antioxidant, anti-cancer, antispasmodic, anti-asthmatic, antidepressant, and anxiolytic properties[9].

Most studies on *Piperine's* anticancer potential have focused on cancers such as breast, lung, colon, and prostate, with limited research on its effects in oral cancer. Furthermore, variations in dosages and exposure times across different studies make it difficult to establish a standardized therapeutic dose. While some research has explored *Piperine's* cytotoxic properties, where there is still a lack of in-depth mechanistic studies specifically targeting oral cancer cell lines. This study aimed to analyse the anticancer potential by observing the expression analysis of *Piperine* in Oral cancer cell line.

MATERIALS AND METHODS

Chemicals

For this study, *Piperine* was procured from Sigma-Aldrich. Essential cell culture reagents, including trypsin-EDTA, fetal bovine serum (FBS), antibiotic-antimycotic solution, Dulbecco's Modified Eagle's Medium (DMEM), and phosphate-buffered saline (PBS), were obtained from Gibco, Canada. The JC-1 dye(5,5,6,6-tetrachloro-1,1,3,3-tetraethyl benzimidazole carbocyanine iodide) and real-time PCR kits (MESA Green) were sourced from Invitrogen, USA. All chemicals utilized in the study were of high analytical grade to ensure precision and reliability.

Procurement and Culture of Oral Cancer Cells

The Oral cancer cell line used in this study was acquired from the National Centre for Cell Science (NCCS), Pune, India. The cells were cultured following standard protocols, utilizing Minimum Essential Medium (MEM) supplemented with 10% FBS, and maintained at 37°C in a humidified atmosphere with 5% CO_2 to support optimal growth conditions.

Cell Viability Assay

Oral cancer cells were seeded in 96-well plates at a density of 5×10^5 cells per well and allowed to adhere overnight. Following incubation, the cells were exposed to varying concentrations of *Piperine* in triplicate and maintained for 24 hours at 37°C in a humidified incubator with 5% CO_2 . Subsequently, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well, and the cells were incubated for an additional four hours at 37°C. The resulting formazan crystals were dissolved in 200 µL of dimethyl sulfoxide (DMSO), and the optical density (OD) was measured. The standard deviation (SD) was also calculated for each set of replicates. The entire experiment was conducted three times to ensure reproducibility. The percentage of cell growth inhibition was calculated using the following formula:

*%Growth Inhibition= (1−OD of extract-treated cells/OD of negative control)*100*

Gene Expression Analysis by Real-Time PCR

The expression levels of target mRNA were quantified using real-time PCR. Total RNA was extracted using Tri Reagent (Sigma), and cDNA synthesis was performed using the Superscript III First-Strand cDNA Synthesis Kit (Invitrogen, USA) according to the manufacturer's instructions. For

gene expression analysis, real-time PCR reactions were carried out on an MX3000p PCR system (Stratagene, Europe) using the MESA Green PCR Master Mix (Eurogentec, USA), which contains all essential PCR components along with SYBR Green dye. The specificity of the amplified products was confirmed through melting curve analysis for each primer pair. Data analysis was conducted using the comparative Ct method (ΔΔCt approach), and fold-change expression was determined using the 2−ΔΔCt method, as described by Venkata et al. (2022)[11].

Statistical analysis

Cell viability at varying concentrations of *Piperine* was assessed, and the fold change in the expression levels of Bcl-2, Bax and p53 was calculated, and the corresponding mean ± SEM values were determined. To evaluate statistical significance, one-way analysis of variance (ANOVA) was performed to compare cell viability across the three different concentrations of *Piperine*, assessing potential differences in fold change at various treatment levels.

RESULTS

The effect of *Piperine* on KB oral cancer cells was analyzed at different concentrations (25 µM, 50 µM, and 100 µM) using an inverted light microscope at 10× magnification. In the control group (untreated cells), the cells exhibited normal morphology, with a high density, adherent nature, and intact cellular structure, indicating healthy proliferation. However, upon treatment with *Piperine* at 25 µM, a slight reduction in cell density was observed, with some cells displaying rounding and detachment, suggesting early apoptotic changes.

At 50 µM, the cytotoxic effects of *Piperine* became more pronounced, with a marked decrease in cell density and a higher number of detached cells. Many cells exhibited membrane shrinkage and irregular morphology, indicating an increased rate of apoptosis. The 100 µM concentration of *Piperine* demonstrated the most substantial cytotoxic effect, as the majority of cells appeared shrunken and detached, with notable membrane blebbing and irregular structures, suggesting a combination of apoptotic and necrotic cell death. These findings suggest a dose-dependent cytotoxic effect of *Piperine* on KB oral cancer cells, with higher concentrations (50 µM and 100 µM) inducing significant morphological alterations associated with apoptosis and loss of viability (Figure 1).



Figure 1. The images show morphological changes in KB oral cancer cells treated with *Piperine* (25 µM, 50 µM, and 100 µM) compared to the untreated control. A dose-dependent reduction in cell density was observed, with cell shrinkage, rounding, and detachment, indicating apoptosis. At 100 µM, extensive cell death, membrane blebbing, and loss of adherence confirm *Piperine*-induced cytotoxicity.

The MTT assay results demonstrate a dose-dependent cytotoxic effect of *Piperine* on KB oral cancer cells, as indicated by the progressive decline in cell viability with increasing concentrations of *Piperine*. In the untreated control group, cell viability was the highest, approximately 90%, indicating normal cell proliferation in the absence of treatment. However, upon exposure to 25 µM of *Piperine*, a slight reduction in viability (~75–80%) was observed, suggesting mild cytotoxicity at this concentration.

At 50 µM, a significant decline in cell viability (~50–55%) was noted, indicating an increased cytotoxic response, likely due to the induction of apoptosis or cell death mechanisms. The most substantial reduction in cell viability was observed at 100 µM, where only ~40% of cells remained viable, highlighting the potent anticancer potential of *Piperine* at higher concentrations. These findings confirm that *Piperine* effectively inhibits KB oral cancer cell proliferation in a dose-dependent manner, with higher concentrations (50 µM and 100 µM) inducing strong cytotoxic effects. The results suggest that *Piperine* may exert its anticancer activity by triggering apoptosis or other cell death pathways (Figure 2).

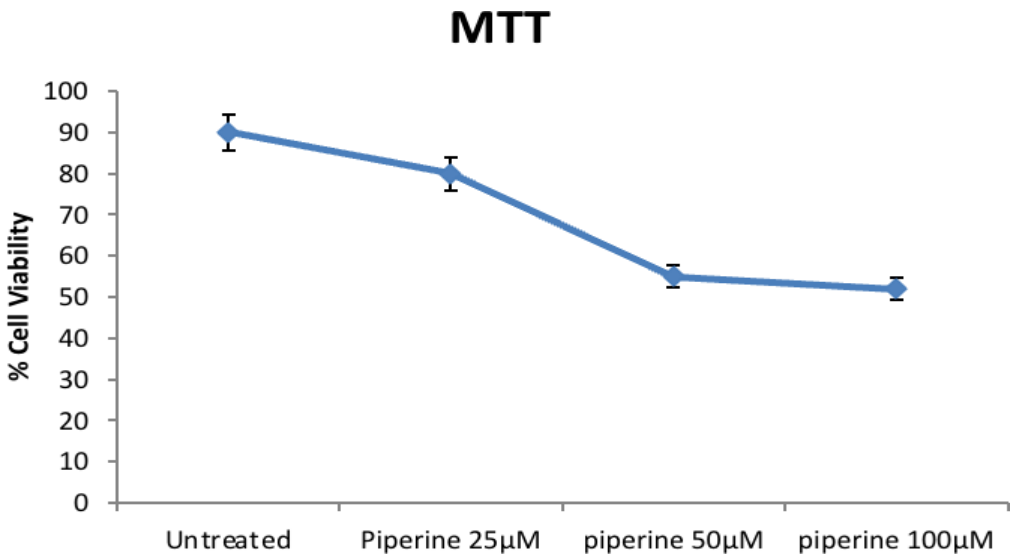


Figure 2. The graph shows the percentage cell viability of KB oral cancer cells treated with *Piperine* (25 µM, 50 µM, and 100 µM), assessed by the MTT assay. A dose-dependent decline in viability indicates *Piperine's* cytotoxic effect, with a significant reduction at 50 µM and 100 µM, suggesting

The expression levels of Bcl-2, an anti-apoptotic gene, in KB oral cancer cells following treatment with different concentrations of *Piperine* (25 µM, 50 µM, and 100 µM) compared to the untreated control group. The results indicate a dose-dependent reduction in Bcl-2 expression, suggesting that *Piperine* induces apoptosis by downregulating this survival promoting gene. In the untreated control group, Bcl-2 expression was the highest, set as baseline (1.0 mean expression value), indicating that the cancer cells maintain a strong survival mechanism in the absence of treatment. Upon exposure to 25 µM of *Piperine*, Bcl-2 expression decreases to 0.74 ± 0.09, indicating the initiation of apoptotic signalling. A further reduction was observed at 50 µM (0.60 ± 0.05) and 100 µM (0.59 ± 0.05), suggesting a significant suppression of Bcl-2 with increasing *Piperine* concentrations (table 1).

Table 1. Expression analysis of *Piperine* in Oral carcinoma cell line

Expression analysis	**Control (untreated KB Cells)**	**Oral carcinoma cells + *Piperine* (50µM)**	**Oral carcinoma cells + *Piperine* (100µM)**	**Oral carcinoma cells + *Piperine* (200µM)**
Bcl2 mRNA	1±0	0.74±0.09	0.60±0.05	0.59±0.05
Bax mRNA	1±0	1.1±0.07	1.3±0.06	1.4±0.06
p53	1±0	1.2±0.09	1.6±0.12	1.6±0.11

**Values are given as: Mean ± S.E.M*

The decline in Bcl-2 expression confirms that *Piperine* promotes apoptosis in KB oral cancer cells by inhibiting anti-apoptotic pathways, thereby making cells more susceptible to programmed cell death. This trend aligns with the MTT assay results, which showed a dose-dependent reduction in cell viability, further supporting *Piperine's* anticancer potential (Figure 3).

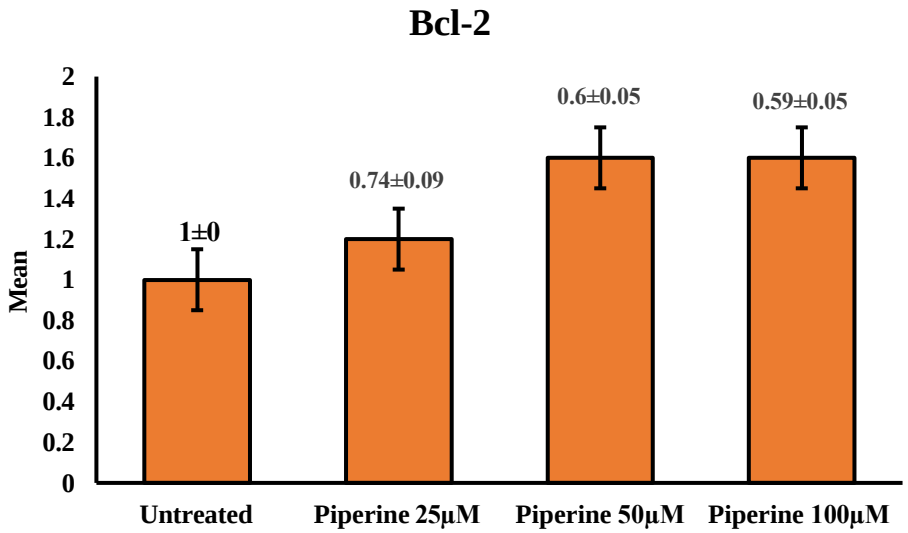


Figure 3. The y-axis represents the mean Bcl-2 expression levels, while the x-axis denotes the different treatment groups, with error bars indicating the standard error of the mean (SEM). A dose-dependent decrease in Bcl-2 expression was observed, with the highest reduction at 100 µM (0.59 ± 0.05), indicating that *Piperine* suppresses anti-apoptotic signalling. These findings suggest that *Piperine* induces apoptosis in KB cells by downregulating Bcl-2, thereby shifting the balance toward cell death pathways.

Bax, a pro-apoptotic gene, in KB oral cancer cells treated with different concentrations of *Piperine* (25 µM, 50 µM, and 100 µM) compared to the untreated control group. The results indicate a dose-dependent upregulation of Bax expression, suggesting that *Piperine* induces apoptosis by promoting the activation of this pro-apoptotic pathway. In the untreated control group, Bax expression was at its baseline (~1.0 mean expression value), indicating a normal level of apoptotic signaling in cancer cells. Following treatment with 25 µM *Piperine*, Bax expression increases to 1.1 ± 0.07, suggesting the initiation of apoptosis. At 50 µM, Bax expression rises further to 1.3 ± 0.06, and at 100 µM, it reaches its highest level at 1.4 ± 0.06, indicating a significant induction of apoptosis at higher *Piperine* concentrations.

The progressive increase in Bax expression with increasing *Piperine* concentrations confirms that *Piperine* triggers apoptosis in KB oral cancer cells by activating the pro-apoptotic pathway. This trend was consistent with the Bcl-2 expression results, which showed a dose-dependent downregulation of Bcl-2, leading to a higher Bax/Bcl-2 ratio a critical factor for apoptosis activation. These findings reinforce *Piperine's* anticancer potential and suggest that its apoptotic effect may be mediated through the mitochondrial (intrinsic) pathway (Figure 4).

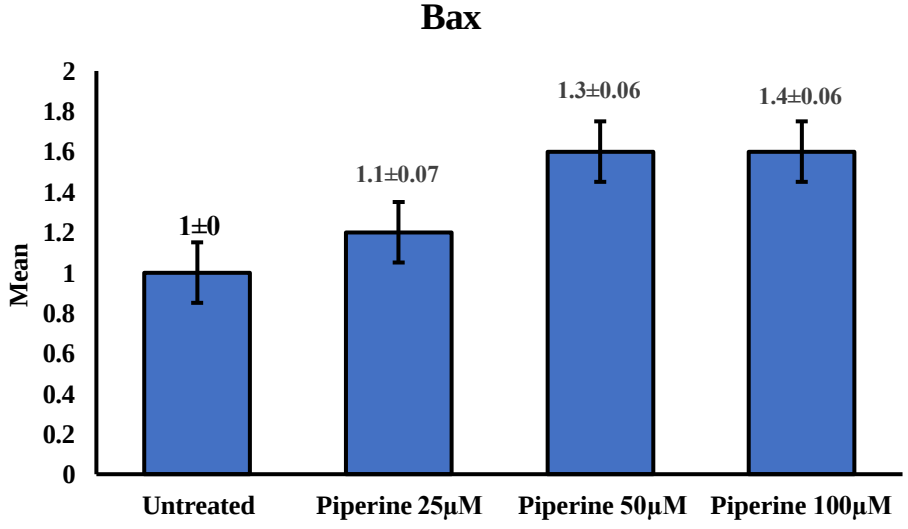


Figure 4. The expression of Bax, a pro-apoptotic gene, was analyzed in KB oral cancer cells after treatment with

different concentrations of *Piperine* (25 µM, 50 µM, and 100 µM) in comparison to the untreated control. The y-axis denotes mean Bax expression levels, while the x-axis represents the treatment groups. A dose-dependent increase in Bax expression was observed, with the highest level at 100 µM (1.4 ± 0.06), confirming the apoptotic potential of *Piperine*. These results suggest that *Piperine* induces apoptosis in KB cells by upregulating Bax, promoting a shift towards programmed cell death.

A dose-dependent elevation in p53 expression was observed following *Piperine* treatment. Compared to the untreated control, where p53 expression was at its lowest, an increase to 1.2 ± 0.09 was noted at 25 µM, suggesting the initiation of p53-mediated apoptotic signaling. A further substantial upregulation was detected at 50 µM and 100 µM with Mean ± S.D as 1.6 ± 0.12 and 1.6 ± 0.11, indicating that *Piperine* induced a sustained activation of p53-dependent cell death pathways. Since p53 played a pivotal role in DNA damage response, cell cycle arrest, and apoptosis, its upregulation supported the notion that *Piperine* triggered apoptotic mechanisms to inhibit oral cancer cell survival.

These findings suggested that *Piperine* effectively enhanced p53 expression, reinforcing its role in tumour suppression by potentially promoting apoptosis and inhibiting uncontrolled proliferation. The consistency in p53 expression levels at 50 µM and 100 µM implied that beyond a certain threshold, *Piperine* may have maximized its stimulatory effect on p53 activation (Figure 5).

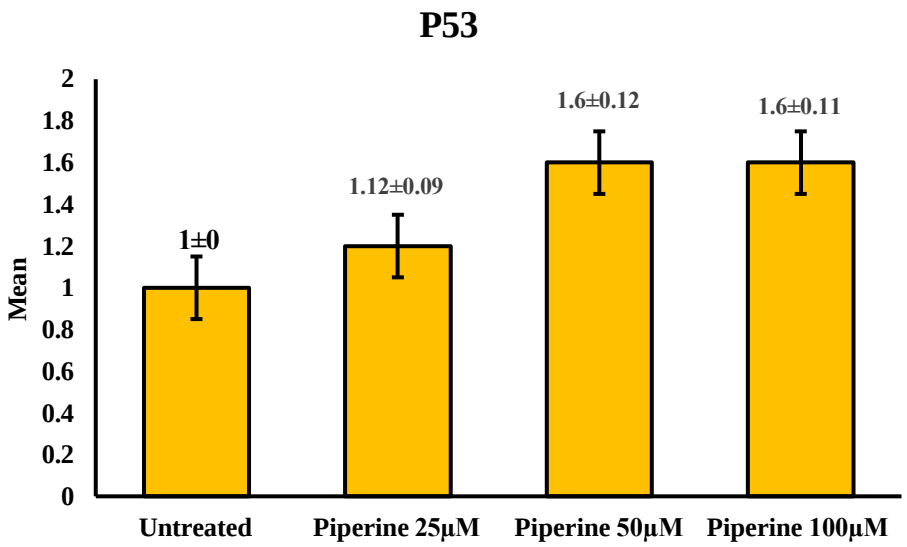


Figure 5. The y-axis denoted the mean p53 expression levels, while the x-axis represented the treatment groups. A dose-dependent increase in p53 expression was observed, with the highest levels at 50 µM (1.6 ± 0.12) and 100 µM (1.6 ± 0.11), suggesting that *Piperine* enhanced p53-mediated apoptotic signaling. These results indicated that *Piperine* upregulated p53 expression, promoted apoptosis, and potentially contributed to tumour suppression in KB oral cancer cells.

DISCUSSION

The present study demonstrated that *Piperine* induces apoptosis in KB oral cancer cells in a dose-dependent manner, as indicated by a reduction in Bcl-2 expression and an upregulation of Bax and p53. These findings are consistent with recent research highlighting *Piperine's* anticancer properties in various malignancies, particularly its role in apoptotic pathway activation and tumour suppression mechanisms. A recent study by Guo et al. (2021) reported that *Piperine* induces apoptosis in gastric cancer cells by increasing Bax/Bcl-2 expression ratio, ultimately leading to mitochondrial membrane depolarization and caspase activation[12]. Similarly, Shaheer et al. (2024) found that *Piperine* inhibits proliferation and promotes apoptosis in breast cancer cells via p53-mediated signaling, further supporting the results obtained in this study[13]. Our findings align with these studies, as we observed a significant increase in Bax and p53 expression, confirming *Piperine's* role in intrinsic apoptotic pathway. Despite these promising results, limited research has

specifically investigated *Piperine's* effects on oral squamous carcinoma cell lines. A study by Siddiqui et al. (2017) examined the cytotoxic effects of *Piperine* on oral cancer cells and found that it induces G2/M cell cycle arrest and promotes apoptosis via ROS generation and caspase activation[14]. The current study extended these findings by demonstrating a dose-dependent decrease in Bcl-2 expression, further supporting *Piperine's* role in downregulating anti-apoptotic mechanisms in oral cancer.

In addition to apoptotic pathway activation, our study highlights *Piperine's* potential for clinical application. Recent evidence suggests that *Piperine* enhances the efficacy of conventional chemotherapeutic agents. Mohammed et al. (2024) reported that *Piperine* synergistically enhances the anticancer effects of cisplatin in tongue squamous cell carcinoma by increasing ROS production and apoptosis markers[15]. These findings suggest that *Piperine* could be a valuable adjunct in combination therapies for oral cancer treatment, reducing the required dose of conventional chemotherapy while mitigating side effects.

Furthermore, bioavailability remains a critical challenge in *Piperine*-based treatments. Bolat et al. (2020) emphasized the need for nano formulations and delivery systems to improve *Piperine's* pharmacokinetics and therapeutic efficacy[16]. Future studies should focus on nanoparticle-based *Piperine* formulations to enhance targeted drug delivery and maximize its anticancer potential in oral squamous cell carcinoma.

CONCLUSION

This study confirms that *Piperine* effectively induces apoptosis in KB oral cancer cells by regulating key apoptotic markers such as Bcl-2, Bax, and p53. The results suggest that *Piperine* has significant therapeutic potential and could be explored as a treatment strategy for oral cancer. However, further preclinical and clinical studies are required to assess its bioavailability, pharmacokinetics, and long-term therapeutic impact.

DECLARATIONS

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Conflict of interest

All the authors declare that there was no conflict of interest in the present study.

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RESEARCH ARTICLE

A COMPARATIVE ASSESSMENT OF ADJACENT TEETH MORPHOLOGIES, ANGULATIONS AND INCLINATIONS IN BUCCAL AND PALATAL IMPACTED CANINES- AN OBSERVATIONAL STUDY

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ABSTRACT

Objectives: The study aimed to compare and evaluate the morphologies, angulations, and inclinations of lateral incisors, central incisors, and first premolars in of buccal and palatal impacted maxillary canines. **Materials and Methods:** The retrospective observational research utilized cone beam computed tomography (CBCT) images from 45 patients, categorized into three groups: Buccal Impacted Canines (BIC), Palatal Impacted Canines (PIC), and a Control Group (CG) devoid of impactions, including 15 patients in each group. Parameters, including crown and root widths and lengths, angulations, and inclinations of adjacent teeth, were measured. Statistical analyses involved one-way ANOVA to compare PIC, BIC and CG. The paired t-test was employed to compare the parameters between the ipsilateral and contralateral sides within the PIC and BIC. *p* values less than or equal to 0.05 were considered significant.

Results: Notable differences were noted primarily in the lateral incisors among the three groups under study. Palatal impacted canines were associated with shorter root lengths of lateral incisors (p=0.04), while buccal impacted canines showed increased angulation and inclination of lateral incisors compared to PIC and CG (p=0.013). Within the PIC group, the affected side exhibited narrower buccolingual width and shorter crown length of central incisors (p<0.001, p=0.005) and reduced root lengths of lateral incisors (p=0.019). Conversely, the BIC group demonstrated increased buccolingual width and greater inclination of lateral incisors on the impacted side (p=0.007, p=0.003). ICC values indicated moderate-good reliability.

Conclusion: Palatal and Buccal impacted maxillary canines were found to be associated with distinct morphological and angulation changes in adjacent lateral incisors.

Keywords: Cuspid, Cone beam computed tomography, Impaction, Orthodontics, Incisors

INTRODUCTION

Maxillary canines are among the most commonly impacted teeth, second only to third molars, with an incidence of 1.7%–4.7%, and are twice as prevalent in females. The prevalence of maxillary canine impaction is nearly double that of mandibular impaction.[1,2]

Maxillary canine impactions may occur either in buccal or palatal aspects with palatal impactions far more common than buccal impactions. Buccal or palatal impaction can result in cosmetic and functional problems, such as resorption of adjacent teeth. Therefore, the management of canine impaction necessitates specialized surgical and orthodontic procedures for optimal results.[3-5] The early detection and prediction of maxillary canine impaction is essential to avert severe effects, such as root resorption, and to initiate prompt orthodontic treatments. Early recognition of maxillary canine impaction can reduce the duration, complexity, and cost of treatment.[6] Hence, identification of the aetiology of the impaction is crucial for its prevention and management.

The aetiology of canine impactions remains disputed. Concerning palatal impacted canines, two hypotheses have been proposed to elucidate its development: the genetic theory and guidance theory. Guidance theory posits that morphological changes in lateral incisors lead to the loss of the eruptive guidance for canines.[7] Increased space within the dental arch along with variations of the lateral incisors are typically correlated with palatal impacted canines.[8]

Previous research by Luik *et al*.[9] established that the orientation and position of palatal impacted canines are associated with the inclination and dimensions of lateral incisors. Another school of thought is the 'sequential theory', which delineates a pattern wherein the two most widely acknowledged hypotheses may operate at distinct phases in the development of the maxillary canine and its adjacent components. Additional characteristics, including as the mesial displacement of the maxillary first premolar, root dilacerations and the width of the maxillary arch, have also been linked to the occurrence of palatal impacted canines.[10]

However, with regard to the buccal impacted canines, dental crowding, constriction of the maxillary arch, and lack of space for eruption are commonly attributed etiologies.[7] However, the investigation by Light *et al*.[11] observed changes in the inclination of the ipsilateral teeth in cases of both buccal and palatal canine impactions.

While most previous studies have focused on the shape, dimensions, and morphology of the lateral incisor pertaining to the palatal impaction of the maxillary canine, very few investigations have explored the angulation and inclination of both ipsilateral and contralateral central incisors, lateral incisors, and first premolars.[7, 9, 11-13] Furthermore, there is a dearth of research that has thoroughly evaluated the influence of the three-dimensional positioning of neighboring tooth structures on the type of impaction among the native South Indian population. Hence, the objective of this study is to assess the crown and root morphologies, angulation, and inclinations of the central incisor, lateral incisor, and first premolar in unilateral buccal and palatal canine impactions, while the secondary objectives are to compare the presence of factors like retained deciduous teeth, dilaceration and supernumerary teeth between palatal and buccal canine impactions.

MATERIALS AND METHODS

The retrospective observational investigation was conducted at Saveetha Dental College and hospitals. This study complied with the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines, ensuring comprehensive and transparent documentation of the research methodology, analysis, and results. Authorization for the study's conduct was obtained from the institutional review board (SRB/SDC/ORTHO-2306/24/019) prior to the study commencement. In this study, the raw data were first retrieved from institutional archives, and patient identifiers, including names, patient IDs, and mobile phone numbers, were anonymized. Informed consent from the patient was secured for the utilization of the data in the study.

Patient Selection

Cone beam computed tomography (CBCT) images was obtained from the institutional database based on an established inclusion and exclusion criteria. The inclusion criteria consisted of patients with permanent dentition, unilaterally impacted canines in the maxillary arch, with or without retained primary maxillary canines. The exclusion criteria were patients with craniofacial anomalies and syndromes, pathologies associated with impacted canines, patients who underwent prior orthodontic

treatment, substandard quality CBCT images, bilateral canine impactions, mandibular canine impactions, multiple missing teeth in either arch, or significant resorption of the maxillary teeth.

CBCT images that were acquired between 2021 and 2024 were chosen and retrieved. The records were categorized into two groups: Buccal impacted canines (BIC) and Palatal impacted canines (PIC). A separate control group (CG) without impacted canines was used in the current investigation for enhanced comparability. Sample size determination was conducted utilizing G Power software (version 3.0.10) predicated on the effect size derived from a prior investigation.[12]

To obtain an effect size of 0.763, an alpha error of 0.05, and a power of 0.95, a total of 45 patients were required, allocated over three groups with 15 patients in each group. All CBCT files were acquired using the CS 9600 (Carestream Dental LLC) under regular settings, with the patient positioned upright. The field of view (FOV) of the obtained scans was 8x8, offering a three-dimensional viewpoint of the maxilla. The scan parameters were: 120 kV, 150 seconds, 4.0 mA, and 75 mGycm².

Parameters assessed

The obtained CBCT data was examined using the CS 3D Imaging software (v3.10.21, Carestream Dental, LLC). Axial, coronal, and sagittal reconstructions were utilized to localise the canines. The scans were examined at a slice thickness of 0.4 mm, and all measurements were conducted by a single operator. Measurements related to crown and root morphology, inclinations, and angulations were carried out for PIC, BIC, and CG. The measured parameters are outlined in Table 1 and Figure 1.

The evaluated parameters were derived from prior investigations.[14, 15] Alongside the quantitative characteristics evaluated, variables such as the existence of dilaceration, supernumerary teeth, retained deciduous teeth, and congenitally missing lateral incisors were obtained for the PIC and BIC groups.

Statistical Analysis

Statistical analyses were performed utilizing the Statistical Package for the Social Sciences (SPSS, version 23.0, Armonk, New York). Normality of the data was assessed using Shapiro-Wilk test. One-way ANOVA was employed to compare the parameters among the three groups. For the BIC and PIC, the ipsilateral side was analysed. However for the CG, right or left side was assigned for each sample randomly. Paired t-tests were employed to compare the teeth parameters between the ipsilateral and contralateral sides within the PIC and BIC groups. Un-paired t-test was used to compare the alpha angle of the impacted canines between PIC and BIC. Descriptive statistics were employed to describe data on the canine sector, the presence or absence of dilaceration in canines and lateral incisors, peg laterals, and retained deciduous canines. The occurrence of dilacerations, supernumerary teeth, retained deciduous teeth and the type of impaction was assessed using Chi square test. Intra examiner reliability for consistency and reliability of the quantitative parameters were assessed using the Intra-class correlation coefficient (ICC) based on values taken one month apart by the same investigator on ten randomly selected samples. A significance level of $p \leq 0.05$ was considered significant for all tests.

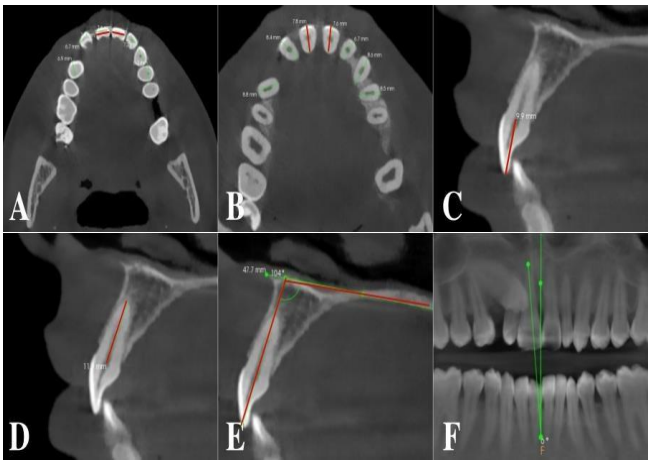


Figure 1. The parameters measured in the CBCT images: A- Mesiodistal width; B- Buccolingual width; C- crown height; D- Root height; E- Tooth angulation; F- Tooth inclination.

RESULTS The current investigation was done over a six-month period from May to October 2024. The current study analyzed a total of 45 CBCTs, which included 15 records in each group. The average age of participants in the BIC was 21.87 ± 5.40 years, in the PIC was 22.40 ± 5.45 years, and in the CG was 23.26 ± 6.57 years. The age disparities across the groups were not statistically significant ($p = 0.803$). The BIC comprised seven females and eight males, whilst the PIC consisted of nine females and six males. The CG included ten females and five males. Regarding impaction, 60% (n=9) of BIC exhibited right-sided impaction, whereas 60% (n=9) of PIC demonstrated left-sided impaction.

Table 1. Parameters assessed in the test groups.

The following parameters were measured on the ipsilateral and contralateral central incisor, lateral incisor and first premolars		
Parameter	**CBCT View Used**	**Description**
Mesiodistal Width (mm)	Axial View	Measured as the distance between the most mesial and distal points on the crown contour, taken at the widest part of the tooth.
Buccolingual Width (mm)	Axial View	Measured as the distance between the most buccal and lingual points on the crown contour at the greatest convexity.
Crown Height (mm)	Sagittal View	Measured as the vertical distance from the cementoenamel junction (CEJ) to the most incisal or cuspal tip of the crown.
Root Length (mm)	Sagittal View	Measured as the distance from the cementoenamel junction (CEJ) to the apex of the root along the central long axis of the tooth.
Tooth Inclination (°)	Curved Slice	Measured as the angle formed between the long axis of the tooth and the facial midline.
Tooth Angulation (°)	Sagittal View	Measured as the angle between the tooth's long axis and the ANS-PNS line.
Angle of dilaceration (°)	Sagittal View	Measured as the angle formed between the long axis of the crown and the long axis of the curved portion of the root. Dilaceration refers to deviations in the root portion of a tooth and is defined as an angulation of 20 degrees or more of the root's apical end from the tooth's normal long axis.[12]
The following parameters were measured with relation to the impacted canines		
Sector Alpha Angle (°)	Sagittal View	The angle formed between the long axis of the impacted canine and the inter-incisor median line
Sector Classification	Panoramic View	Lindauer's modification[13] of Ericson and Kurol's definition of sectors is as follows: • **Sector I** is located distal to a line tangent to the distal crown and root of the lateral incisor. • **Sector II** encompasses the area between the tangent on the distal surface and a midline bisector of the lateral incisor. • **Sector III** covers the region from the midline bisector to a tangent on the mesial surface of the lateral incisor's crown and root. • **Sector IV** includes all areas mesial to Sector III.

Comparison of parameters between the three groups

The comparison of tooth morphologies, inclinations, and angulations among buccal, palatal, and control groups using ANOVA revealed significant differences exclusively in the lateral incisors. Specifically, lateral incisors in PIC had a statistically significant short root length when compared to BIC and controls. The differences between the groups were statistically significant (p=0.04). Additionally, BIC showed significantly higher angulation and inclination of the lateral incisors compared to PIC and control groups. There was a statistically significant difference in the lateral incisor inclination (p=0.013) and angulations (p=0.013) between the groups. No significant differences were observed in morphometric parameters of central incisors, canines, or premolars across the buccal, palatal, and control groups (Table 2).

Table 2. The mean and standard deviation (SD) of the parameters assessed for BIC, PIC and CG. One-way ANOVA was used to compare the group. * indicates a *p* value ≤ 0.05

Teeth	Parameter	Mean and SD			p value
		BUCCAL	PALATAL	CONTROL	
CENTRAL INCISOR	Mesiodistal Width	8.28±0.87	8.07±0.76	8.49±3.3	0.321
	Buccolingual Width	8.55±1.16	8.07±0.99	8.56±3.27	0.346
	Crown Length	10.18±1.33	10.62±0.5	10.97±4.16	0.083
	Root Length	13.17±1.6	12.24±1.52	12.23±4.45	0.165
	Inclination	116.35±11.65	115.81±8.97	117.95±45.31	0.832
	Angulation	8.07±8.26	6.25±7.31	8.91±2.46	0.63
LATERAL INCISOR	Mesiodistal Width	6.89±0.68	6.47±0.67	6.46±2.39	0.202
	Buccolingual Width	7.81±1.18	7.1±0.83	7.22±2.55	0.096
	Crown Length	8.87±1.03	8.51±0.9	8.66±2.92	0.692
	Root Length	12.45±2.17	10.78±1.93	12.18±4.48	0.04*
	Inclination	124.59±11.15	113.7±13.78	114.29±44.5	0.013*
	Angulation	20.31±12.27	11.89±10.89	9.41±4.44	0.013*
CANINE	Mesiodistal Width	7.55±0.51	7.91±0.91	7.71±2.75	0.381
	Buccolingual Width	8.4±0.41	8.59±2.26	8.25±3	0.799
	Crown Length	9.18±1.55	9.38±1.08	9.73±3.72	0.484
	Root Length	13.47±1.68	14.03±1.55	14.56±6	0.833
PREMOLAR	Mesiodistal Width	6.83±0.54	6.93±0.5	7.09±2.78	0.384
	Buccolingual Width	9.41±0.61	9.15±0.48	9.39±3.46	0.409
	Crown Length	7.27±2.16	7.5±1.21	7.82±2.95	0.667
	Root Length	11.57±2.46	12.27±1.89	11.91±4.25	0.612
	Inclination	92.77±10.74	95.3±8.93	94.87±35.97	0.713
	Angulation	9.83+8.52	10.97±6.46	10.17±3.2	0.906

Table 3. Intergroup comparison done using Bonferroni post-hoc test. * indicates a *p* value ≤ 0.05.

Parameter	Groups		Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Lateral incisor root length	BIC	PIC	.68119	.05*	-.0253	3.3720
	BIC	CG	.68119	1.000	-1.4253	1.9720
	PIC	CG	.68119	.138	-3.0987	.2987
Lateral incisor Inclination	BIC	PIC	3.95621	.026*	1.0212	20.7521
	BIC	CG	3.95621	.038*	.4345	20.1655
	PIC	CG	3.95621	1.000	-10.4521	9.2788
Lateral incisor Angulation	BIC	PIC	3.68976	.083	-.7810	17.6210
	BIC	CG	3.68976	.015*	1.7056	20.1077
	PIC	CG	3.68976	1.000	-6.7144	11.6877

Inter-group comparison was done for significant parameters using Bonferroni test. For lateral incisor root length, a significant difference was observed between the BIC and PIC groups ($p = 0.05$), with no significant differences involving the CG. In terms of lateral incisor inclination, the BIC group showed significant differences when compared to both the PIC ($p = 0.026$) and CG ($p = 0.038$). For lateral incisor angulation, significant differences were identified between the CG and BIC ($p = 0.015$), whereas comparisons involving the PIC group did not reach statistical significance (Table 3).

Ipsilateral side vs contralateral side in PIC and BIC

Paired t-test was used compare the parameters between ipsilateral and contralateral sides within PIC and BIC. In PIC group, significant differences were observed between ipsilateral and contralateral sides, with ipsilateral side showing a narrower buccolingual width of the central incisors ($p < 0.001$), shorter crown length ($p = 0.005$), and altered angulation ($p = 0.02$). Additionally, the lateral incisors on the ipsilateral side had reduced root lengths ($p = 0.019$), and impacted canines exhibited smaller mesiodistal widths ($p = 0.03$). In BIC group, significant differences included increased buccolingual width of the lateral incisors ($p = 0.007$), greater inclination ($p = 0.003$), and higher angulation ($p = 0.015$) on the ipsilateral side. Furthermore, canines showed longer root lengths ipsilaterally ($p < 0.001$), and premolars had larger mesiodistal width ($p = 0.039$) and shorter root lengths ($p = 0.047$) ipsilaterally. Other measured parameters did not exhibit significant differences between ipsilateral and contralateral sides in either BIC or PIC (Table 4). The PIC showed a higher mean alpha angle of 41.3 ± 14.25^0 while the mean alpha angle in BIC was 38.9 ± 26.2^0. However the differences were not statistically significant (p=0.75).

Table 4. The mean and standard deviations (SD) of measurements and comparison between ipsilateral and contralateral teeth in BIC and PIC groups. A paired t-test was used to compare the groups. * indicates a *p* value ≤ 0.05.

Group	Tooth	Parameter	Ipsilateral	Contralateral	p-value
			MEAN±SD	MEAN±SD	
PIC	CENTRAL INCISOR	Mesiodistal Width	8.07±0.76	8.21±0.65	0.057
		Buccolingual Width	8.07±0.99	8.43±0.78	<0.001*
		Crown Length	10.62±0.5	10.9±0.47	0.005*
		Root Length	12.24±1.52	11.88±1.22	0.091
		Inclination	115.81±8.97	117.51±8.72	0.02*
		Angulation	6.25±7.31	6.25±3.79	1
	LATERAL INCISOR	Mesiodistal Width	6.47±0.67	6.41±0.82	0.484
		Buccolingual Width	7.1±0.83	7.05±0.79	0.832
		Crown Length	8.51±0.9	8.77±0.78	0.16
		Root Length	10.78±1.93	11.45±1.04	0.019*
		Inclination	113.7±13.78	114.31±6.81	0.81
		Angulation	11.89±10.89	11.03±6.75	0.659
	CANINE	Mesiodistal Width	7.91±0.91	7.57±0.76	0.03*
		Buccolingual Width	8.59±2.26	8.17±0.51	0.328

		Crown Length	9.38±1.08	9.56±1.32	0.484
		Root Length	14.03±1.55	13.54±3.98	0.497
	PREMOLAR	Mesiodistal Width	6.93±0.5	6.97±0.45	0.668
		Buccolingual Width	9.15±0.48	9.19±0.71	0.646
		Crown Length	7.5±1.21	7.43±1.36	0.73
		Root Length	12.27±1.89	12.49±1.78	0.487
		Inclination	95.3±8.93	97.45±6.15	0.208
		Angulation	10.97±6.46	10.57±5.58	0.81
BIC	**CENTRAL INCISOR**	Mesiodistal Width	8.28±0.87	8.33±0.84	0.52
		Buccolingual Width	8.55±1.16	8.52±0.98	0.722
		Crown Length	10.18±1.33	10.36±1.27	0.392
		Root Length	13.17±1.6	13.09±1.13	0.534
		Inclination	116.35±11.65	115.79±9.74	0.662
		Angulation	8.07±8.26	9.94±8.55	0.253
	LATERAL INCISOR	Mesiodistal Width	6.89±0.68	6.8±0.68	0.342
		Buccolingual Width	7.81±1.18	7.47±1.05	0.007**
		Crown Length	8.87±1.03	8.78±1.59	0.686
		Root Length	12.45±2.17	18.77±22.24	0.128
		Inclination	124.59±11.15	108.93±26.74	0.003*
		Angulation	20.31±12.27	14.67±7	0.015*
	CANINE	Mesiodistal Width	7.55±0.51	7.45±0.35	0.352
		Buccolingual Width	8.4±0.41	8.47±0.45	0.522
		Crown Length	9.18±1.55	8.96±1.28	0.442
		Root Length	13.47±1.68	15.06±2.07	<0.001*
	PREMOLAR	Mesiodistal Width	6.83±0.54	6.65±0.48	0.039*
		Buccolingual Width	9.41±0.61	9.51±0.52	0.233
		Crown Length	7.27±2.16	6.98±1.34	0.409
		Root Length	11.57±2.46	12.73±1.36	0.047*
		Inclination	92.77±10.74	94.16±7.65	0.457
		Angulation	9.83+8.52	7.95±8.27	0.469

On comparing the prevalence of lateral incisor agenesis and lateral incisor root dilaceration, PIC group presented with only one case in each and it was not statistically significant (p=0.5). 53.3% of patients with PIC presented with a retained deciduous while 33.3% of patients in BIC had retained deciduous canines (p=0.29). There was no significant difference in the distribution of the sector classification in both BIC and PIC (Table 5).

Table 5. Comparison of clinical parameters between BIC and PIC. Chi-square test was used to compare the prevalence of the parameters. * indicates a *p* value ≤ 0.05.

Parameters	BIC	PIC	p-value
Lateral Incisor Agenesis	0(0%)	1 (6.67%)	0.5
Dilaceration Of Lateral Incisor	0(0%)	1 (6.67%)	0.5
Deciduous Canine	5 (33.3%)	8 (53.3%)	0.296
Sector Classification:			
Sector I	1 (6.67%)	0(0%)	0.5
Sector II	1 (6.67%)	3 (20%)	0.299
Sector III	7(46.6%)	4 (26.6%)	0.256
Sector IV	6 (40%)	8 (53.3%)	0.464

All the quantitative measurements were repeated after a month on 10 randomly selected patients. The ICC for the tooth measurements (Mesiodistal width, Buccolingual width, crown height and root length) was good (0.799-0.854). ICC for inclination and angulation showed moderate-good reliability (0.735-0.786).

DISCUSSION

The present study aimed to compare the morphological and angulation differences of maxillary lateral incisors, central incisors, and first premolars in patients with buccal and palatal impacted canines. This comprehensive assessment contributes to the understanding of localized effects in maxillary canine impactions. The current study revealed that in PIC groups, the roots of the lateral incisors on the side of impaction were shorter than those on the contralateral side and shorter than those in the BIC and CG groups. The BIC group showed significant higher inclination and angulation of the lateral incisors in the ipsilateral side when compared to PIC and CG. On comparing the ipsilateral and contralateral sides, the buccolingual width, of the lateral incisor was larger on the impacted side. Kucukkaraca[12] examined the morphological traits and positions of lateral incisors and canines in buccal and palatal impacted canines. The study revealed that the lateral incisor length and root length on the side of impaction was shorter compared to the buccal instances similar to the present study. The study also revealed that canine angulation in palatal impactions was greater than in buccal impactions Previous studies[16-18] indicated that palatal impacted canines correlate with reduced dimensions of lateral incisors. Nevertheless, the current study did not identify a significant variation in the widths of lateral incisors among BIC, PIC, and CG, in contrast to earlier investigations.Controversy persists about the influence of lateral incisor dimensions on impactions, since a recent study identified no association between the dimensions of the lateral incisor and palatal canine impactions.[19] Previous research indicate that shorter roots of lateral incisors are associated with impacted canines on the impacted side when compared to contralateral side.[20, 21] Nevertheless, these investigations have not evaluated palatal and buccal displacements independently.

The angulation of lateral incisors has also been linked to impacted canines. The research conducted by Kanavakis *et al.*[22] indicated that in palatal impacted canines, the lateral incisor exhibited a mesial angulation of at least 2.5^0 greater than that on the non-impacted side, unlike the results of the current study. In the current study, the BIC group exhibited a greater distal angulation compared to the PIC and CG groups. PIC showed slightly more distal angulation than CG, however the differences were not significant. Prior study indicates that distal crown tilting and increased incisor inclination are markers of buccal canine impaction.[9] The study indicated that the angulation of palatal displaced canines increased when the lateral incisors were less mesially inclined, as shown in the current investigation. Light *et al.*[11] investigated the inclinations of teeth in buccal and palatal canine impactions. Buccal impactions were associated with higher inclination, indicating a more palatal positioning of the roots of lateral and central incisors, whereas palatal canine impactions demonstrate a more labial location of the roots of lateral incisors with differing inclinations. These findings align with the conclusions of the present research. Comparable results concerning the inclination of the lateral incisor were also seen in another investigation.[23] The mesiodistal width of the impacted canine was increased in the PIC group. Similar findings were seen in another study.[24] In

contrast, a previous study revealed no differences in the mesiodistal width of the canine between the impacted and non-impacted sides, unlike the present results.[25] The present study found short lateral incisor roots in patients with palatal impacted canines supporting the guidance theory of canine impaction to an extent.[2] The buccal canine impactions were associated with proclined and distally angulated incisors. Hence, these parameters can be used early in diagnosis for prediction of canine impactions.

The primary strength of the current investigation was the utilization of accurate 3D measures derived from CBCT data for complete examination of all neighboring tooth structures. CBCT provides an accurate representation of the dental structures unlike traditional 2D imaging.[26] Nonetheless, specific limitations existed. The limited sample size, due to the study being conducted at a single center, may diminish the generalizability of the results. Consequently, additional multicentric research with a bigger sample size must be conducted. The investigation was done retrospectively, a method prone to bias due to potential variations in the quality and completeness of the CBCT images. Future research should focus on prospective longitudinal studies that incorporate genetic factors to provide a thorough knowledge of the aetiology of impactions.

CONCLUSION

This study reveals that buccal and palatal impacted maxillary canines were associated with distinct alterations in the morphology and angulation of ipsilateral lateral incisors. Specifically, palatal impactions are linked to shorter root lengths of lateral incisors, while buccal impactions exhibit increased angulation and inclination of incisors. Additionally, in the palatal impaction group, the central incisors on the impacted side showed narrower buccolingual width and shorter crown length, whereas the buccal impaction group demonstrated increased buccolingual width and greater inclination and angulation of lateral incisors on the impacted side compared to the contralateral side. These findings underscore the importance of a thorough assessment of neighboring tooth structures

DECLARATIONS

Conflicts of interest and financial disclosures

The author declares that they have no conflict percent and there was no external source of funding for the research in question.

Ethical approval

This study was approved by the Institutional Review Board of Saveetha Dental College, (SRB/SDC/ORTHO-2306/24/019) and was conducted in accordance with the Declaration of the World Medical Association.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Informed Consent

All participants signed an informed consent form. Informed consent in regional language Tamil or English

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LITERATURE REVIEW

FLUORIDE PENETRATION DEPTH AND ITS IMPACT ON WHITE SPOT LESIONS AND MOLAR INCISOR HYPO-MINERALIZATION: A LITERATURE REVIEW

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ABSTRACT

Background: Fluoride (F) is an effective anticaries agent and can be delivered through various mediums at different concentrations. A pivotal aspect of fluoride's effectiveness is its ability to penetrate enamel, impacting its remineralizing potential but limited comparative data exists on their effects on lesion depth.
Objective: To evaluate the efficacy of topical fluoride on lesion depth specifically by comparing its effects on white spot lesions and Molar Incisor Hypo-mineralization (MIH).
Material and Methods: Literature review included 16 references dated from 2021 to 2024. The articles were divided into 6 clinical studies analyzing the Fluoride effect on enamel lesions and 4 articles treating its effect on MIH lesions. In addition, 4 studies revealing depth of enamel lesions and a reviewing of EAPD and ICDAS international guidelines.
Results: In healthy enamel and early carious lesions such as white spot lesions (WSLs), fluoride primarily acts on the superficial layers, typically within the outer 30-50 μm, promoting remineralization and inhibiting demineralization. However, deeper penetration is often limited, making fluoride less effective in reversing more advanced subsurface demineralization. In contrast, molar incisor hypo-mineralization (MIH) affected enamel is structurally compromised, with porosities extending much deeper (often beyond 300 μm), making it less receptive to conventional fluoride treatments.
Conclusion: Although Fluoride is frequently recommended, its remineralization effect on MIH lesions is limited, whereas it has been proven effective on white spot lesions.

Keywords: Lesion depth, remineralization, demineralization, fluoride, white spot lesion, MIH.

INTRODUCTION

Enamel demineralization is a critical issue in modern dentistry, leading to aesthetic and structural damage to teeth. Two common enamel conditions are white spot lesions (WSLs) and molar-incisor hypo-mineralization (MIH**)**. While both conditions weaken the enamel, their structural differences impact how well they respond to topical fluoride treatments.

White Spot Lesions

WSLs are the initial stage of dental caries, and their prevalence has been rising in recent years, ranging from 10% to 49%. They are commonly observed in patients undergoing orthodontic treatment, primarily due to the accumulation of bacterial plaque in the cervical region of the tooth (Fig1).[1] Clinically, the lesion appears as an opaque white area due to an optical phenomenon resulting from mineral loss and the difference in the refractive index of water and air filling the spaces within the enamel. As a result, the lesion exhibits a whitish color with reduced translucency due to increased enamel porosity.[2]

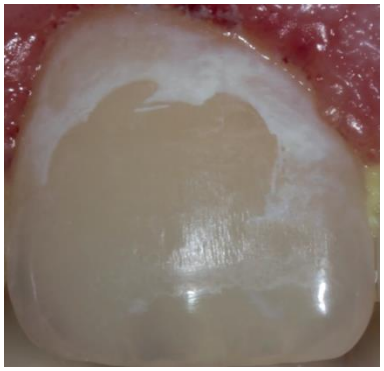


Figure 1. A clinical photo of WSL

According to the International Caries Detection and Assessment System (ICDAS), WSLs are classified as follows.[3]

Code 1	Code 2
Visible only on dry surfaces	visible on wet surfaces

Histologically, WSLs display four zones: (A) The demineralized translucent zone is characterized by the loss of the enamel structure by a 1% pore volume from the initial mineral loss.
(B) The dark zone is characterized by a 2 - 4% pore volume filled with air.
(C) The demineralization core zone is situated approximately 15 - 30 µm beneath the overlying intact enamel surface. It is characterized by a structureless enamel, with the highest pore volume 5-25%.
(D) The remineralization surface zone forms the outermost intact surface overlying the lesion, with < 5% pore volume (10 to100µm).[4]
In this context, accurate diagnostic tools are essential for measuring enamel demineralization depth. Silvabraga et al. evaluated OCT and TMR for measuring enamel caries depth in demineralized bovine enamel. OCT ranged from 24.0–174.0 µm, TMR from 59.2–198.0 µm, showing a strong correlation (rho = 0.75, $p < 0.001$), supporting OCT's reliability.[5]

Molar Incisor Hypo-mineralization

The term molar-incisor hypo-mineralization (MIH) was initially introduced by Weerheijm et al. in 2001, where it was defined as "hypo-mineralization of systemic origin, characterized by demarcated, qualitative defects in the enamel of one to four first permanent molars (FPMs), often associated with affected incisors.
The histopathological data relating to MIH reveals that, unlike other types of enamel defects, *hypo-mineralization* in MIH begins at the amelodentinal junction (ADJ) and not at the surface of the enamel." For example, Incisor enamel thickness ETH ranged between 0.60 and 0.84 mm (600 and 840 µm), so it's expected that lesion will fall within this range.



Figure 2. MIH figure representation (6)

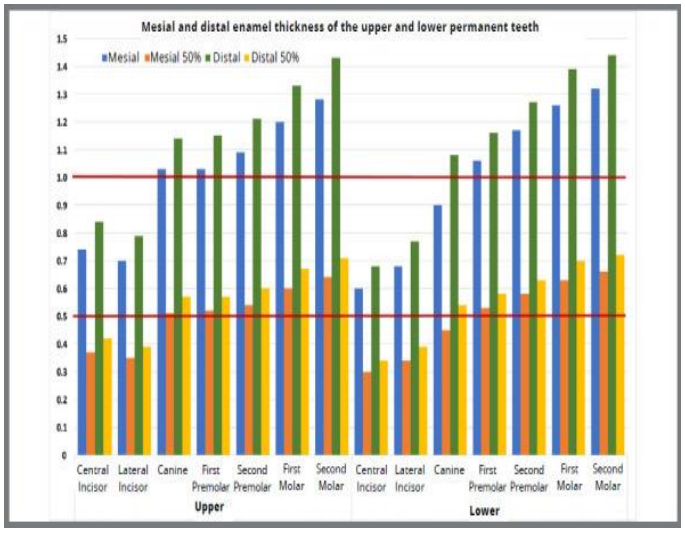


Figure 3. Enamel thickness on the mesial and distal surfaces of the upper and lower permanent teeth. Lower horizontal line indicates value limits up to 0.5 mm and upper horizontal line indicates value limits up to 1 mm. [7]

The European Academy of Pediatric Dentistry (EAPD) has established diagnostic criteria for assessing dental defects. These criteria are based on the description of the defect, including the affected teeth, presence of demarcated opacities, sensitivity, atypical restorations, and severity classification. The severity is categorized into two levels: mild and severe lesions.
Meanwhile, the EAPD outlines treatment options involving fluoride and their effects, as detailed in the table below.

Table 1. Grade Ratings for Remineralization Management in MIH

Interventions for remineralization	No. of studies	No. of teeth	GRADE of evidence quality	Strength of Recommendation
Topical CPP-ACP can be used to remineralize affected teeth	3	61	Moderate	Conditional
Topical CPP-ACFP/NaF 4–5% with and without tricalcium phosphate can be used to remineralize affected teeth	3	88	Very low	Conditional

Given the limited capacity for enamel recovery, the EAPD guidelines do not recommend fluoride-based treatments as a primary approach for MIH management, as shown in table 2.[8]

Table 2. Diagrammatic Management Summary for MIH-Affected Incisors

Severity	Clinical examination	Problems	Therapeutic approach
Severe ↑ Mild	Opacities + Loss of enamel structure	Risk of tooth fracture + Aesthetic concerns	Localised macro-abrasion/micro-abrasion + Resin infiltration + Composite restoration
	Opacities on the incisal edge	Aesthetic concerns ± Risk of tooth fracture	Localised macro-abrasion/micro-abrasion + Resin infiltration + Composite restoration
	Brown/yellow opacities	Aesthetic concerns	Localised macro-abrasion/micro-abrasion + Resin infiltration + Composite restoration
	White/creamy opacities	Aesthetic concerns	Regional whitening +/- Micro-abrasion +/- Resin infiltration OR don't do anything

Therefore, distinguishing between the two lesions is crucial for selecting the appropriate treatment. Table 3 presents a comparative analysis of WSL and MIH.

Table 3. Comparative analysis of WSL and MIH

WSL	MIH
Initial stage of dental caries	Hypo-mineralization begins at the amelodentinal junction,(lesions> 1 mm)
Lesion appears opaque white	White-creamy to yellow-brown opacities
Cervical region of the tooth (Fig1)	Incisal edge of the incisors (lesions do not involve the cervical third of affected teeth) (Fig2)
Due to the accumulation of bacterial plaque	Hypo-mineralization of systemic origin
Lesion depth varied from 24.0 to 198.0 µm	Lesion depth ranged between 600 and 840 µm
All teeth can be affected	Permanent molars, often associated with affected incisors

This study comprehensively evaluates the efficacy of fluoride treatment on enamel lesions by examining lesion structure, clinical results, and advancements in treatment approaches. The comparison of fluoride treatments based on their depth of penetration provides practical guidance for clinical decision-making. Moreover, the findings establish a crucial baseline for future research and the formulation of evidence-based guidelines for enamel lesion management.

MATERIALS AND METHODS

This literature review covered the field of WSL, MIH and remineralization by using varnish fluoride. It was reported per the PRISMA guidelines and outlined the research question, objectives, inclusion and exclusion criteria, and the methodological approach. The review included articles from Google Scholar, Medline, Scopus, Web of Sciences, and PubMed. Two review authors screened the results of the searches against inclusion criteria, extracted data and assessed the risk of bias independently and in duplicate.

This review presents a comparative overview of remineralization studies between WSL and MIH focusing on NaF (5%) as an active ingredient.

- The literature search included sixteen articles from the period 2021 to 29 December 2024.
- Scientific articles published in the English language.
- Role of caries in lesion formation.
- Six Clinical studies that mention the effect of fluoride varnish on Enamel lesions.
- Four studies mention the effect of fluoride varnish on MIH lesions
- EAPD and ICDAS Guidelines.

Experience revealing the depth of enamel lesions:

- Radiographic evaluation of enamel thickness

- Enamel caries lesion depth (Aline Silva Braga).

- Findings from Chris Ivanoff's study about fluoride penetration into enamel.

RESULTS

The results are divided into WSL-remineralization studies and MIH remineralization using in both studies FV 5%NaF. Although fluorides are frequently recommended for MIH patients, the clinical evidence for using fluoride for remineralization of MIH is very low (table1).

Six studies were found analyzing the WSL remineralization effect using 5% sodium FV (table 4), And four studies evaluating the effect of same FV on MIH patients (table5).

Table 4. Overview of studies analyzing the WSL remineralization effect using 5% sodium FV

Articles	Age	Patients	Applications range	Duration	Diagnosis method	Results
Effectiveness of Fluoride Varnishes for White Spot Lesion Prevention and Remineralization during Orthodontic Treatment: A Randomized Controlled Trial. [9]	Adolescents	65	Weekly for the first 4 weeks and then after 6 and 12 months	12 months	-ICDAS -Quantitative light-induced fluorescence -ANOVA -Kruskall-Wallis tests	Decline of WSL at 12 months
Fluoride varnish, ozone and octenidine reduce the incidence of white spot lesions and caries during orthodontic treatment.[10]	16 - 50	30	5 % NaF Every 4 weeks	12 months	WSL index	Reduce the incidence of WSLs comparing to control group
White spot lesions: diagnosis and treatment – a systematic review. [2]	12 - 22	110	Every month	6 months	Diagnodent	WSLs decreased at 3-month and more at 6-month
Systematic review of preventive and treatment measures regarding orthodontically induced white spot lesions.[11]	12 - 25	240	2 app at 3 months and 6 months	6 months	Quantitative light-induced fluorescence	Treatment can induce the greatest amount of remineralization in comparison with control group
Systematic review of preventive and treatment measures regarding orthodontically induced white spot lesions. [12]	13 - 20	12	2 app at 1 week interval 1 week 1 month 2 months 3 months	3 months	Diagnodent	Capable of controlling the WSLs adjacent to bracket
Analyzing Effectiveness of Different Delivery Methods for Remineralization Agents in Pediatric Dental Health: A Systematic Review. [12]	6 - 7	12	Applied at baseline, 1 month, and 3 months	3 months	Diagnodent	showed significant remineralization at 3 and 6 months
Analyzing the Effectiveness of Different Delivery Methods for Remineralization Agents in Pediatric Dental Health: A Systematic Review.[12]	8 - 15	21	5 treatments (weeks 1-4 and week 12	12 weeks	Visual assessment Diagnodent	Visual appearance Laser fluorescence showed significant remineralization in all treatment groups (p <

						0.001).
Analyzing the Effectiveness of Different Delivery Methods for Remineralization Agents in Pediatric Dental Health: A Systematic Review.[12]	12-20 years	240	No specific duration mentioned, treatment was applied during monthly visits	Once a month	QLF imaging, assessing DF (%), area (mm^2), and DQ (mm^2 × %)	significant improvements in demineralization metrics (DQ,DF, area) after 3 and 6 months
Methods for Remineralization Agents in Pediatric Dental Health: A Systematic Review.[12]	12-36 Months	140	Fluoride varnish is applied every 4 months: 1minute per session	12 months	Visual inspection of WSL area. DMFT index	Significant reduction in WSL after 12 months 51% decrease in WSL
Evaluation of the preventive effect of two concentrations of xylitol varnish versus fluoride varnish on enamel demineralization around orthodontic brackets: a randomized controlled trial.[13]	Orthodontic patients	30	App at T1(before ortho treatment) T2 at 3 months T3 at 6 months T4 after treatment	Not mentioned	Diagnodent	lower mineral loss than the placebo group at T2 and T3

Table 5. Overview of studies analyzing the MIH-remineralization effect of 5% sodium fluoride.

Article (year of publication)	Age	Patients	Applications range	Duration	Diagnosis method	Results
Remineralization Strategies for Teeth with Molar Incisor Hypomineralization (MIH): A Literature Review.[6]	6-17	29	3 app of 5% sodium fluoride varnish (Colgate Duraphat®) for 1 minute; T0 T1= day15 T2= day30	45days	Diagnodent	Effective in moderate lesions
Treatment Approaches to Molar Incisor Hypomineralization: A Systematic Review.[14]	9-12y	51	4 app of 5% NaF (Duraphat) varnish, with a 1-week interval	4 Weeks	Quantitative Light- Induced Fluorescence QLF. The data were analyzed by repeated measures ANOVA and Tukey's	No significant changes

Structural integrity of MIH-affected teeth after treatment with fluoride varnish or resin infiltration: An 18-Month randomized clinical trial. [15]	6-12	51 (86 teeth)	FV Duraphat	18 months	Fisher's Exact was used to test the association of treatments with PEB, the Kaplan-Meyer and Cox DMFT	loss of integrity due to post-eruptive enamel breakdown (PEB)
Effects of different remineralization agents on MIH defects: a randomized clinical study.[16]	6-9	49	FV no clarification	24 months	Diagnodent	No statistically significant difference

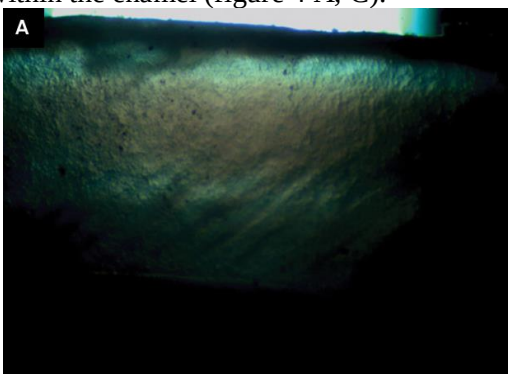
DISCUSSION

Recently, non-invasive approaches to managing early carious lesions for preserving healthy tooth substance and preventing lesion progression have become important. In this regard, fluoride has been shown to increase remineralization and fluoride varnishes have gained importance.

In this regard, this literature review represents a comparative analysis of the remineralization effects of sodium fluoride (NaF) varnishes on white spot lesions and molar incisor hypo-mineralization (MIH). It examines the efficacy of NaF varnishes in promoting enamel remineralization in both conditions, providing insights into their clinical implications and potential benefits.

NaF Varnish (5%) is a commonly used fluoride varnish that has been evaluated by similar studies, and it increases the fluoride content of the enamel by releasing sufficiently high levels of fluoride ions. In the present study, Duraphat, the gold standard fluoride varnish, containing NaF alone, was used. White spot lesions (WSL) and molar incisor hypo-mineralization (MIH) differ in their response to remineralization with sodium fluoride (NaF) varnishes due to variations in their etiology, enamel structure and depth of the lesion inside.

An image with polarized light microscope demonstrates the effectiveness of fluoride penetration within the enamel (figure 4 A, C).



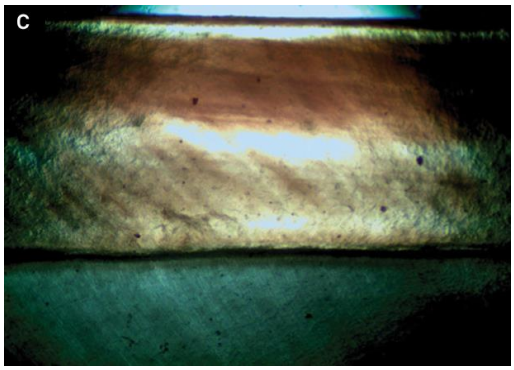


Figure 4. Polarized light microscopy images showing the subsurface caries lesion, as well as the region of the most mineralized surface, resulting from varnish application. A) dark color in the subsurface area (the most demineralized); C) Duraphat group.[17]

The distinct contrast observed in the treated areas highlights the mineral deposition facilitated by fluoride, reinforcing its role in enamel remineralization. The image provides visual evidence of fluoride's ability to integrate into demineralized regions, enhancing enamel structure and reducing lesion porosity. Such microscopic analysis is crucial for understanding the depth and extent of fluoride uptake, particularly in different enamel conditions like white spot lesions and molar incisor hypo-mineralization.

In the present study, to monitor mineralization changes in both cases, laser fluorescence scanning method with a Diagnodent device was used in most studies.

In most clinical cases of white spot lesion (WSL), sodium fluoride (NaF) Varnish has shown a significant reduction in lesion size and severity at both 3- and 6-month follow-up visits (Table 4).

The effectiveness of NaF in WSL is attributed to its ability to enhance remineralization by depositing fluoride ions, as deep as possible, which integrate into the enamel and strengthen its structure.

However, in cases of molar incisor hypo-mineralization. (MIH), clinical studies have reported minimal or no significant improvements following NaF varnish application (Table5).

This lack of response, along with experimental studies analyzing the depth of fluoride penetration into enamel, inspired me to undertake this article.

Dr Chris Ivanoff has conducted significant research on enhancing fluoride penetration into dental enamel to improve the treatment of lesions and reduce their depth. Traditional methods of fluoride application often result in limited penetration, typically not exceeding 50 micrometers (μm) into the enamel. To address this limitation, Dr Ivanoff explored the use of Di electrophoresis (DEP) and alternating current electroosmosis (ACEO) as techniques to facilitate deeper fluoride delivery.

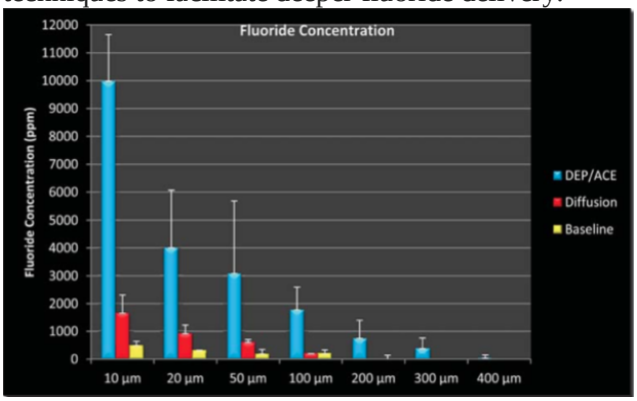


Figure 5. Chart showing how fluoride concentrations vary at different depths within tooth enamel[18]

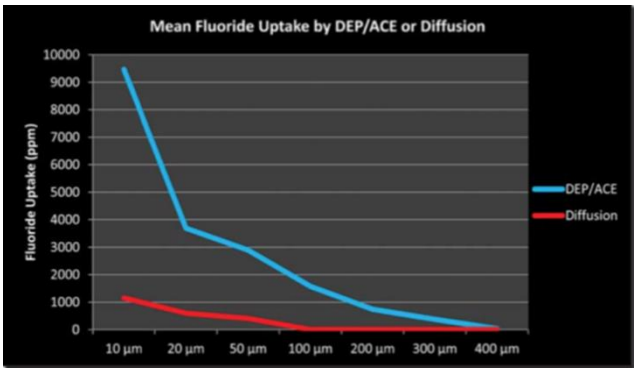


Figure 6. Chart showing Mean fluoride Uptake by DEP/ACE or diffusion (18)

CONCLUSION

In summary, the comparison of enamel conditions such as white spot lesions (WSLs) and molar-incisor hypo-mineralization. (MIH) reveals significant differences in their structural characteristics and treatment responses, particularly concerning fluoride varnish applications. WSLs, being an initial stage of dental caries, demonstrate a robust capacity for remineralization through topical fluoride treatment, leading to a marked reduction in lesion size and severity. In contrast, MIH, characterized by a systemic origin of hypo-mineralization and significant structural deficits beginning at the amelodentinal junction, shows a limited response to fluoride varnish treatments.

This literature review highlights that while sodium fluoride (5% NaF) varnishes have proven effective in the management of WSLs, their clinical efficacy in MIH presents minimal improvement or no significant change. The findings underscore the necessity for tailored management strategies for these enamel conditions, focusing on the inherent differences in lesion depth, structural integrity, and underlying pathology. Moreover, advancements in fluoride delivery methods, such as those explored by Dr. Chris Ivanoff, offer promising avenues for enhancing fluoride penetration and efficacy in treating deeper enamel lesions. The potential application of these innovative techniques may improve the clinical outcomes for MIH patients, warranting further exploration and research.

As evidence-based guidelines continue to evolve, it is crucial for practitioners to be informed about the comparative effectiveness of fluoride treatments in managing enamel defects. Future studies should aim to establish standardized protocols for addressing MIH while assessing new technologies that may augment treatment efficacy. Overall, this review provides foundational insights into enamel remineralization strategies, reinforcing the importance of individualizing treatment approaches to optimize patient outcomes in modern dental practice.

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Declaration of competing interest
The authors declare None of the authors have any relevant financial relationship(s) with a commercial interest.

Data Availability
Not applicable

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ORIGINAL ARTICLE

A STUDY ON HEMOSTASIS AND HEALING: COLORADO MICRODISSECTION NEEDLE VS SCALPEL FOR INTRAORAL INCISIONS

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ABSTRACT

Background: Various armamentarium used for intraoral incisions in maxillofacial surgery ranges from simple scalpel to lasers, electrosurgical tip. Few of them have disadvantages such as being non hemostatic, requiring training or which are costly and are sophisticated. The quest for a better tool for intraoral incisions has lead to the use of Colorado microdissection needle which achieves hemostasis, requires less equipment and easy to handle. **Aim:** To evaluate the effectiveness of the Colorado microdissection needle over the scalpel during intraoral incisions for impacted mandibular third molar surgery.

Methods and Materials: A total of 30 patients were randomly categorized into the Group A using Scalpel and Group B using Colorado microdissection needle for Terrance ward's Incisions. The parameters assessed were Incision time, Quantity of blood loss, Wound healing on 7th and 14th postoperative day. Statistical analysis used were a parametric independent - T test and a non-parametric test (Mann-Whitney U test). **Results:**The results showed that, apart from wound healing there was no statistically significance difference in incision time and blood loss between the groups.

Conclusion: The Colorado microdissection needle can be used as an alternative for incisions, as it appears to cause less tissue damage while providing tissue healing comparable to that of a scalpel.

Keywords: Colorado tip, third molar surgery, Wards incision, scalpel

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INTRODUCTION

Third molars are the most commonly impacted teeth in the oral cavity, with 33% of the population having one tooth impacted. Surgical removal of third molars is the most commonly performed procedures in the Oral and Maxillofacial practice, to prevent and treat a variety of pathogenesis associated with impacted teeth[1]

Incision which is an integral part of third molar surgeries is often accomplished with stainless steel scalpels over the decades because of its accuracy, ease of handling, and minimal damage to the tissues, but with the incisions marked by scalpel, excessive blood flows to the operative field leading to poor visibility.

To overcome the disadvantages, Electrocautery was introduced in the early 20th century into the clinical practice, with the emergence of modern surgical diathermy units, this technique is becoming popular due to its hemostasis, faster incision, and decreased overall operative blood loss[2,3].

Though this hemostatic ability represents a major advantage, these electrocautery units are associated with certain drawbacks because of its larger tip size, thus lead to the emergence of a microdissection needle with fine electrode tips[4,5]

The Colorado microdissection needle(CMN) is one such (Stryker-Leibinger, Freiburg-im-Breisgau, Germany) (figure1) which has been introduced into the clinical practice in 1997, with the primary feature of an ultra-sharp tungsten tip that concentrates the waveform to a very small spot and allows for the use of extremely low wattage, resulting in precise cutting, cauterization, and less tissue damage and postoperative pain[5].

Because of these properties, the microdissection needle has a wide range of applications in the fields of neurosurgery, ophthalmology, and others[4,8,12]. There are limited literature available regarding the use of colorado microdissection needle for intraoral incisions. Thus, the purpose of the study is to evaluate the effectiveness of colorado microdissection needle during intraoral incisions for the mandibular third molar surgery.

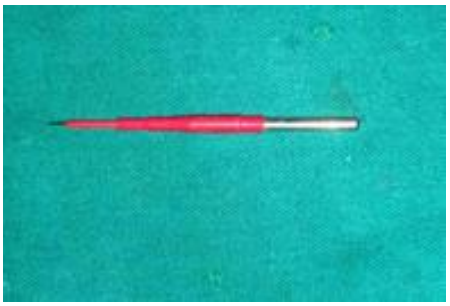


Figure 1. Colorado microdissection needle(type N 103 A)

MATERIALS AND METHODS

This prospective randomized comparative study was done in 30 patients who reported to the Department between May 2021 and June 2022 with slightly or moderately difficult impacted lower third molar according to modified pederson's index, were taken into the study. After fulfilling the inclusion criteria, the selected patients underwent surgical removal of impacted mandibular 3rd molar.The nature and design of the clinical study were explained to all the patients and informed consent was taken from them for the study. The clearance from the ethical committee was taken for this study.

Inclusion criteria:

- Healthy patients ASA I
- Age between 18-45 years
- Patients who are indicated for surgical extraction of impacted third molar teeth
- Patients who have signed the informed consent and who are willing to participate in the study

Exclusion criteria:

- Medically compromised patients
- Patient on anticoagulant and anti-platelet therapy
- Patient with bleeding disorders
- Patient on Corticosteroid therapy
- Patient with local inflammation and infections

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STUDY GROUPS

Patients who visited the Department of Oral and Maxillofacial Surgery for surgical extraction of impacted mandibular third molar were randomly categorized into Group A and Group B, randomization is carried out by taking every alternate patient with the inclusion criteria into Group A and Group B. In Group A Terrance wards Incision was given using Scalpel (NO 15) and in Group B using Colorado Microdissection Needle.

EVALUATION OF STUDY OUTCOMES

SURGICAL PROCEDURE

Following the standard precautions of scrubbing and draping the patient under aseptic conditions, classical IAN, lingual, and long buccal nerve blocks were given with 2% lignocaine containing 1:80,000 epinephrine. Local infiltration was avoided in both groups. Before the start of the procedure weight of dry gauze was measured using a digital weighing device(Gravimetric method). After achieving sufficient anesthesia, in Group **A** Terrance wards incision was given with the surgical scalpel (no 15 BP blade).In Group **B** Terrance wards incision was given using a 3 cm long straight CMN (Type N103A) (figure 2) attached to an electrocautery unit (BASCO INDIA - 250 surgical diathermy) which was used at a power setting of 3 in cutting and coagulation mode and no suction for evacuation of blood was done while giving the incision. A full thickness mucoperiosteal flap was elevated using no 9 molts periosteal elevator and the weighed dry gauze were used till the entire mucoperiosteal flap was raised. The circumferential buccal bone guttering was done with no. 702 and 703 straight fissure carbide bur using motor-driven handpiece under copious saline irrigation. Extraction of the tooth was performed either by simple elevation or odontectomy according to the impacted tooth. The socket was debrided with the povidine iodine solution, the wound was approximated and sutured using a 3-0 silk suture. The blood-soaked gauze swabs were weighed, and differences between the soaked gauze and dry gauze were recorded to calculate the amount of blood loss. Post-extraction instructions and medications were given to both groups. Parameters such as Incision time and quantity of blood loss were measured intra-operatively. Wound healing was assessed using Modified Landry's scale on 7th and 14th postoperative days.



Figure 2. Terrance wards Incision with Colorado Microdissection Needle

Parameters

The Incision time was calculated in minutes/seconds using stop watch. The Time taken for incision is calculated from the start of incision to the complete elevation of the mucoperiosteal flap.

Quantity of blood loss was measured by Gravimetric method[10] (figure 3), which is based on the use of gauze swabs employed for collecting blood and weighing them after use. A standard prepacked sterile gauze of size 7.5cm X 7.5cm was taken and the weight was measured using the digital weighing device (ATOM Electronic compact scale SF-400D). Immediately after their use, the weight of used gauze pieces was measured so that evaporation doesn't become a critical factor. The difference between the two measurements was considered the total amount of blood loss. One gram of blood was regarded as the equivalent of 1ml of blood loss.

Blood loss = (total weight of wet gauze)- (total weight of dry gauze) in grams 1 gm = 1 ml of blood loss.



Figure 3. Postoperative weight of Gauze

Wound healing was assessed by Modified Landry's scale[11] on 7th and 14th postoperative days (Figure 4).

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Figure 4. Wound healing on 14th postoperative day

Statistical analysis

The data were analyzed in SPSS-V28. Descriptive statistics were represented with percentages and mean with standard deviation. A parametric test independent - T test is used for the statistical analysis of incision time. A non-parametric test (Mann-Whitney U test) is used for the analysis of quantity of blood loss and wound healing.The results were averaged (mean + standard deviation) for parameters of each group. Results were considered significant if the p values were less than 0.05.

RESULTS

A total of 30 patients were taken into the study, who were randomly divided into two equal groups. Mean value of incision time in Group A is 158 sec and in Group B is 139.33 sec. There is no statistically significant difference in mean Incision time between Group A and Group B (P=0.459). The results states that, there is no difference in time taken for incision with the colorado microdissection needle to that of scalpel for giving an intraoral Terrance wards incision(table 1).

Table 1. Comparison of mean Incision time between Group A and Group B

	Groups	N	Mean	SD	t	P Value
INCISON TIME	Group A	15	158.00	73.11	0.751	0.459
	Group B	15	139.33	62.60		

Mean amount of blood loss is 1.47ml in Group A and 1.22 ml in Group B. The results shows that there is no statistically significant difference in mean rank of quantity of blood loss (P=0.443) between the Group A and Group B. From the above result it can be inferred that the amount of blood loss is almost equal when Colorado microdissection needle or scalpel is used for Terrance wards incision(table 2)

Table 2. Comparison of mean rank of quantity of blood loss between Group-A and Group-B

	Groups	Normal	Mean	SD	Median	Mean rank	P Value
QUANTITY OF BLOOD LOSS	Group-A	15	1.47	1.26	0.83	16.73	0.443
	Group-B	15	1.22	0.95	0.75	14.27	

The mean rank of wound healing on 7th day in Group A is 3.8 and in Group B is 3.73 and the mean rank on 14th day in Group A is 4.07 and in Group B is 4.2. The result shows that there is no statistically significant difference in wound healing on 7th day (P=0.671) and 14th day (P=0.355) between both the Groups. From the result it can be inferred that no difference is seen in wound healing when Colorado microdissection or scalpel is used for intraoral Terrance wards incision (table3).

Table 3. Comparison of mean rank of wound healing on 7th and 14th days between Group-A and Group-B

		N	Mean	SD	Median	Mean rank	P Value
WOUND HEALING ON 7TH DAY SCORE	Group -A	15	3.8	0.41	4	16	0.671
	Group -B	15	3.73	0.46	4	15	
WOUND HEALING ON 14TH DAY SCORE	Group -A	15	4.07	0.26	4	14.47	0.355
	Group -B	15	4.2	0.56	4	16.53	

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Table 4 shows that there is a statistically significant improvement (P=0.008) in wound healing is seen from 7th day to 14th day in Group B. From the above results, it can be inferred that wound healing in Colorado microdissection needle was better on 14th day in comparison to scalpel for intraoral Terrance wards incision.

Table 4. Pairwise comparison of rank of wound healing between 7thand14thday among GroupA and Group B

	Wound healing	Ranks	N	Mean Rank	Z	P Value
Group A	14th day- 7th day	Negative Ranks	0	0	-2	**0.046***
		Positive Ranks	4	2.5		
Group B	14th day- 7th day	Negative Ranks	0	0	-2.64	**0.008***
		Positive Ranks	7	4		

*statistical significance

DISCUSSION

Surgical removal of third molar is commonly performed procedure in Oral and Maxillofacial Surgical practice, of which incision and flap reflection are integral part for the success of the procedure. Electrosurgery refers to passage of high frequency alternating current through the tissue to obtain a predetermined surgical effect, with currents up to 500 mA can be passed safely through the patient[3]. The hemostatic ability represents a major advantage of the standard electrode tip, there are certain drawbacks includes delay in healing process and thermal injury.Studies have shown that heat produced by electrocautery unit is influenced by certain factors such as current intensity, contact duration between the tissue and electrode, and the size of electrode tip[8,14,15]. When standard electrode tip is used, there is increased use of operating power density, more latent heat production there by more damage to the tissues. Apart from these, there is decrease in wound tensile strength, increased inflammation and tissue adhesion. Thus, the concept of electrocautery instruments with fine tipped electrodes emerged. The presence of ultra sharp tungsten tip, allows precision cutting, cauterization, use of low wattage, resulting in less tissue damage. This tip ensures a constant, high intensity heat concentration which allows for clean microdissection without affecting adjacent areas[8,9,15].

In the present study the time taken for incision is less with colorado microdissection needle in comparison to scalpel, but the difference was not statistically significant.A study reported by Peneva Margarita et al[17],stated that the speed of incision was slightly more in electrosurgical microneedle but was not statistical significant. Another study reported by Milan Modi et al[12] stated that the incision time was significantly less with CMN[4]. According to the study reported by Sheik B[14] for the use of CMN in neurosurgery, the speed of incision was significantly less when using colorado microdissection needle

Bleeding during incision and surgery significantly influence accessibility of the surgical field and also the outcome of the procedure[19]. Rampalli Viswa Chandra et al[16], compared the incisions made by scalpel, electrocautery tip and colorado microdissection needle for periodontal surgeries, stated no statistical significance in quantity of blood loss among the groups. A study reported by Ali S.Al-Qabtani[18], stated that intra-operative blood loss was significantly less with the microdissection needle when compared with cold scalpel during tonsillectomy procedures. Another study done by Praveen sathish kumar et al[13], stated that the intra-operative blood loss was significantly less during incision with colorado microdissection needle compared to scalpel for neck dissections. Sheikh.B[14] reported that the approximately 4 times more blood loss is seen when scalpel is used for skin incisions in

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neurosurgery.According to the study reported by Milan Modi[9]et al stated that the blood loss was significantly less in colorado microdissection needle compared to scalpel. Peneva Margarita et al[17], reported that the blood loss was significantly less in electrosurgical microneedle. In this study the results showed the blood loss is less with colorado microdissection needle clinically, but not statistically significant.

Wound healing is a normal biological process consisting of four phases: haemostasis, inflammation, proliferation and remodeling[20,21]. The results obtained, indicates that no statistical significant difference is seen in wound healing on 7th and 14th day between scalpel and colorado microdissection needle. An study reported by P. E. M. Butler et al[23] stated that the incisions marked by modified electrode heal better than the standard electrosurgical electrode, as they have a histological response close to that of steel scalpel wounds. Study reported by B.Sheik[14] for skin incisions during neurosurgery where the wound was inspected on 1,3,14 days post-operative for colour, presence of charcoaling effect,viability and dermal peeling. Stated that, no difference was seen between the skin edges of incised by the steel scalpel and micro-needle electrocautery. Study shows not much difference in wound healing on 7th and 14th postoperative days, but ironically improved healing observed on 14th day compared to 7th day in colorado microdissection needle, this can be likely due to the fineness of tip, decreased surface area and less tissue distortion.

CONCLUSION

Colorado microdissection needle and scalpel are effective for incisions in mandibular third molar surgeries , from our observations the Colorado microdissection needle offers superior incision precision and improved hemorrhage control.

DECLARATIONS

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None

Competing and conflicting Interests

The authors declare no conflicts of interest.

Ethical approval

Ethical clearance for the present study was given by the institutional review board and the ethical committee of GITAM Dental College and Hospital.

Consent to participants

A written informed consent was obtained from the individual

Data availability

The data is available upon the considerable request to the corresponding author

Informed consent

The interns who consented to take part were enrolled after being informed of the study objectives through a participant information form.

Abbreviation

CMN- Colorado Microdissection Needle

IAN-Inferior Alveolar Nerve

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RESEARCH ARTICLE

COMPARATIVE EVALUATION OF MASTICATORY EFFICIENCY AND NUTRITIONAL STATUS OF PATIENTS BEFORE AND AFTER A FULL MOUTH REHABILITATAION -PROSPECTIVE CLINICAL TRIAL

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ABSTRACT

Aim: To evaluate masticatory efficiency and nutritional status prior and subsequent to a full mouth rehabilitation therapy

Materials and Methods: The study followed the CONSORT guidelines and was a prospective clinical trial. This trial was conducted on ten patients who were undergoing full mouth rehabilitation. Masticatory efficiency was calculated before the start and 1 month post completion of rehabilitation where the subjects were given 9 grams of peanuts (3 grams - 3 times). The participant was instructed to complete 40 chewing strokes. The masticated food was subsequently collected in a disposable container. Additionally, the participant rinsed their mouth with water twice, and the resulting rinsate was combined with the chewed food in the same disposable container. Following thorough mixing of the chewed food using a glass rod, the mixture was strained through a 10 mesh sieve. This specific test serves the purpose of quantitatively assessing the chewed food. The strained liquid, referred to as the filtrate, was then carefully transferred into tubes and subjected to centrifugation at 3000 revolutions per minute for a duration of 5 minutes. This centrifugal process effectively separated the filtrate into two distinct components: a solid pellet and a liquid supernatant. The supernatant, or the liquid portion, was subsequently discarded, while the pellet, or the solid residue, was weighed using a digital scale. In this study, masticatory performance was quantified according to the methodology proposed by Yurkstas and Manly. This evaluation encompassed assessing particle size, as determined by the size of the sieve employed, by calculating the ratio of the weight of the test food that passed through the sieve to the total weight of the test food that was recovered. This ratio was then expressed as a percentage. The initial wet weight of the materials was computed as the starting point for these calculations. The specimens were then placed in a hotair oven at 40℃ for 8 hours and again the dry weights were measured. Fasting venous blood samples from each participant were collected for means of measuring hemoglobin, total serum calcium, total serum protein, and total cholesterol levels as indicators of nutritional status. The tests were carried out at similar intervals as chewing efficiency.

Results: All of the patients showed a statistically significant improvement in masticatory efficiency post full mouth rehabilitation (p <.001) which was calculated with Yurkstas and Manly formula. There was no significant relationship between age, gender and occlsal scheme provided to the masticatory efficiency pre and post FMR. Paired sample t test revealed that haemoglobin, total serum protein and total ionic calcium showed a significant improvement post full mouth rehabilitation at 1 month follow up (p<0.05). There was no statistically significant difference in cholesterol values.

Conclusion: We can conclude that full mouth rehabilitation leads to a notable increase in masticatory efficiency. A significant improvement in nutritional status was also seen post completion of therapy. There was no influence of age, gender and occlusal scheme on chewing efficiency.

Keywords: *Chewing efficiency, full mouth rehabilitation, nutritional status*

INTRODUCTION

Full mouth rehabilitation, also known as full mouth reconstruction, is a comprehensive and complex dental treatment that aims to restore the function and aesthetics of the entire mouth. This type of treatment is typically recommended for patients who have extensive damage or decay to their teeth, gums, or jaws, or those who suffer from complex occlusal discrepancies Full mouth rehabilitation involves a combination of different dental procedures, which may include crowns, bridges, veneers, dental implants, orthodontics, and periodontal therapy. The specific treatments that are required will vary depending on the individual patient's needs and the extent of their dental problems.[1,2]

Indications for full mouth rehabilitation include a variety of dental issues mainly involving loss of vertical dimension of occlusion. These factors can have a significant impact on a patient's quality of life, including their ability to speak, chew and smile comfortably. Additionally, untreated dental problems can lead to more severe health issues such as infections, chronic pain, and even systemic diseases.[3]

Masticatory efficiency is defined as the number of strokes needed to achieve a certain particle size reduction . Various factors affect chewing efficiency, such as tooth morphology, occlusal contacts, and jaw movements . Studies indicate that compromised chewing efficiency can have adverse effects on nutrition, overall health, and quality of life. Comparing to an ideal dentate state, edentulism leads to a significant decrease in bite force , comminution of food and Body Mass Index (BMI) Improvement in masticatory efficiency as an outcome of a prosthetic rehabilitation is still highly debated in current literature, where there is a significant improvement for completely edentulous people, the results for partially edentulous people are still controversial.[4]

To assess masticatory performance, various tests are performed that involve standardized food items such as chewing gums, carrots, peanuts or meat substitutes. The size of the particles and texture produced is evaluated to determine the quantitative measure of chewing efficiency . The results obtained from these tests can be used to compare pre and post-treatment outcomes. Electromyography (EMG) and bite force analysis are some of the other techniques commonly used to assess masticatory muscle activity patterns, coordination and maximum bite force exerted .[5]

Research has demonstrated that poor masticatory efficiency can lead to a variety of nutritional deficiencies, such as low levels of protein, calcium, and vitamins . This can result in a range of health problems, including malnutrition, osteoporosis, and cardiovascular disease, especially in older population with worn and torn dentition . Furthermore, compromised chewing ability can lead to digestive problems and reduced appetite, further promoting nutritional deficiencies. Full mouth rehabilitation can address these issues by restoring the natural structure, function and esthetics of the oral cavity.[6]

There have been many researches done on comparing masticatory efficiency prior and subsequent to implant and removable rehabilitation (fixed and removable) but none on effect of a full mouth rehabilitation on the outcome. Thus, the aim of this study is to compare masticatory efficiency and nutritional status of patients prior and subsequent a full mouth rehabilitation.

MATERIALS AND METHODS

Study Design

The current study is a prospective clinical trial that followed the standards published by Consolidated Standards of Reporting Trials. Ethical clearance was obtained from Ethical Committee of Saveetha Dental College and Hospitals, Chennai-SRB/SDC/PROSTHO-2003/22/TH-025.

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Sample Size Calculation

The sample size for the present trial was based on the methodology and results of the study by P.Pera et al.[7] A power analysis was established by G*Power version 3.0.1(Franz Faul universitat , Kiel, Germany). Total minimum calculated sample size of 5 patients rounded to final 10 patients to accommodate an attrition rate of 15% would yield 98 % power to detect significant differences, with effect size of 2.208 and significance level at 0.05.

Recruitment of Participants

The present study took place from March 2021 to January 2023. In accordance with the ethical standards laid down in the 1964 declaration of Helsinki and its later amendments, this prospective clinical trial was conducted with a cohort of ten participants within the age range of 41 to 70 years. The selection of these participants adhered to predefined inclusion and exclusion criteria. Before their formal inclusion in the study, each participant provided informed written consent. A comprehensive briefing was provided to the participants, elucidating the study's nature, the treatments administered, the expected duration of follow-up, as well as potential advantages and risks associated with the treatment. Participants were duly informed of their absolute freedom to discontinue their participation in the study at any point, without any adverse impact on the treatment course they might require. In accordance with the 'CONSORT' guidelines (fig. 1), each phase of this clinical trial was meticulously executed and reported. This meticulous approach was undertaken to ensure the highest standards of research ethics and transparency in the study's execution and documentation.

Inclusion Criteria

- Patients with loss of vertical dimension (Turner and Missirlian Classification).
- Patients with severe attrition.
- Patients with occlusal plane discrepancy.
- Patients with esthetic concerns like Amelogenesis Imperfecta.
- Patients with not >1 teeth missing in one sextant.

Exclusion Criteria

- Long span edentulous area.
- Patients requiring implant placement or a removable prosthesis.
- Mentally unstable patients.
- Patients with temporomandibular joint disorders.

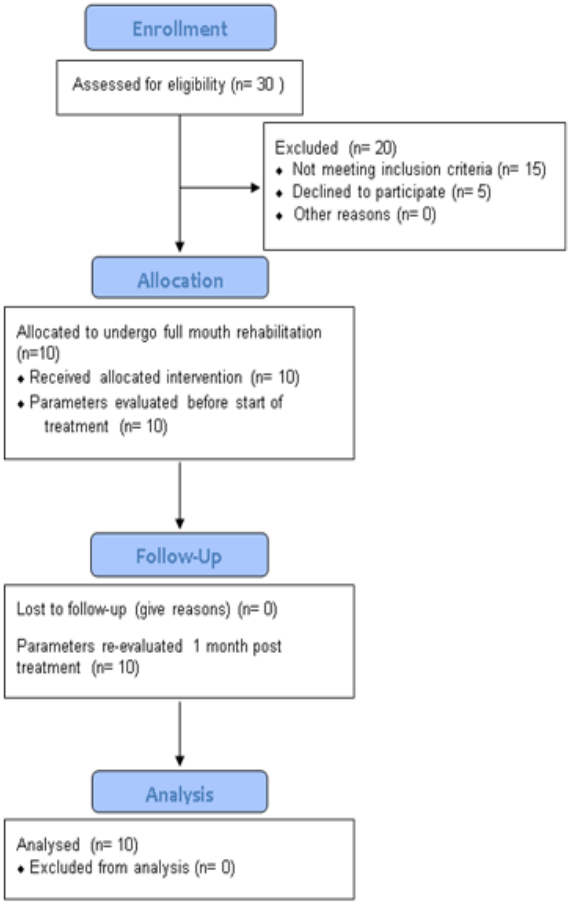


Figure 1. CONSORT Flowchart

Outcome Measures

Nutritional Status (Blood Tests)

In the current trial, fasting venous blood samples from each participant were collected for means of measuring hemoglobin, total serum calcium, total serum protein, and total cholesterol levels as indicators of nutritional status. The tests were carried out before the start of full mouth rehabilitation and 1 month after the therapy was completed. This was done as the turnover rate of albumin (protein) in serum is 3 weeks.[8]All the parameters reflect the dietary intake of the patient and the body's homeostasis.

Masticatory Efficiency

The subjects were then given 9 grams of peanuts (3 grams - 3 times). The subject was then asked to perform 40 masticatory strokes. The masticated food was gathered and placed within a disposable container. The participant was then provided with water for mouth rinsing, a process repeated twice, and the resultant rinsate was likewise introduced into the same disposable vessel. Following thorough mixing of the chewed food facilitated by a glass rod, it underwent filtration through a mesh sieve with a size of 10. This test helps to determine the quantitative estimation of the chewed food. The filtrate was then collected in tubes and centrifuged at 3000 rpm for 5 minutes. The filtrate split into a solid pellet and a liquid supernatent. The supernatent was then discarded and the pellet was measured in a digital scale. Chewing performance in current study was calculated by the method suggested by Yurkstas and Manly.[9]

Performance assessment encompassed an evaluation for any particle size, as determined by the size of the sieve employed. This evaluation was achieved by dividing the weight of the test food that successfully passed through the sieve by the total weight of the test food that was collected. This resulting fraction was subsequently expressed as a percentage. Initially, the first wet weight was meticulously determined. The test specimens were subsequently subjected to a hot air oven set at a temperature of 40℃ for a duration of 8 hours. Following this drying process, the dry weights of the specimens were reevaluated and recorded. This was done both before the start and 1 month after completion of full mouth rehabilitation.

Statistical Analysis

Descriptive and Inferential statistics were analyzed by IBM SPSS version 22.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). Descriptive statistics such as mean and standard deviation were calculated for quantitative variables. Qualitative data was represented using percentage/proportion.

The p value was fixed at 0.05. Data normality was checked using the Shapiro Wilk test and it revealed that all variables follow normal distribution. Therefore parametric tests are applied to analyze the data. Paired student t-test was done to compare masticatory efficiency and nutritional status (Haemoglobin, total serum protein, total serum cholesterol, total ionic calcium) before and after full mouth rehabilitation for all the subjects. One way ANOVA was carried out to evaluate relationship between the occlusal schemes, age and gender to the difference in chewing efficiency pre and post treatment.

Ethics Statement

This study was conducted in accordance with the ethical guidelines set forth in the Declaration of Helsinki (1964) and its later amendments. Ethical approval was obtained from the Ethical Committee of Saveetha Dental College and Hospitals, Chennai (Approval number: SRB/SDC/PROSTHO-2003/22/TH-025). The research adhered to the Consolidated Standards of Reporting Trials (CONSORT) guidelines for clinical trials.

Informed Consent Statement

Prior to their participation, all subjects were provided with a detailed explanation of the study's objectives, procedures, potential benefits, and risks. Each participant voluntarily signed a written informed consent form before enrollment in the study. They were informed of their right to withdraw from the study at any stage without any impact on their treatment. Confidentiality of personal data was strictly maintained throughout the study.

RESULTS

Descriptive

The present study recruited a total of 10 participants, 5 females and 5 males with 40% of patients falling under 51-60 years of age group (Figure 2).

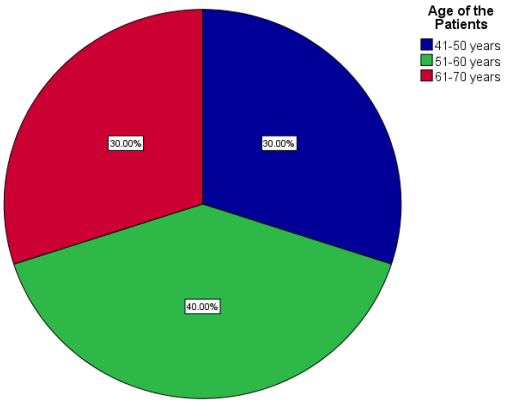


Figure 2. Pie chart representing the distribution of study participants based on age

Each participant underwent a fully tooth supported full mouth rehabilitation therapy by various post-graduate students under the guidance of faculty in the Department of Prosthodontics and Implantology.

Canine guided occlusion was given in 7 cases and group function in others.

Chewing Efficiency

All of the patients showed a statistically significant improvement in masticatory efficiency post full mouth rehabilitation (p= .000) which was calculated with Yurkstas and Manly formula (Table 1,2 and Figure 3).

Table 1. Values of dry weight, wet weight of the chewed peanuts in grams with standard deviation and chewing efficiency of patients pre and post full mouth rehabilitation.

Patients No	Tooth Supported Full Mouth Rehabilitations					
	Pre- FMR			Post- FMR		
	Wet Weight ± SD (gm)	Dry Weight ± SD (gm)	Masticatory Efficiency	Wet Weight ± SD (gm)	Dry Weight ± SD (gm)	Masticatory Efficiency
1	1.663± 0.15	0.742± 0.11	52.3%	1.851± 0.19	0.884± 0.13	58.1%
2	1.548± 0.21	0.627± 0.10	50.8%	1.694± 0.22	0.726± 0.17	56.4%
3	1.552± 0.13	0.597± 0.09	48.5%	1.705± 0.20	0.661± 0.09	55.3%
4	1.613± 0.20	0.693± 0.12	54.2%	1.692± 0.31	0.804± 0.19	61.2%
5	1.479± 0.17	0.642± 0.08	49.6%	1.642± 0.25	0.735± 0.14	54.4%
6	1.619± 0.24	0.755± 0.13	47.9%	1.802± 0.22	0.977± 0.15	55.7%
7	1.702± 0.25	0.791± 0.18	46.8%	1.930± 0.26	0.985± 0.20	55.8%
8	1.397± 0.19	0.585± 0.10	51.5%	1.612± 0.18	0.744± 0.11	58.2%
9	1.461± 0.16	0.618± 0.08	48.2%	1.667± 0.15	0.794± 0.07	53.6%
10	1.654± 0.23	0.703± 0.14	46.9%	1.884± 0.28	0.899± 0.17	54.3%

Table 2. Paired t test with df and p values for comparison of pre and post FMR chewing efficiency

Chewing Efficiency	Mean	Standard deviation	Standard Error Mean	p value
Pre FMR	49.67	2.46	0.77	0.000
Post FMR	56.20	2.34	0.74	

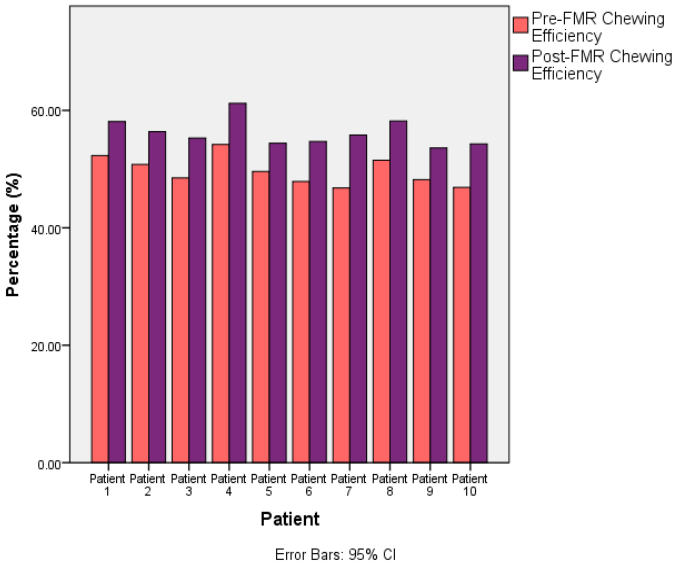


Figure 3. Bar graph depicting the pre and post masticatory efficiency of all the patients included in the study

There was an increase both in the wet weight and dry weight post FMR as well. There was no significant relationship between age, gender and occlusal scheme provided to the masticatory efficiency pre and post FMR (p>0.05).

Nutritional Status

Paired sample t test revealed that haemoglobin, total serum protein and total ionic calcium showed a significant improvement post full mouth rehabilitation at 1 month follow up (p<0.05). There was no statistically significant difference in cholesterol values (Table 3,Table 4 and Figure 4).

Table 3. Descriptives of haemoglobin, total protein, serum ionic calcium and total cholesterol with normal values pre and post full mouth rehabilitation for all the patients.

S.no.	Haemoglobin (12-16gm/dL)		Total Protein (6-8.5gm/dL)		Serum Ionic Calcium (4.4-5.4mg/dL)		Total Cholesterol (<200mg/dL)	
	Pre-FMR	Post-FMR	Pre-FMR	Post-FMR	Pre-FMR	Post-FMR	Pre-FMR	Post-FMR
1	11.80	12.20	7.0	8.0	4.9	5.1	154	146
2	10.50	11.00	6.8	7.9	4.1	4.7	162	158
3	11.00	11.60	5.8	7.5	4.0	4.8	134	146
4	13.00	13.50	6.0	7.8	4.9	5.4	180	175
5	12.50	12.50	6.4	7.6	4.0	4.3	165	160
6	13.00	13.40	5.5	7.2	4.5	5.0	153	146
7	14.00	14.70	5.8	8.1	4.0	4.5	129	135
8	13.00	13.80	6.3	7.5	4.5	4.9	145	145
9	12.20	12.90	6.0	8.3	4.2	5.0	175	178
10	11.00	12.00	5.5	7.1	4.0	4.4	155	161

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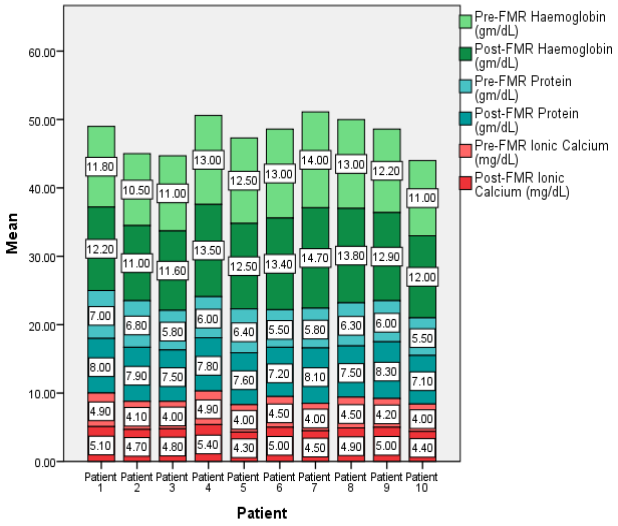


Figure 4. Stacked bar graph depicting the pre and post FMR values of haemoglobin, total protein and serum ionic calcium for all patients.

Table 4. Paired sample t test with df and p values for all the parameters included for nutritional status (p<0.05 is taken as significant)

Nutritional Parameters	Mean	Standard Deviation	Standard Error Mean	p value
Pre-FMR Haemoglobin (gm/dL)	12.20	1.11	.35	.000
Post-FMR Haemoglobin (gm/dL)	12.76	1.11	.35	
Pre-FMR Protein (gm/dL)	6.11	.51	.16	.000
Post-FMR Protein (gm/dL)	7.70	.38	.12	
Pre-FMR Ionic Calcium (mg/dL)	4.31	.36	.11	.000
Post-FMR Ionic Calcium (mg/dL)	4.81	.34	.10	
Pre-FMR Cholesterol (mg/dL)	155.20	16.31	5.15	.927
Post-FMR Cholesterol (mg/dL)	155.00	13.91	4.39	

DISCUSSION

Full mouth rehabilitation is a comprehensive dental treatment approach aimed at restoring the function, aesthetics, and occlusal stability of the entire dentition. It involves a combination of restorative, prosthetic, and periodontal procedures tailored to address various dental issues, such as extensive tooth loss, occlusal discrepancies, and functional impairments. By considering the unique needs and goals of each patient, full mouth rehabilitation aims to improve oral health, enhance masticatory function, and ultimately enhance the individual's overall quality of life. The complexity and individualized nature of full mouth rehabilitation make it a multifaceted and challenging endeavor, requiring careful planning, collaboration among dental specialists, and a comprehensive understanding of the patient's specific dental needs and expectations.[10]

Effect of FMR on Masticatory Efficiency

Masticatory efficiency refers to the ability to effectively chew and grind food, leading to the formation of a bolus that can be easily swallowed and digested. It plays a vital role in nutrient intake, digestion, and overall nutritional status.[11,12] Evaluating masticatory efficiency provides insights into the functional outcomes of full mouth rehabilitation, especially in cases where individuals have experienced significant dental issues or have undergone extensive dental procedures.

The present study is a prospective clinical trial carried out in 10 adults who underwent full tooth supported full mouth rehabilitation by different post graduate students. An individual pre and post treatment comparision was done thereby reducing risk of bias.

The assessment of masticatory efficiency can be performed using various methods, including subjective assessment (patient-reported outcomes), objective measurements (particle size distribution analysis), or functional tests (such as chewing performance tests using artificial test foods). These assessments help quantify the effectiveness of mastication, the degree of impairment, and potential improvements resulting from full mouth rehabilitation. In current study particle size distribution of peanuts was evaluated after specific masticatory strokes, when passed through a 10 mesh sieve. Particle size distribution analysis stands out as the preferred method for calculating masticatory efficiency due to its ability to capture the complexity, heterogeneity, and dynamic nature of the mastication process. Its ability to provide detailed information regarding food particle breakdown during chewing allows for a comprehensive assessment of masticatory efficiency.[13]

All the patients showed a significant increase in masticatory efficiency along with the wet and dry weight ($p<0.05$).

Although no previous studies have evaluated

chewing efficiency for FMR patients, there have been multiple publications on comparison of chewing efficiencies pre and post prosthodontic rehabilitation. Providing support to full prosthetic dental solutions through oral implants appears to yield favorable impacts on parameters such as the thickness of the masseter muscle, the maximum bite force, and overall chewing efficiency. However, it's worth noting that the specific design and nature of the implant-supported prosthetic apparatus employed may exert varying degrees of influence on the extent of these effects.[14-18]

Differences in various attachment systems have also been seen in implant supported overdentures.

There is contradictory evidence on the need for a shortened dental arch or restoration with a removable partial dentures, where some studies demonstrate that only implant supported replacement beyond SDA show significant improvement in chewing efficiency on other hand some have established that a removable prosthesis beyond SDA helps in improving chewing efficiency [19] and some say that SDA is equivalent to an RPD .[20]

Effect of Full Mouth Rehabilitation on Nutritional Status

The current literature is highly contradictory on the effects of various prosthetic rehabilitations on nutrient intake.[21-23] This is predominantly because nutritional intake is shaped not solely by the function of chewing but also by a multitude of other factors, including habits, preferences, cultural practices, as well as financial and logistical considerations . However, it's noteworthy that the findings from the current study revealed a substantial rise in protein, calcium, and iron (related to hemoglobin) consumption following full-mouth rehabilitation, even in the absence of any dietary guidance from the research team. This is in agreement with previous studies on change in dietary intake.[24-25]

Furthermore, individuals who wear removable dentures and experience reduced chewing efficacy are at a heightened nutritional risk, primarily because they tend to have a lower frequency of consuming fruits and vegetables. It appears that enhanced masticatory capability is linked with a higher likelihood of making healthier dietary selections among patients who have undergone prosthodontic rehabilitation. The augmented calcium intake subsequent to the utilization of Full Mouth Rehabilitation (FMR) is closely linked to enhanced bone health, fortifying both the structure and functionality of bones, consequently mitigating the risk of osteoporosis and associated fractures . Elevated iron levels may be correlated with an increased consumption of meat, a factor that could potentially elevate cholesterol levels.[26]

However, it's noteworthy that no discernible variation in cholesterol levels was observed following FMR, suggesting that the heightened iron intake might be attributed to an increased consumption of vegetables and/or lean meats.

Effect of Age and Gender on Masticatory Efficiency

The ability to orally process foods can affect food intake and food choices among vulnerable populations, including elderly and consumers with decreased eating capability. Masticatory function is frequently impacted in the elderly population. In fact, research indicates that as individuals age, there is a progressive rise in the number of cycles required to adequately chew a standard piece of food. This aging-related phenomenon is associated with greater particle size reduction during chewing and an extended duration of the chewing sequence.[27] In a study by Ikebe et al[28] showed that age does not directly impact masticatory performance within an individual's own set of teeth. This discovery implies that the decline in masticatory performance associated with age can be mitigated if one retains their natural teeth. The influence of age is presently understood as the consequence of a cumulative effect of various stressors on orofacial structures and function. In current study no difference was found between masticatory efficiency and age groups of patients. It should be noted that unlike other cross-sectional studes the age gap between the subjects was not huge.

Regarding gender, the influence on chewing efficiency is less clear. Some studies have suggested that there may be gender differences in masticatory performance, with males generally exhibiting higher chewing efficiency than females. These differences have been attributed to variations in muscle mass, bite force, and jaw morhology between genders.[29,30] However, conflicting findings have also been reported, and the influence of gender on chewing efficiency remains a subject of debate.[31,32] In current trial there was no difference seen ($p>0.05$).

Effect of Occlusal Scheme on Masticatory Efficiency

Several investigators have studied the effect of occlusal schemes on chewing ability, both objectively and subjectively in complete denture patients. Results of studies have shown different results for different study designs, which have led to

controversy in the outcomes [33]no difference was noted between canine guided and group function occlusion in current trial.

In conclusion, evaluating masticatory efficiency and nutritional status in full mouth rehabilitation plays a critical role in assessing the functional outcomes and nutritional well-being of patients. Objective assessment methods and biochemical markers help quantify the impact of full mouth rehabilitation. In the context of evaluating different types of prostheses, paired studies represent a more robust design. This choice minimizes the impact of intraindividual confounding factors, enhancing the validity of the comparisons being made.

It is essential to acknowledge and consider potential limitations that may affect the study's findings and conclusions. Some possible limitations include a small sample size which may limit the statistical power and the ability to detect significant differences between the pre and post-intervention groups accurately. The trial's findings may be influenced by selection bias as the participants might not be representative of the broader population. Differences in patient demographics, treatment techniques, and clinical settings could affect the generalizability of the results to other populations or contexts. The trial's duration may impact the ability to capture long-term changes in masticatory efficiency and nutritional status accurately. Short-term assessments may not fully reflect the sustained effects of full mouth rehabilitation on these outcomes. Recognizing and transparently discussing these limitations is essential for researchers and clinicians to understand the potential impact and applicability of the study's conclusions.

Future scope involves inclusion of other types of full mouth rehabilitations (tooth tissue supported vs. shortened dental arch) with a larger sample size and longer follow ups so that results are more indicative of generalized population.

CONCLUSION

Within limitations of the current trial, we can conclude that full mouth rehabilitation leads to significant increase in masticatory efficiency. A significant improvement in haemoglobin, serum total protein and serum ionic calcium levels post completion of therapy. There was no influence of age, gender and occlusal scheme on chewing efficiency.

Author Contributions

- **Varun Wadhwani**: Conceptualization, Data Collection, Analysis, and Manuscript Writing
- **Deepak Nallaswamy**: Study Design, Supervision, and Critical Review of the Manuscript
- **Suresh Venugopalan**: Methodology, Statistical Analysis, and Interpretation of Results
- **Saravanan Sekaran**: Statistical Analysis, and Interpretation of Results
- **Dhanraj Ganapathy** (Corresponding Author): Project Administration, Final Manuscript Editing, and Approval

DECLARATIONS

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Ethical Approval

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Informed Consent

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CASE REPORT

SURGICAL TREATMENT OF MULTIPLE GINGIVAL RECESSIONS WITH TUNNEL TECHNIQUE USING FREE GINGIVAL GRAFT: CLINICAL CASE SERIES

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ABSTRACT

Background: Mucogingival periodontal surgery is a surgical procedure to correct defects in the morphology, position, and/or quantity of soft tissue and underlying bone support around teeth. Various methods can be performed for the surgical management of the recession defects of gingiva. Different surgical techniques have been advocated for root coverage, like free soft tissue graft procedures, free gingival graft and sub-epithelial connective tissue graft, pedicle soft tissue graft, rotational flap and flap advancement, pouch and tunnel techniques and guided tissue regeneration. Free gingival graft is a reliable mucogingival surgical procedure to cover localized gingival recessions.

Objective: The aim of this case series was to evaluate the results obtained by the surgical treatment of multiple gingival recessions with tunnel technique using free gingival graft.

Materials and Methods: This case series included 18 patients with localized multiple gingival recessions who underwent surgical treatment using free gingival graft from 2023 to 2025. The Miller and Cairo classifications was used to classify gingival recession. The following clinical parameters were measured:Probing pocket depth (PD);Clinical attachment level (CAL);Recession depth (RD) ;Recession width (RW). Professional hygiene was performed before surgical treatment.

Free gingival graft was used to close the recession in all patients. Evaluations of clinical changes (recession depth, height of keratinized tissue) and patient satisfaction were performed over a follow-up period of 12 months.

Results: Adequate keratinized gingival width, thickening of the gingival phenotype, and treatment of recession were observed at follow-up appointments on the 3rd month and 12th month. At baseline, six months, and one year postoperatively, clinical measurements demonstrated the effectiveness of free gingival graft in improving soft tissue parameters in cases of gingival recession. All patients complained of hypersensitivity before surgery, and six months after treatment, a third percentage of patients reported mild hypersensitivity complaints. All patients were satisfied with the aesthetic results of the procedure.

Conclusion: Free gingival graft can be used as a predictive treatment modality for recession defects and the results can be well maintained with optimal care and patient satisfaction.

Keywords:Free gingival graft, gingiva, gingival recession, mucogingival surgery, tunnel technique

INTRODUCTION

Gingival recession is a common pathology that occurs when the gingival margin migrates apical to the cementoenamel junction (CEJ) of one or more teeth. Recession is a decrease in the volume of gum recession, which leads to exposure of the neck and roots of the tooth.[1]

The etiology of gingival recession is multifactorial and may be caused by a variety of factors.[2-4]

Etiological factors gingival recession:

- Periodontal disease;
- Mechanical forces;
- Iatrogenic factors;
- Anatomical factors.

Recession can be caused by such problems as crowded teeth and their incorrect positioning. It develops due to overload of individual teeth.

Orthodontic treatment is used to eliminate it. Incorrect structure and attachment of the frenulum. Recession in this case develops due to injury to the area of attachment of the frenulum to the gum. The cause is eliminated with the help of frenuloplasty. Violation of hygiene causes inflammatory diseases of the periodontium, such as gingivitis, periodontitis. In this case, tooth mobility, formation of pockets, bad breath, and suppuration from the gums can be observed. As a result of the destruction of bone tissue and gums around the tooth, recession appears. One of the common causes of recession is trauma to the mucous membrane. Incorrect aggressive brushing and flossing, using toothpicks, bad habits are common causes.

Gingival recession can be painful due to hypersensitivity of the dentin of the exposed root surfaces of the tooth and can also cause a deterioration in the aesthetic appearance.

Classification of gingival recession

Miller (1985)[5] proposed a classification of recession based on the height of the interdental bone adjacent to the defective area (table 1)and the relationship of the gingival margin to be used as a basis for determining treatment options.[6]

Table 1. Miller's classification of gingival recession defects (1985).

Class I	Recession within attached gingiva. No loss of interdental bone and soft tissue papillae covering interdental bone at full height
Class II	Recession extending to or beyond the mucogingival junction. No loss of interdental bone and soft tissue papillae covering interdental bone at full height
Class III	Recession extending to or beyond the mucogingival junction. Loss of interdental bone but interdental bone height coronal to apical extent of recession defect. Reduction in height of the soft tissue papillae covering interdental bone
Class IV	Recession extending to or beyond the mucogingival junction. Loss of interdental bone apically to recession defect. Gross flattening of interdental soft tissue papillae

Initial recession (IR): Measured in mm from the CEJ to the most apical gingival margin of the recession.

When choosing a treatment method for gum recession, it is important to consider the etiological factors and the degree of tissue involvement.

The following types of operations are used to eliminate recession:

CAIRO CLASSIFICATION (2011) Gingival recession based on the Assessment of CAL at both buccal and interproximal sites.

Type 1: Gingival recession with no loss of interproximal attachment. Interproximal CEJ was clinically not Detectable at both mesial and distal aspects of the tooth.

Type 2: Gingival recession associated with loss of interproximal attachment. The amount of interproximal attachment Loss (measured from the interproximal CEJ to the depth of the interproximal Pocket) was less than or equal to the Buccal attachment loss (measured from the buccal CEJ to the depth of the buccal Pocket)

Type 3: Gingival recession associated with loss of interproximal attachment. The amount of interproximal attachment Loss (measured from the interproximal CEJ to the depth of the pocket) was Higher than the buccal attachment loss (measured from the buccal CEJ to the Depth of the buccal pocket)

1.Pedicle soft tissue grafts

Closing the recession with local tissues. In this case, the mucous membrane is moved from areas adjacent to the tooth. Additionally, special membranes are used to restore tissue. This technique can be used for mild to moderate severity of the disease. Procedures for transplanting soft tissues on the leg. These types of grafts remain attached to their base and presuppose the placement of soft tissue over the recessional defect; they save their blood supply when moving to a new place. Examples include:

• Procedures with rotating flap, including lateral flap, double papillary flap.

• Procedures for moving the flap, including the flap for repositioning the crown.

2. Free gingival grafts

Closing the recession with a free palatal flap. In this case, a mucous graft is taken from the palate. Artificial membranes are also used. The technique of transferring soft tissues from a distant area of recession is used when there is a lack of donor tissue near the recipient site.

3.Coronally advanced flap

The coronal flap is used to treat Miller class I and II recession defects.[10] This technique is however, only when adequate keratinized tissue is present.[11]It is relatively simple, providing good aesthetic results. It is performed by making two vertical incisions extending beyond the mucocutaneous junction and

lifting a full-thickness flap, which is repositioned in a coronal position and sutured in place.[12]

This surgical approach allows for restoration of the original gingival morphology, provides color to the resected area of the recession, and has a low morbidity rate, with patients undergoing this procedure.

4.Semi-lunar flap

A modified version of Coronally advanced flap is the crescent semi-lunar flap procedure, first proposed by Tarnow in 1986. This technique allows for partial thickness flap resection to avoid alveolar bone flaps.

Laterally positioned grafts

This method is indicated for narrow defects with attached gingivae at the donor site.[13]To provide root coverage, the marginal gingival tissues around the tooth are incised with an oblique incision at the base of the gingival pocket. Incisions are made in the gingival margin and interdental papilla of the donor site.[14,15]

Free gingival graft transplantation Procedures

Free gingival grafts are used for:

• Increasing the amount of keratinized tissue (more specifically attached gingiva)

• Increasing the vestibular depth

• Increasing the volume of gingival tissues in edentulous spaces (preprosthetic procedures)

• Covering roots in areas of gingival recession.

Free gingival grafts, include:

- Connective tissue grafts.
- Subepithelial tissue graft.

The technique is used for moderate to severe disease.

The choice of surgical technique for recession is dictated by a number of factors, including the size of the tooth recession defect, the presence or absence of keratinized tissue adjacent to the defect, the width and height of the interdental soft tissue, the depth of the vestibule or the presence of a frenula.[7] The desired aesthetic outcome and the predictability of the expected results are also important.[8,9]

Before recession closure, root surfaces are mechanically thoroughly cleaned with ultrasonic or hand instruments and irrigated with sterile saline. They are then treated with citric acid or tetracycline hydrochloride.

Free soft tissue grafts lack a blood supply and, as an embedded graft, rely on the nutrient supply of serum from their recipient's bed. Although free gingival grafts are usually harvested from the palate and sutured into position at the recipient site, the recipient bed should extend at least 3 mm lateral and apical to the recession defect, as this will be the only nutrient supply to the graft during the initial healing phase. Care should be taken to avoid palatine blood vessels.[16]

Good adaptation of the flap to the underlying tissues is essential for adequate diffusion, so care should be taken in preparing the recipient's bed and cutting the graft to the exact size required to ensure good fit.

Free gum grafting can compromise esthetics due to the color difference between the graft and the recipient tissue, as well as the painful appearance of a large exposed area in the palate and discomfort to the patient.

In contrast to this method, in the case of connective tissue (CT) grafting, the color match with the recipient tissue is much better. There is less pain at the donor site because the wound surface is closed, which reduces the patient's discomfort. Connective tissue graft is harvested from the palate if there is an adequate thickness of tissue. IF the space is sufficient, it can also be taken from the Retro-molar pad area due to the thickness of the submucosal membrane in this area.

Among the various surgical approaches used to treat gingival recession, connective tissue grafting combined with a gingival flap can be considered the gold standard for the treatment of recession defects.[17,18]

Since the success rate of root capping depends on the survival of the graft tissue itself, it has been suggested that the capping should cover the majority of the graft. This is thought to provide sufficient blood supply to nourish the lower part of the graft on the exposed root.[19]

Allen (1993) proposed the use of a technique in which a connective tissue graft is placed in a tunnel preparation.[20]

This technique allows for the preservation of a thicker cap at the apex of the recession to cover the exposed root surface in the case of multiple adjacent recession defects.[21-24]

One of the concerns about harvesting the graft tissue is the potential risk of damaging the greater palatine artery due to variation in the anatomy of the palatal vault.[25]

Additive treatments:

- Root surface modification agents;
- Enamel matrix proteins;
- Guided tissue regeneration;
- Nonresorbable barrier membrane;
- Resorbable barrier membrane.

After periodontal surgery, it is very important to monitor the wound healing process. It is necessary to apply a topical preparation that will protect the

wound from the harmful effects of oral microflora and enhance wound regeneration, such as the properties of hyaluronic acid. In dentistry, it was first used in the treatment of periodontal diseases (gingivitis).[26-28]

Hyaluronic (HA) acid plays a multifunctional role in wound healing processes. Hyaluronic acid can increase local immunity in the oral cavity, enhance the antibacterial function of cells, improve tissue regeneration, which has a positive effect on the healing process, and can be a good drug in the treatment of oral wounds. It enhances tissue regeneration, wound healing properties and stimulates the migration of fibroblasts and cell proliferation.[29,30]

Hyaluronic acid is a polysaccharide, a polymer of glucuronic acid and N-acetylglucosylamine, and a member of the high molecular weight glycosamine family. Hyaluronic acid (Hyaluronan) is an unchanged, indispensable component of healthy gingival and oral mucosal tissue.[31]

It has many properties that make it a good drug for wound healing, promoting early granulation tissue formation, inhibiting inflammation, stimulating epithelial turnover, and also angiogenesis of connective tissue.

Various authors have reported the effectiveness of the use of hyaluronic acid in periodontal therapy: it reduces pain and swelling and accelerates the regenerative process in soft tissues.[32,33]

Considering these properties of hyaluronic acid, we have included Gengigel® in postoperative period.

The aim of this case series was to evaluate the results obtained by the surgical treatment of multiple localized gingival recessions with tunnel technique using free gingival graft.

MATERIALS AND METHODS

This case series included 18 patients with localized multiple gingival recessions who underwent surgical treatment with tunnel technique using free gingival graft from 2023 to 2025. The Miller and Cairo classification was used to classify gingival recession.

Inclusion criteria:

- Patients without systemic diseases;
- Miller's gingival recession of different classes in one or more teeth;
- Shallow vestibule (<5 mm);
- No active signs of periodontal disease;
- Do not smoke.

Exclusion criteria:

- Patients with systemic diseases or taking medications contraindicated for periodontal surgery;
- Active signs of periodontal disease;
- Para-functional habits;
- Poor oral hygiene;
- Presence of cervical caries;
- Pregnant or breastfeeding patients.

All subjects were informed about the study and clinical procedures and provided written consent.

The following clinical parameters were measured: Probing pocket depth (PD);Clinical attachment level (CAL);Recession depth (RD);Recession width (RW).

4 weeks before surgery, professional hygiene was performed and patients underwent professional oral hygiene instructions.

Surgical Procedure

After local anaesthesia, root planing of the exposed root surfaces was performed by means of hand instruments (Gracey Curettes, Hu-Friedy, Chicago, IL, USA). Subsequently, intrasulcular incisions were placed and mucoperiosteal flaps were raised using sharp tunnel elevators (Hu-Friedy, Chicago, IL, USA) The flaps were extended beyond the mucogingival junction and under each papilla to allow passive, tension-free mobilization in coronal direction .Remaining collagen bundles on the inner surface of the flap were carefully cut using Gracey curettes until passive coronal displacement of the flaps and papillae was obtained. Great care was taken to avoid flap perforation.

A FGG immediately harvested from palatal side using N°15 blade after tunnel preparation..

Immediately after FGG harvesting, the donor site was sutured with continuous suture using 5/0 nonabsorbable sutures and hemostatic sponge was placed. FGG was deepithelialized with 15c blade.

CTG was inserted under the tunnelled flap by starting at the deepest recession (Figs. 2b and 3b). Subsequently, the grafts were pulled laterally towards each end of the tunnel by means of mattress sutures with 5/0 absorbable suture(LUXCRYL PGA: Weiswampach, Luxemburg). Finally, the flap was positioned coronally to the CEJ using sling sutures.(Figs. 2c and 3c). Anchored sutures were placed to keep the flap coronally .

Post-surgical protocol

Post-surgically, all patients were given analgesics (3 9 50 mg Cataflam, Budapest, Hungary) for 3 days and antibiotics (3 9 625 mg Augmentin, Pfizer KFT, Budapest, Hungary) for 7 days due to university

regulation for implantable biological materials. Patients were instructed to rinse their mouth with a 0.2% chlorhexidine solution, two times a day for 1 min. for 3 weeks. Patients were instructed to avoid brushing in the operated area until suture removal at 2 weeks. Patients underwent manual supragingival tooth cleaning twice a week until suture removal. At suture removal 2 weeks after surgery, all patients were controlled and instructed in mechanical tooth cleaning of the operated areas using a soft tooth brush and a roll technique. All patients were recalled after 28 days, 3, 6 and 12 months and received one session of prophylaxis, including reinforcement of oral hygiene, supragingival debridement and tooth polishing. The study was completed in 1 year.

Results: No intraoperative complications occurred, and postoperative swelling was rare, no partial graft necrosis was observed, and mild varicocele pain in the donor site was reported in all patients. Adequate keratinized gingival width, thickening of the gingival phenotype, and treatment of recession were observed at follow-up appointments on the 3rd month, and 12th month.At baseline, six months, and one year postoperatively, clinical measurements demonstrated the effectiveness of free gingival graft in improving soft tissue parameters in cases of gingival recession. All patients complained of hypersensitivity before surgery, and six months after treatment, a third percentage of patients reported mild hypersensitivity complaints. All patients were satisfied with the aesthetic results of the procedure. The results of all clinical parameters obtained are summarized in Table 2.

Table 2. Clinical parametrs patienents (n=18) after 3 months, 6 months, 1 year

Clinical parametrs	Baselin	After 3 months	After6 months	After One year
Probing pocket depth (PD)	1	0	0	0.8
Recession depth (RD)	4	0	0	0
Recession width (RW)	3	0	0	0

Case Report 1

This clinical case report is about a 33-year-old woman whose main complaint was 23 tooth sensitivity and esthetic problems. The patient had no history of systemic health or abnormal habits. On periodontal examination and radiographic evaluation, the patient was diagnosed with 23 tooth Miller Class III gingival recessions,24 tooth Miller Class II gingival recessions,25 and 26 tooth Miller Class I gingival recessions (fig.1).



Figure 1. Preoperative view gingival recession in 23,24,25,26 teeth.

The patient was presented with the treatment protocol and after obtaining written consent, the treatment process began. Before periodontal surgery, professional dental hygiene was performed.

Clinical Records

Clinical examination revealed a healthy periodontium with gingiva showing no signs of bleeding on probing. The following clinical parameters were measured:

Probing pocket depth (PD)was measured with a standard periodontal probe to the nearest millimeter from the gingival margin to the bottom of the sulcus;

Recession depth (RD) was measured from the CEJ to the gingival margin;

Recession width (RW) was measured across the buccal surface at the CEJ.

Surgical Procedure

Recipient Site Preparation

A horizontal intrasulcular incision was made at the CEJ level (figure 2) Mucoperiosteal flap was raised using sharp tunnel elevators (, Hu-Friedy, Chicago, IL, USA) The flaps were extended beyond the mucogingival junction and under each papilla to allow passive, tension-free mobilization in coronal direction .Remaining collagen bundles on the inner surface of the flap were carefully cut using Gracey curettes until passive coronal displacement of the flaps and papillae was obtained (figure 3).





Figure 2. Intrasulcular incision
Figure 3. Separation of the flap

A 2 mm thick graft consisting of epithelium and underlying connective tissue layer was harvested from the palate donor site (Figure 4,5).

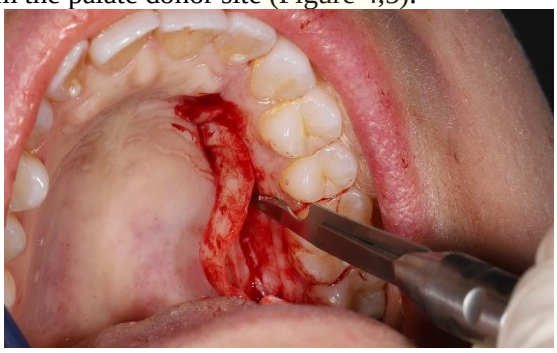


Figure 4. Harvesting a free gingival graft from the palate



Figure 5. Free gingival graft
Palatal side was sutured with 5/0 nonabsorbable suture and covered with hemostatic sponge (figure 6)



Figure 6. Palate after suturing

After de-epithelialization free gingival graft was then placed in the tunnel and sutured to the lateral margins. Finally, the flap was positioned coronally to the CEJ using sling sutures. Anchored sutures were placed to keep the flap coronally (figure 7). Periodontal dressing Gengigel® (0.8% hyaluronic acid) was placed over the donor site and recipient site. A periodontal dressing was applied to the surgical site for 10 days.



Figure 7. Free gingival graft sutured
The patient was advised not to brush the treated site for 4 weeks. 0.12% chlorhexidine mouth rinse was prescribed twice a day for 4 weeks. Antibiotics and analgesics were administered as needed. Sutures were removed 3 weeks after surgery. Patient was examined weekly for the 1st month and then once a month for the next 3 months (figure 8,9), one year postoperative. Patient was recalled at 3 months intervals for oral hygiene instructions.

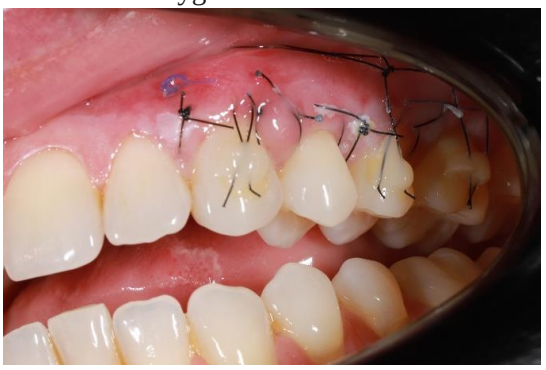


Figure 8. 2 weeks postoperative



Figure 9. 10 weeks postoperative



Figure 10. One year postoperative

After treatment, all parameters improved There was a significant reduction in RD, RW and probing PD were observed at the end of 1 year compared to baseline values.

Case Report 2

The patient is a 21-year-old female patient complaining of esthetics of the frontal part of the lower jaw. There is recession Miller Class III 31 tooth, Miller Class II 41 tooth teeth (fig.11).



Figure 11. Preoperative view gingival recession in 31,41 tooths.

The patient was presented with the treatment protocol and after obtaining written consent, the treatment process began. Before periodontal surgery, professional dental hygiene was performed.

Clinical Records

Clinical examination revealed a healthy periodontium with gingiva showing no signs of bleeding on probing. The following clinical parameters were measured;Probing pocket depth (PD); Recession depth (RD); Recession width (RW).

Surgical Procedure

Recipient Site Preparation

A horizontal intrasulcular incision was made at the CEJ level (figure 11) Mucoperiosteal flap was raised using sharp tunnel elevators (Hu-Friedy, Chicago, IL, USA). The flaps were extended beyond the mucogingival junction and under each papilla to allow passive, tension-free mobilization in coronal direction.Remaining collagen bundles on the inner surface of the flap were carefully cut using Gracey curettes until passive coronal displacement of the flaps, papillae was obtained and prepared tunnel (figure 12,13,14).



Figure 12. Intrasulcular incision



Figure 13 Tunnel design with a gum knife



Figure 14. Prepared tunnel

Harvesting a free gingival graft from the palate

A 2 mm thick graft consisting of epithelium and underlying connective tissue layer was harvested from the palate donor site and de-epithelialization of the transplant (figure 15,16,17).



Figure 15. Free gingival graft harvested from the palate



Figure 16. The donor area in the palate after the transplant was taken.



Figure 17.De-epithelialization of the transplant

Free gingival graft from the palate was then placed in the recipient site and sutured and fixation in the tunnel (figure 18,19,20). The graft was held firmly in place using digital pressure for 5 minutes, and periodontal dressing Gengigel® (0.8% hyaluronic acid) was placed over the donor site and recipient.

A periodontal dressing was applied to the surgical site for 10 days. The patient was advised not to brush the treated site for 4 weeks. 0.12% chlorhexidine mouth rinse was prescribed twice a day for 4 weeks. Antibiotics and analgesics were administered as needed. Sutures were removed 10 days after surgery.



Figue 18**.** Placement of the transplant in the recipient area and fixation in the tunnel



Figure 19. The wound was closed with parasitoid sutures; composite sutures were placed on teeth 32, 31, 41, and 42 to fix the flap in place.



Figure 20. 3 days later

Patient was examined weekly for the 1st month and then once a month for the next 3 months (figure 21, 22). Patient was recalled at 3 months intervals for oral hygiene instructions.



Figure 21. After 13 days postoperative



Figure 22. Palate after 13 days

After treatment, all parameters improved. There was a significant reduction in RD, RW and probing PD were observed at the end of 1 year compared to baseline values (figure23,24).



Figure 23. 6 months later postoperative.



Figure 24. One year postoperative

DISCUSSION

Currently, free gingival grafting is considered a predictable and effective procedure for covering bare root surfaces.[34-36] This grafting method relies on the blood plasma circulation of the gingival tissue to the periosteum of the recipient bed, there should be no dead space between the graft and the recipient area for the survival of the free flap, and immobilization of the graft is important.[5] Treatment of a patient with gingival recession should begin with correction of etiological factors. Numerous therapeutic solutions for recession defects have been proposed in the literature and have been modified with time according to the evolution of clinical knowledge. Various surgical procedures, including the lateral sliding flap, coronal progressive flap, and their combinations with barrier membranes or subepithelial connective tissue grafts, have been proposed for the treatment of single-tooth gingival recession, and the selection of effective procedures and the skill of the surgeon are essential for successful results. [37]

Revascularization of the graft takes several days, and good adaptation of the flap to the underlying tissues is necessary to maintain tissue viability. Attention must be paid to preparing the recipient bed and the

exact size of the graft cut. Proper flap design is also an important step in obtaining satisfactory flap results. The bag and tunnel techniques have been developed to increase the efficiency of the procedure.

The envelope technique was proposed by Raetzke (1985) to eliminate vertical incisions, which has the advantages of preserving the flap's blood supply, close adaptation to the graft, and reducing postoperative discomfort and scarring.[38] Main disadvantage of FGG is two donor site, donor site morbidity. For the treatment of multiple-tooth gingival recession, various types of allografts or substitute biomaterials have been used instead of connective tissue grafts to reduce patient discomfort.

Subepithelial connective tissue graft has exhibited the optimal clinical outcome.[39-43]There are limitations when multiple teeth are recession-prone and their roots need to be covered or when the palatal tissue thickness is very thin. Due to the color mismatch of the free gingival grafts were deepithelialized.

Zucchelli et al. reported that deepithelialized free gingival grafting for root coverage was as effective as the subepithelial connective tissue grafting technique.[7]

This case series study predictability of free gingival graft in management of gingival recession. The aim of this paper is to present18 cases for the management of gingival recession and increasing the width of attached gingiva in Miller's class II and III recession using free gingival graft. Non-surgical phase along with scaling and root planning was conducted and patient was educated for plaque control. Recipient site prepared by giving one horizontal incision and two vertical incisions up to the vestibule from mesial and distal line angle of adjacent tooth with 15c blade, graft is closely adapted over the recipient and sutures, periodontal dressing use of hyaluronic acid(Gengigel®).

The current study has limitations due to the small number of cases presented, which did not cover all aspects of the classification of mucosal deformities. Therefore, there is a need for a larger number of cases and a longer observation period.
A satisfactory outcome can be achieved for surgical intervention for recession if an objective classification is made, with a thorough understanding of the etiological origin and the selection of appropriate surgical procedures.

Within the limitations of the present case report, free gingival graft can be used as a predictive treatment modality for recession defects and the results can be well maintained with optimal care and patient satisfaction.

DECLARATIONS

Competing interests
No competing interests
Ethics approval and consent to participate
The study was reviewed and approved by the Ethics Committee, Yerevan State Medical University and in accordance with those of the World Medical Association and the Helsinki Declaration. Informed consent
Patients were informed verbally and in writing about the study and gave written informed consent.
Consent for publication
Not Applicable.
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CASE REPORT

HIDDEN WITHIN THE JAW: AN UNUSUAL FINDING OF OSTEOSARCOMA FOLLOWING MANDIBULAR TRAUMA

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ABSTRACT

This case report focuses on the aggressive nature of osteosarcoma in head and neck region which poses a diagnostic challenge and effort of maxillofacial surgeons to provide better prognosis by identifying in early stages.

Background:A 24-year-old male patient presented with post traumatic swelling in left mandible with aggressive growth in just few weeks. He was initially reluctant to undergo the procedure as he overlooked the severity of the tumour.

Diagnosis: Patient was diagnosed with parosteal juxtacortical osteosarcoma of the left mandible on clinicoradiohistopathological diagnosis and had no distant metastasis.

Treatment: Multi-modality treatment implemented with hemi-mandibulectomy tumour free margins followed by radiochemotherapy.

Outcome: There was no evidence of local recurrence and distant metastasis after one year of follow up period.

Conclusion: Osteosarcomas of head and neck region are less than 2-10% of all cases. Emphasis on early diagnosis, negative surgical margins and inclusion of radio and chemotherapy provides disease free survival.

Key words: Osteosarcoma, Mandible, Juxtacortical, Parosteal

INTRODUCTION

Osteosarcomas are rare malignant connective tissue tumours. Out of the all-primary tumours, osteosarcomas account for 20% which are mostly confirmed by the biopsy[1] 4-6 % occurs in jaw bones.[2] Osteosarcomas are of two types, primary and secondary. Etiology for the primary tumour is not known but genetic and environmental factors have a great influence. Secondary type is seen in Paget's disease, Fibrous dysplasia, craniofacial irradiation cases, Li-Fraumeni syndrome and Rottmund-Thomson syndrome.

As these tumours occur in metaphyseal growth plate of long bones, rapid bone growth acts as a major etiological factor. Other factors include genetic mutation of P53 tumour suppressor gene and mutated retinoblastoma.[1]

It occurs in 30-40 years of age groups with more of male predilection. Treatment protocols include combination of surgery, radiotherapy and chemotherapy and the 5-year survival rate for these patients is 50 to 70%.[3]

We report a case of a 24-year-old male diagnosed with osteosarcoma of left mandible accidentally found after a road accident.

Case report

A 24-year-old male patient reported with a complaint of swelling in the lower jaw on the left side of the face for one month. Swelling is not associated with pain. Patient gave a history of road traffic accident and had injury on the left side of lower jaw one month back. Patient had no difficulty related to trauma but developed a small swelling next day of trauma which grew to the size of 6x4 cm (figure 1).



Figure 1. Extraoral photograph showing swelling at the left body of the mandible

Patient reported to Department of oral and maxillofacial surgery a week after the trauma due to the swelling. On examination, general physical examination was within normal limits.

Vitals are normal. Examination of head and neck revealed facial asymmetry on left side of the lower jaw. There is a solitary oval shaped swelling of size 6 x 4 x 3 cm occupying the body of left mandible extending intraorally from 31 to 38 teeth, medially abutting the floor of the mouth and laterally gingivobuccal sulcus, inferior 3cm crossing the lower border of the mandible with no involvement of overlying skin and mucosa, tongue and floor of the mouth (figure 2). Clinically palpable supple nodes level IA and IB. Otherwise rest of the oral cavity and oro-pharyngeal examination is normal. No paresthesia or anesthesia of lower lip and chin noted. Lower lip deviated due to swelling. Margins of the swelling are distinct. Surface is slightly lobulated. No ulcerations seen extra orally or intraorally. There is no mobility or displacement of the teeth. No pus discharge. No inflammatory signs over the swelling. There is no lingual cortical expansion seen.



Figure 2. Intraoral swelling extending from 31 to 38 teeth

Panoramic radiograph, mandible showed mixed radiolucent and radiopaque pattern in the lower left quadrant in the region of 34, 35, 36 teeth. Widening of the periodontal ligament space and attenuation of the lamina dura is seen in above mentioned teeth which is the most common radiographic finding in osteosarcoma. Occlusal x-ray of mandible showed expansion of buccal cortical plate and bony spicule radiating perpendicular from the cortex of alveolar bone (figure 3).

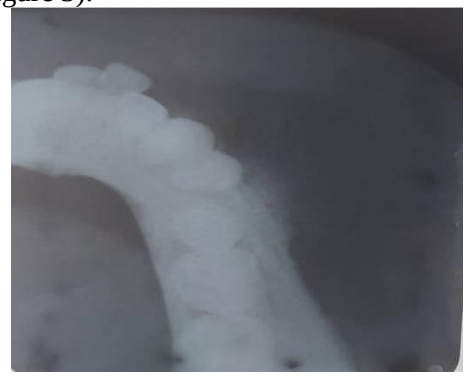


Figure 3. Occlusal radiograph of the lesion present in the left body of the mandible

Computed tomography (CT) findings suggestive of well-defined hypodense juxta cortical lesion seen encircling body of left hemimandible with irregular periosteal reaction and perpendicular bony spicules radiating from cortex, sun ray appearance suggesting parosteal osteosarcoma (figure 4).

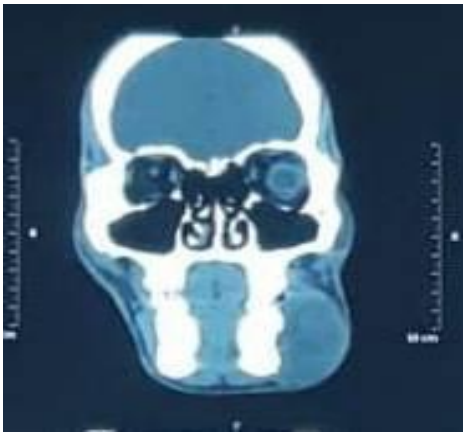


Figure 4. Coronal section of computed tomographic scan showing swelling in the left body mandible

Incisional biopsy confirmed parosteal type of osteosarcoma. Positron emission tomography (PET) scan was suggested for staging. It showed metabolically active lesion in the left half of mandible, mildly active cervical lymphadenopathy. No active regional or distant metastasis. Based on the above clinical, radiological and histopathological findings, the tumour was classified under TNM classification as T2N0M0, we maxillofacial surgeons with the help of the oncologists, left hemi-mandibulectomy was planned with normal margins of 1.5 cm along with left modified neck dissection with preservation of internal jugular vein and spinal accessory nerve (figure 5,6) and reconstruction with pectoralis major myocutaneous pedicled flap for intraoral defect coverage.

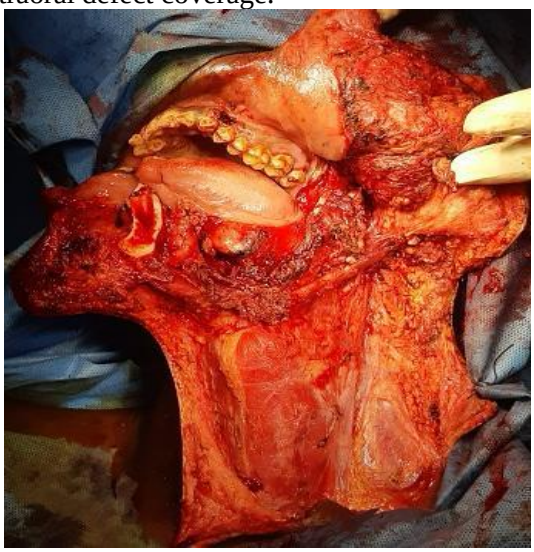


Figure 5. Left Composite resection hemi-mandibulectomy and modified neck dissection

Post-operative period was uneventful. Specimen was sent for histopathological examination to see surgical margins are free of tumour remnants. Excisional biopsy also revealed parosteal osteosarcoma T2N0M0.

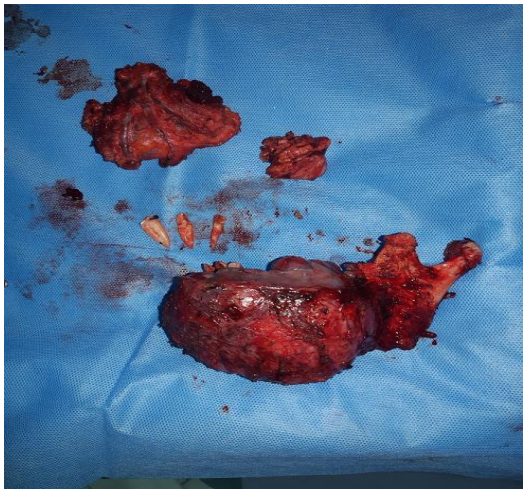


Figure 6. Specimen after composite hemi-mandibulectomy and modified neck dissection

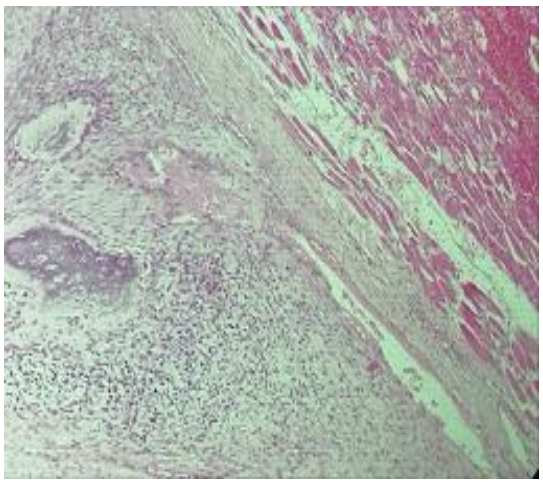


Figure 7. Histopathological section of the specimen

Radiotherapy was given for 6 weeks, 60 gray each session. Adjuvant chemotherapy with 5 cycles of CISPLATIN 120mg/m2 IV D1, ADRIAMYCIN 100mg/m2 IV D1 for improved prognosis(figure 8, 9)..

One year follow up period patient showed no signs of recurrence.

There is a plan to take up for second stage surgery for reconstruction with fibula bone graft.



Figure 8. Patient images for Adjuvant therapy



Figure 9. Intraoral images for Adjuvant therapy

DISCUSSION

This case is very peculiar and surprising that he reported to the Department of oral and maxillofacial surgery with history of road traffic accident and injury to the left side mandible and his orthopantomogram revealed a highly malignant and rare tumour osteosarcoma hidden inside the jaw. He presented with a small swelling with no symptoms at the left body of the mandible. There was rapid growth pattern of the lesion day after day and increased to the size after incisional biopsy.There was no paresthesia of the lower lip in this case, as the involvement of inferior alveolar canal indicates poor prognosis. Studies show that there is microscopic spread seen through mandibular canal, mental nerve and periodontal ligament. Inferior alveolar canal facilitates rapid spread to the adjacent soft tissues.[10] The orthopantomogram, occlusal and CT scan revealed characteristic feature of stippled bone pattern and destruction of the cortical plate and perpendicular bony spicules radiating from the cortex. Characteristic sun ray appearance was seen in this case. CT provides excellent detection of tumour calcification, cortical involvement, soft-tissue and intramedullary extension.[6] MRI is even more effective in demonstrating the intramedullary and soft tissue components. There are two clinical variants i.e., Juxtacortical and Intramedullary. Juxtacortical has parosteal and periosteal types. Histologically based on amount of osteoid cartilage or collagen fibers which are produced by the tumour they are osteoblastic, fibroblastic and chondroblastic.[4,5,7] Incisional biopsy revealed as parosteal chondroblastic variant of osteosarcoma. Chest X-ray and PET-CT of this case revealed no metastasis to the lung or other regions. Metastasis is lower in jaw bones compared to long bones. Metastasis is higher in post irradiation osteosarcoma lesions of jaws.[9] Biological behavior of jaw lesions is different from long bones even though they share similar histological features. For the tumours of maxilla, maxillectomy is performed but difficulties arise due to involvement of structures like maxillary sinus, pterygopalatine fossa and orbital floor. Obturators are used to cover the large defects which are categorized into surgical, post-surgical and definitive.[3]

There are special histological investigations such as bone matrix protein, osteocalcin bone protein which are useful in diagnosing osteosarcomas from other malignant tumours. Anatomical limitations in maxillofacial region cause difficulties in acquiring tumour free margins leading to local recurrence of 33-39% for which multimodality therapy of radical resection with 1.5- 2 cm normal margins followed by radiotherapy and chemotherapy which effects the better prognosis of the lesion.[6,8] Presurgical chemotherapy or radiotherapy is preferred in large tumours to reduce the volume and less aggressive surgical treatment.[8] Radiotherapy is opted for cases with residual, un-ressectable and recurring lesions.[2]

CONCLUSION

This case enlightens us that sudden bony growth in the jaws should alert and be viewed with suspicion of malignant lesion like sarcomas and treated radically with combination therapy for better prognosis and improved survival rate.

DECLARATION

Ethics approval and consent to participate
Not applicable
Patient consent for publication
Not applicable
Animal studies
Not applicable
Acknowledgments
Not applicable
Conflicts of interest and financial disclosures

The author declares that he has no conflict percent and there was no external sourceof funding for the research in question
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ORIGINAL ARTICLE

A COMPREHENSIVE APPROACH TO THE TREATMENT OF PATIENTS WITH PRIMARY GROWTH OF PLEOMORPHIC ADENOMA OF THE PAROTID SALIVARY GLAND

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ABSTRACT

Objective: The aim of the study is to develop a patient management algorithm for pleomorphic adenoma (PA) of the parotid salivary gland (PSG) based on a retrospective analysis of medical histories of patients with parotid gland tumors.

Materials and Methods: An analysis of archival data was conducted to determine the total number of patients with PA who were operated on from 2002 to 2021 (excluding the period from 2006 to 2008) at Department No. 8 of the Research Institute of Stomatology and Maxillofacial Surgery, St. Petersburg State Medical University named after Acad. I.P. Pavlov, Ministry of Health of the RF, as well as the conclusions from the postoperative dispensary follow-up of these patients.

Gor Harutyunyan, Andrei Yaremenko, Margarita Rybakova, Svetlana Kutukova, Ksenia Kazachkova, Gelfiya Nutfullina. A Comprehensive Approach to the Treatment of Patients with Primary Growth of Pleomorphic Adenoma of the Parotid Salivary Gland.Bulletin of Stomatology and Maxillofacial Surgery. 2025;21(3).116-122.doi: 10.58240/1829006X-2025.3-116

Results: 537 patients with neoplasms of the parotid salivary gland (PSG) were identified, of whom 253 (47.1%) were diagnosed with PA based on postoperative morphological findings. In 175 (32.6%) patients, no tumor growth was detected. The concordance rate between preoperative fine-needle aspiration biopsy (FNAB) under ultrasound (US) guidance and postoperative histological findings in patients with PA was 90.9%. During long-term follow-up ultrasound monitoring, recurrence of PA of the PSG was detected in 5 (14.7%) of the 34 examined patients. The average time to recurrence of PA of the PSG in patients with primary tumor growth was 8.9 years.
Conclusions: Based on the analysis of domestic and foreign sources, as well as the data from our study, a management algorithm for patients with primary growth of PA of the salivary glands was proposed. The key points include: mandatory preoperative FNAB under US guidance, exclusion of enucleation as a surgical treatment for PA, an alternative approach for superficially located PAs, removal of PA with a 0.9 cm margin from the fibrous capsule, and lifelong follow-up with ultrasound examination once a year.

Keywords: pleomorphic adenoma, parotid salivary gland, recurrence, fibrous capsule, enucleation, myxoid, morphometry

INTRODUCTION

Pleomorphic adenoma (PA) is the most common benign neoplasm of the salivary glands (SG), with an incidence of up to 80% in major salivary glands.[1] Pleomorphic adenoma occurs in all age groups but is most frequently observed in patients aged 40 to 60 years, with a higher prevalence among females.[2, 15]

PA is a mixed tumor composed of myoepithelial and epithelial cells, which, in turn, are classified into morphological subtypes.[3,4]

The primary preoperative clinical and instrumental diagnostic methods for patients with SG neoplasms include ultrasound (US),computed tomography (CT), and magnetic resonance imaging (MRI), as well as fine-needle aspiration biopsy (FNAB) under US guidance. FNAB is considered the method of choice for preoperative diagnosis and is performed at the initial diagnostic stage.[7]

According to several authors, the accuracy of FNAB under US guidance varies, with false-negative and false-positive rates ranging from 1% to 14%. The reliability of results for benign neoplasms (BN) and malignant neoplasms (MN) of the SG ranges from 81% to 98%, while the specificity of diagnostic methods is between 60% and 75%. False-negative diagnoses are primarily associated with factors such as improper sample collection and the ongoing development of SG tumor classification based on cytological studies.

For BN and MN of the SG, frozen section analysis of postoperative specimens is considered the most reliable method, with an accuracy of up to 98.7% for BN (excluding delayed cases) and up to 85.9% for MN.[5-7]

In recent years, there has been a growing trend toward revising surgical approaches for patients with benign salivary gland neoplasms (BSGN), focusing on organ-preserving surgery. However, the primary surgical treatment methods for BSGN, particularly pleomorphic adenoma, remain extracapsular enucleation/dissection, partial superficial parotidectomy, superficial parotidectomy, and total parotidectomy with or without facial nerve preservation. Additionally, a new trend has emerged involving the removal of salivary gland neoplasms using endoscopic-assisted visualization techniques.[6,7]

At the Congress of the European Society of Sialologists in 2010, a decision was made to divide the parotid salivary gland into five anatomical zones: I–upper lateral (superficial), II – lower lateral (superficial), III – lower deep, IV – upper deep, V – accessory lobe of the gland. The name of the surgical procedure on the parotid salivary gland should be formulated as either "parotidectomy" **or** "extracapsular dissection."

The extent of parotid gland resection is determined by its localization within an anatomical zone and the designation of the removed non-glandular anatomical structure.[8]

When selecting the scope of surgical treatment for pleomorphic adenoma, factors such as tumor size, morphological subtype, integrity and thickness of the fibrous capsule, and the likelihood of satellite nodules must also be considered.[9, 15, 17]

Regarding postoperative follow-up, the 2014 Clinical Protocol for Medical Care of Benign Soft Tissue Tumors of the Head and Neck by the StAR Section "Association of Maxillofacial Surgeons and Dental Surgeons" recommends postoperative follow-up with a maxillofacial surgeon in a consultative and

Gor Harutyunyan, Andrei Yaremenko, Margarita Rybakova, Svetlana Kutukova, Ksenia Kazachkova, Gelfiya Nutfullina A Comprehensive Approach to the Treatment of Patients with Primary Growth of Pleomorphic Adenoma of the Parotid Salivary Gland.Bulletin of Stomatology and Maxillofacial Surgery. 2025;21(3).116-122.doi: 10.58240/1829006X-2025.3-116

diagnostic outpatient clinic or a regional dental clinic. However, the protocol does not specify the timing and frequency of follow-up examinations .[10]

Additionally, the 2014 Clinical Guidelines of the Ministry of Health of the Russian Federation state that the minimum set of postoperative prevention and follow-up measures should include an examination by the attending physician and postoperative scar therapy no later than six months after surgery**,** but they do not specify the frequency of follow-up visits in the long-term postoperative period.[11]

The aim of the study is to develop a patient management algorithm for pleomorphic adenoma (PA) of the parotid salivary gland (PSG) based on a retrospective analysis of medical histories of patients with parotid gland tumors.

MATERIALS AND METHODS

In accordance with the objectives and tasks, an analysis was conducted of archival data from patients who underwent surgical treatment for maxillofacial tumors at the Oncology Department No. 8 (Maxillofacial Surgery) of the Maxillofacial Surgery Clinic at the I.P. Pavlov First St. Petersburg State Medical University of the Ministry of Health of the Russian Federation from 2002 to 2021, excluding the years 2006-2008.

RESULTS

Based on the analysis of the data obtained during this period, 537 patients (figure 1) were identified as having undergone surgical treatment for maxillofacial tumors, of which 364 (67.8%) were female and 173 (32.2%) males.

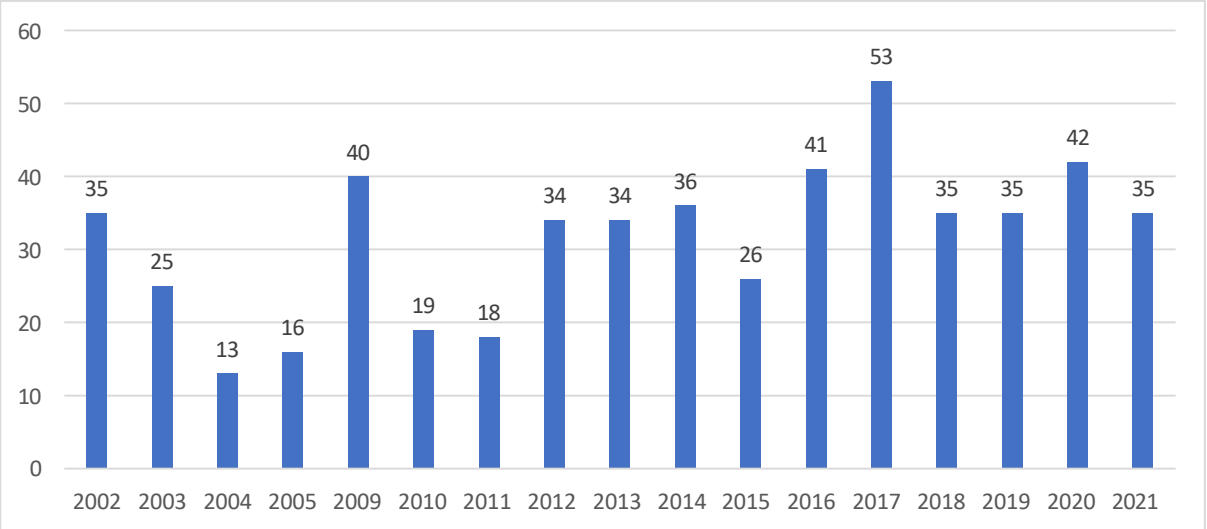


Figure 1. Distribution of 537 patients who received surgical treatment for neoplasms of the parotid gland by years from 2002 to 2021 (excluding 2006–2008).

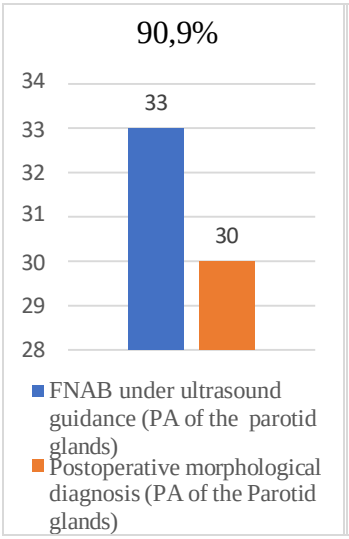
The average age was 51.7 (±15.2, 95% CI 1.29) years (men 51.3±15.5, 95% CI 2.37 years, women 51.9±15.1, 95% CI 1.55 years). All patients underwent ultrasound of the maxillofacial tumors upon admission. Among the aforementioned group of patients with benign maxillofacial tumors, analysis of the postoperative morphological examination reports, according to the 2017 WHO classification of head and neck tumors, revealed that 253 (47.1%) patients had primary pleomorphic adenoma, of which 176 were women and 77 were men. The average age of these patients was 48.2 (±14.4, 95% CI 1.79) years, with women being 49.7 (±14.0, 95% CI 2.08) years and men 44.7 (±14.8, 95% CI 3.42) years. In 143 (26.6%) patients, non-tumorous epithelial lesions of the maxillofacial tumors were identified (lymphoepithelial sialogadenitis, sclerosing polycystic adenosis, hyperplasia of the intercalated ducts) and other types of maxillofacial tumors, including patients with recurrence of pleomorphic adenoma, accounting for 141 (26.3%) patients who did not fall into the main research groups (Table 1).

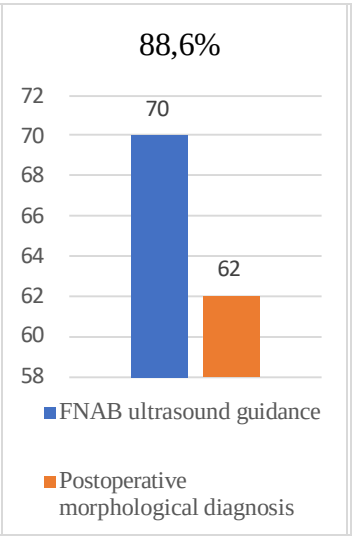
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Table 1. Distribution of patients by type of neoplasm of the parotid gland.

Histological Type	Number of Patients
Pleomorphic Adenoma	253
Non-Tumorous Epithelial Lesions	143
Other Tumors Not Included in the Study	141
Total	**537**

In the overall group of patients (537 individuals), only 70 patients (13%) underwent fine-needle aspiration biopsy (FNAB) under ultrasound guidance in the preoperative period. In 33 out of 70 cases (47.1%), pleomorphic adenoma was detected, while in 37 (52.9%) cases, other types of maxillofacial tumors were identified. Furthermore, an analysis of the cytological examination results revealed that in 30 out of 33 patients with pleomorphic adenoma, the preoperative cytological findings matched the postoperative morphological diagnosis, which is 91% (figure 2). At the same time, an analysis showed that in 175 patients (32.6%) out of 537, the preoperative clinical diagnosis did not match the postoperative morphological diagnosis. Additionally, during the study of preoperative cytological examination data under ultrasound guidance, it was found that the diagnostic accuracy for benign salivary gland tumors, particularly pleomorphic adenomas, was 30 (90.1%). In 3 (3%) cases, the postoperative histological findings revealed a malignant tumor. It is also worth noting that in 8 (11.4%) cases out of 70 cytological conclusions, including those with pleomorphic adenoma, the benign nature of the tumor was not confirmed according to the postoperative morphological diagnosis. Thus, when comparing the discrepancy between the preoperative clinical diagnosis in the overall patient group with the postoperative morphological diagnosis, excluding patients who underwent preoperative FNAB under ultrasound guidance, 175 (37.5%) out of 467 (from 537) patients had discrepancies. In contrast, the group of patients who underwent preoperative FNAB under ultrasound guidance showed significant differences, with a P-value <0.0001 (Fisher's exact test) figure 3,4).





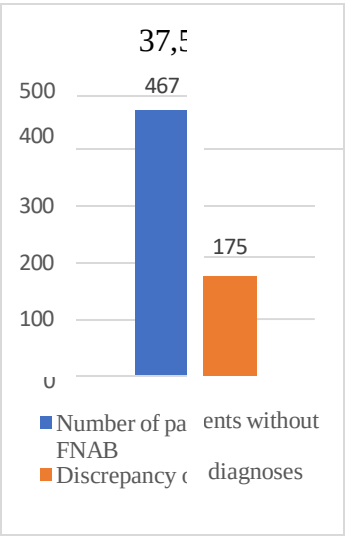


Figure 2. **Figure 3.** **Figure 4.**

Figure 2. Coincidence of the findings of preoperative FNAB and postoperative histological conclusions in patients with pleomorphic adenoma.

Figure 3. Coincidence of the findings of preoperative FNAB and postoperative histological conclusions in patients with neoplasm of the parotid gland.

Figure 4. Discrepancy between the conclusions of preoperative clinical diagnosis in patients without preoperative FNAB and postoperative histological conclusion in patients with neoplasm of the parotid gland.

A detailed analysis of the data from patients with primary pleomorphic adenoma growth also revealed that in 222 (87.7%) patients, the tumor was located in the superficial lobe of the parotid gland. As a result, 129 (50.9%) patients underwent extracapsular enucleation/dissection, 97 (38.3%) patients underwent superficial parotidectomy or subtotal parotidectomy, as the tumor was located at the upper or lower pole of the parotid gland. Complete removal of the parotid gland (extirpation) with preservation of the branches of the facial nerve was performed in 18 patients, where the tumor was located in the deep lobe of the parotid gland. In 4 (1.5%) patients, endoscopic-assisted tumor removal was performed (figure 5).

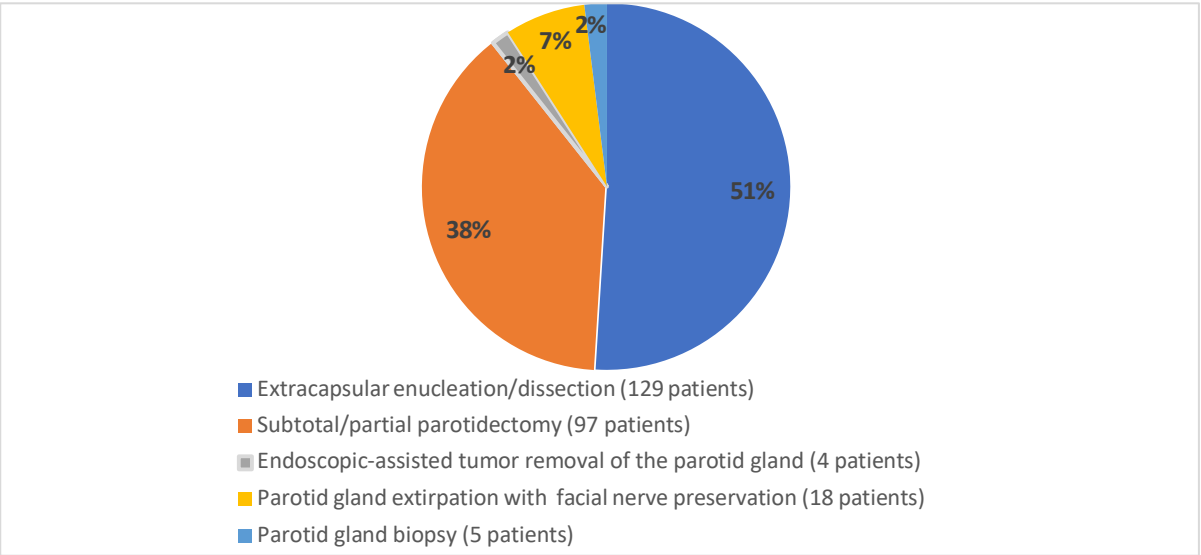


Figure 5. Analysis of the data from patients with primary pleomorphic adenoma

DISCUSSION

Despite the existence of the clinical protocol for medical care in soft tissue head and neck tumors (STAR) from 2014, as well as the clinical guidelines for benign tumors and tumor-like lesions of the parotid gland by the Ministry of Health of the Russian Federation from 2017, the need for improvement and refinement of the existing recommendations remains relevant.

Analyzing archival data from patients with primary tumor growth, particularly pleomorphic adenoma, who underwent surgery in Department No. 8 of the Institute of Stomatology and Maxillofacial Surgery at the I.P. Pavlov First St. Petersburg State Medical University of the Ministry of Health of the Russian Federation, and reviewing publications by foreign authors, we observe the following pattern: preoperative diagnostics, such as ultrasound or MRI, are of limited value in verifying tumor growth in the parotid gland. Based on this, we recommend including mandatory fine-needle aspiration biopsy (FNAB) under ultrasound guidance in the preoperative diagnostic protocol for patients with parotid gland tumors, not only to identify the nature of the tumor growth but also to more accurately plan the surgical treatment strategy.

The analysis of archival data also revealed that 129 (50.9%) patients with primary pleomorphic adenoma underwent extracapsular enucleation, which in turn increases the risk of recurrence. Comparing these data with the recommendations from the European Society of Sialogists from 2010 and our previous research, which indicates that the probability of recurrence of pleomorphic adenoma in the parotid gland is influenced not only by the volume of surgery but also by factors such as the integrity and thickness of the fibrous capsule of the adenoma and the presence of satellite nodules (satellites), which are located 0.5 to 0.85 cm from the fibrous capsule of the adenoma[12-14, 16], we recommend abandoning the extracapsular enucleation approach for removing pleomorphic adenomas.

As an alternative, we propose that in cases where the pleomorphic adenoma is located in the superficial lobe of the parotid gland and has a size of ≤2.5 cm, the tumor should be removed with a margin of 0.9 cm from the fibrous capsule into the "safe zone" with regard to the presence of satellite nodules.

Additionally, the analysis of archival data revealed the following pattern: postoperative follow-up should not have a time limit and should be conducted for life, with annual ultrasound examination of the parotid gland. Based on the analysis of all the above data, we developed a management algorithm for patients with primary tumor growth in the parotid gland, particularly pleomorphic adenoma (figure 6).

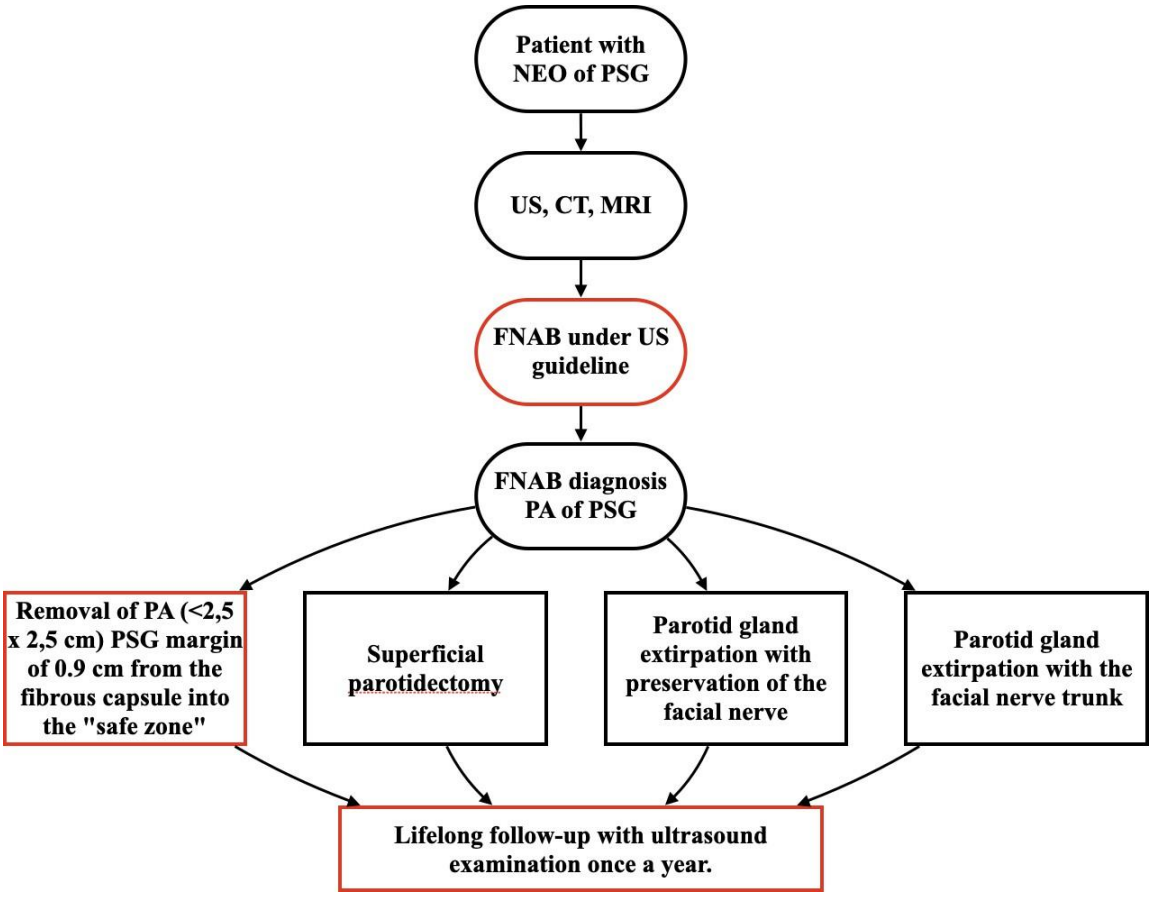


Figure 6. Algorithm for choosing the management and surgical treatment of patients with pleomorphic adenoma of the parotid gland.

DECLARATIONS

Conflicts of interest and financial disclosures: The authors declare that they have no conflicts of interest, and there was no external source of funding for this research.

Ethical approval: The study was approved by the Institutional Ethics Committee and was conducted in accordance with the Declaration of the World Medical Association.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Source of funding: The work was not funded.

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REVIEW ARTICLE

EXPLORING THE ROLE OF LONG NON-CODING RNAs AS BIOMARKERS IN CARDIOVASCULAR DISEASE: A BIBLIOMETRIC ANALYSIS

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Background: Cardiovascular diseases (CVDs) are a leading cause of mortality globally, necessitating novel biomarkers for diagnosis and treatment. Long non-coding RNAs (lncRNAs) have emerged as significant regulatory molecules in cardiovascular biology, yet comprehensive bibliometric analyses in this area are lacking.
Objective: This study aims to conduct a bibliometric analysis of lncRNAs as biomarkers in CVD to identify publication trends, key contributors, and thematic developments from 2008 to 2023.
Methods: A systematic search was performed across PubMed, Scopus, and Web of Science using the keywords "((Long non-coding RNA) OR (lncRNA)) AND (Cardiovascular disease) OR (Cardiovascular disorders) AND (Biomarkers)." A total of 33,805 records were retrieved, with duplicates removed, resulting in 29,545 unique records. After screening, 341 relevant articles were selected for bibliometric analysis using VOSviewer.
Results: A total of 341 publications were analyzed, involving 54 countries/regions, 548 institutions, and 1744 authors across 177 journals. The United States (84 publications) and China (61 publications) were the leading contributors. Zhengzhou University had the highest publication output, while Guo Wei was the most prolific author. Keyword analysis revealed a focus on lncRNAs' roles in the occurrence, development, diagnosis, treatment, and prognosis of CVD.
Conclusion: This bibliometric analysis provides insights into the research landscape of lncRNAs as biomarkers in CVD, highlighting significant trends, influential authors, and collaborative networks. The findings underscore the importance of lncRNAs in cardiovascular research and suggest potential areas for future investigation

Keywords: Biomarker, Long coding RNA, Cardiovascular disease

INTRODUCTION

Cardiovascular diseases (CVDs) remain the leading cause of death worldwide, responsible for approximately 17.9 million deaths annually according to the World Health Organization (WHO).[1,2] The substantial morbidity and mortality associated with CVDs, including heart failure,[3] myocardial infarction,[4] and stroke,[5] emphasize the urgent need for novel biomarkers to enhance early diagnosis, guide therapeutic interventions, and predict prognosis.[6] In recent years, long non-coding RNAs (lncRNAs) have emerged as promising molecular entities in this regard.[7]

LncRNAs are a class of RNA molecules longer than 200 nucleotides that, unlike messenger RNAs (mRNAs), do not code for proteins but play crucial roles in regulating gene expression and various cellular processes.[8] They have been implicated in numerous biological functions, including chromatin remodeling, transcriptional regulation, splicing, and post-transcriptional modification.[7–13] In the context of cardiovascular diseases, lncRNAs have been shown to regulate pathways involved in heart development, vascular function, and pathological remodeling following myocardial injury.[7–10]

The unique properties of lncRNAs, such as tissue-specific expression and stability in body fluids, make them attractive candidates for non-invasive biomarkers. These features provide opportunities for their use in diagnostic assays, which could facilitate early detection and personalized treatment strategies for CVD patients.[14–16] Despite the increasing body of research on lncRNAs in cardiovascular biology, a systematic evaluation of the publication trends, key contributors, thematic developments, and collaborative networks in this field has been lacking. Bibliometric analysis offers a powerful tool to analyze the structure and dynamics of scientific literature, providing insights into research progress and guiding future studies.[17]

This study aims to conduct a comprehensive bibliometric analysis of research on lncRNAs as biomarkers in cardiovascular diseases from 2008 to 2023. By examining publication trends, co-authorship networks, country collaborations, and keyword co-occurrence patterns, this analysis seeks to identify key contributors, thematic foci, and emerging trends. Ultimately, this analysis will provide a detailed overview of the research landscape, identifying influential authors, journals, and institutions while highlighting potential areas for future investigation.

Methodology for Bibliometric Analysis

This bibliometric analysis was conducted to investigate the research landscape of long non-coding RNAs (lncRNAs) as biomarkers in cardiovascular disease. The methodology involved several key steps to ensure a comprehensive and systematic approach to data collection and analysis.(Figure 1)

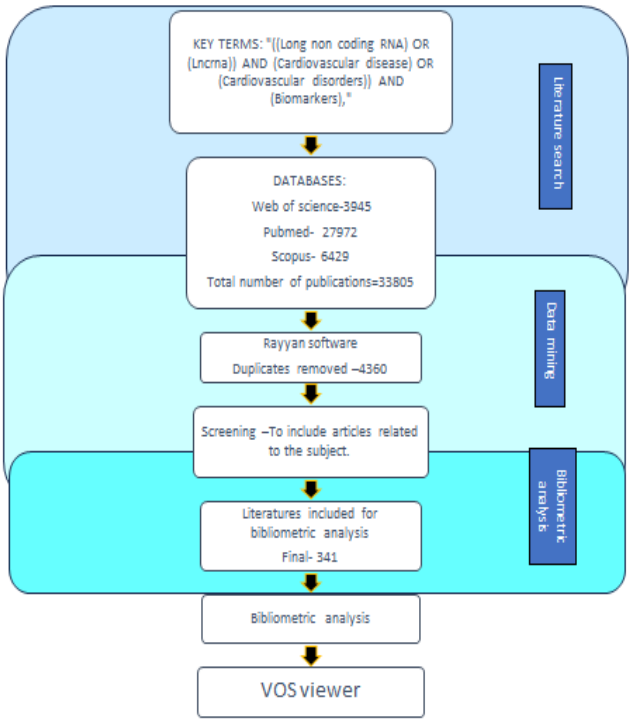


Figure 1. Bibliometric analysis methodology flow chart

Data Sources

To capture the breadth of research on lncRNAs as biomarkers in cardiovascular diseases, data were retrieved from three major academic databases: PubMed (27,972 records), Scopus (6,426 records), and Web of Science (3,945 records). These databases were selected for their comprehensive coverage of biomedical and life sciences research.

Search Strategy

A comprehensive search was performed using the following keywords:

"((Long non-coding RNA) OR (lncRNA)) AND (Cardiovascular disease) OR (Cardiovascular disorders) AND (Biomarkers)"

This search yielded a total of 38,346 records from the three databases mentioned above.

Data Cleaning and selection

After the initial search, the records were screened for duplicates, resulting in the removal of 4,360 duplicate entries, leaving 33,986 unique records. The remaining records were then manually screened for relevance. Only articles directly addressing lncRNAs as biomarkers in cardiovascular diseases were included in the final dataset. This process narrowed the focus to 341 publications, which formed the basis for the bibliometric analysis.

Analysis Tools

The bibliometric analysis was performed using VOSviewer (Version 1.6.20), a software tool specifically designed for constructing and visualizing bibliometric networks. VOSviewer was used to analyze co-authorship networks, citation networks, and keyword co-occurrence patterns. These analyses allowed for the identification of key authors, institutions, journals, and thematic clusters within the research domain.

Metrics and Network Construction

In the analysis, several key metrics were utilized:

- **Degree Centrality*:*** This metric was used to measure the influence of individual authors and institutions within the research network. Degree centrality refers to the number of direct connections an entity has within the network, indicating its centrality and influence.
- **Cluster Analysis*:*** To identify thematic clusters within the research landscape, VOSviewer's clustering algorithm was applied to the co-authorship and keyword networks. Each cluster represents a group of related studies, often centered around a specific subtopic within the field.
- **Citation Analysis*:*** Citation networks were constructed to identify the most influential articles, authors and journals. The strength of the links between nodes in these networks reflects the frequency with which articles are co-cited, providing insight into the most impactful research within the field.
- **Keyword Co-occurrence Analysis**: This analysis was used to identify key themes and emerging trends in the field of lncRNAs and cardiovascular diseases by examining the frequency and co-occurrence of specific keywords within the literature.
- **Bibliometric coupling*:*** Bibliometric coupling is a method used to measure the similarity between two documents based on the number of references they share.

RESULTS

Yearly trends publications

The graph depicts yearly publication trends related to long non-coding RNAs and cardiovascular disease. Research activity saw a sharp rise, peaking in 2021 with 64 publications**,** followed by sustained high output in 2022 and 2020**.**

(Figure 2) Early years, such as 2013 to 2016, had minimal publications, indicating that the research area was emerging. However, starting from 2017**,** there was a marked increase in interest. Recent years show a decline, with 12 publications **in** 2024**,** likely reflecting the year's incomplete status or shifting research priorities. Overall, the field experienced rapid growth but fluctuated in recent years.

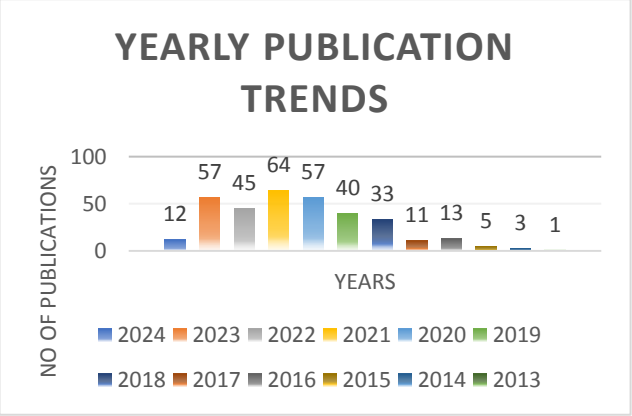


Figure 2. Yearly trend of publications

Analysis of Journals Having Maximum Publications: The Journal of Cardiovascular Research leads with 17 publications in the field of long non-coding RNAs and cardiovascular disease, followed by Frontiers in Cardiovascular Medicine with 11 publications and the International Journal of Genetics with 8 publications**.** (Figure 3) Other journals like the Journal of Cellular and Molecular Medicine and Advances in Experimental Medicine and Biology also contribute significantly.Overall,cardiovascular-specific journals dominate the publication landscape, while genetics and molecular biology journals also play important roles.

Analysis of Publications by Country

The graph highlights the global distribution of research publications on long non-coding RNAs and cardiovascular disease, with the United States leading the way at 84 publications**,** followed by China and England**,** each contributing 61 publications**.** (Figure 4) The United Arab Emirates makes a significant showing with 48 publications, reflecting its growing investment in scientific research and innovation. Switzerland follows with 18 publications**,** showcasing its strong tradition in life sciences and biotechnology. Countries like Brazil and Australia**,** with 13 and 8 publications respectively, also contribute meaningfully to the field, despite facing different levels of research funding and resources. Smaller contributors such as India**,** Turkey**,** Serbia**,** Luxembourg**,** Iran**,** Singapore**,** South Korea, and Poland have published between 1 to 6 papers, indicating a global interest in this emerging area of study. Overall, the distribution reflects both the dominance of a few key nations and the broad international engagement in understanding the role of long non-coding RNAs as biomarkers in cardiovascular disease.

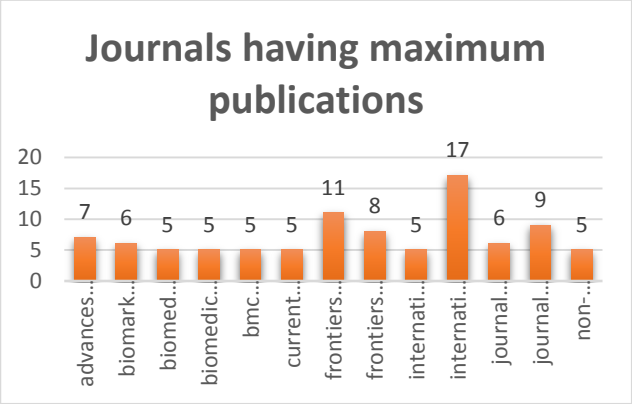


Figure 3. Analysis of journals having maximum publications

Co-Author Network Analysis:
Co-Author Network Analysis: Specific Authors in Key Domains

The image provided is a co-authorship network analysis visualized using VOSviewer, depicting the collaborative relationships between authors within a specific research domain.

(Figure5A) Each node in the network represents an author, while the edges (links) between them represent co-authorship relationships. The size of the nodes typically indicates the number of publications or the impact of the author, while the thickness of the edges reflects the strength of the collaboration (e.g., how often authors have co-published). The color gradient from blue to yellow shows the temporal evolution, with blue indicating earlier collaborations and yellow indicating more recent collaborations.

Network Metrics

Degree Centrality:

- Thomas Thum, Stefanie Dimmeler, and Lars Maegdefessel have high degree centrality, reflecting their extensive collaboration networks. These authors are likely key influencers or leaders in the research domain.

Cluster Analysis:

In this co-author network, several distinct clusters represent different research domains within the broader study of long non-coding RNAs (lncRNAs) and their role as biomarkers in cardiovascular disease (CVD). Below is an interpretation of the authors within each domain, grouped by their clusters.

A. Largest Cluster - Cardiovascular Biomarkers and Therapeutics

This cluster likely centers on translational research related to the therapeutic application of lncRNAs in cardiovascular disease. Authors like Thomas Thum and Stefanie Dimmeler are recognized leaders in this space, contributing to studies on lncRNAs as biomarkers for heart failure, myocardial infarction, and other cardiovascular conditions.

B.Yellow Cluster - Molecular Mechanisms and Regulatory Pathways

This cluster appears to focus on the molecular biology of lncRNAs, particularly their regulatory roles in cellular pathways involved in cardiovascular diseases. Yunzhu Peng and Luqiao Wang are likely conducting research on how lncRNAs influence gene expression and cardiovascular pathology.

C.Blue Cluster - Fundamental Research in lncRNA and Cardiovascular Disease

The blue cluster seems to be involved in foundational research regarding the molecular and genetic mechanisms of

lncRNAs in cardiovascular health. Authors like **Zhiguo Wang** and **Yong Zhang** are likely contributing to key insights in gene regulation and the basic biology of lncRNAs.

D.Green Cluster - Emerging Research on lncRNAs and CVD

The green cluster represents emerging researchers in the field. **Guoping Li** and **Ying Liu** are likely newer contributors focusing on the identification and functional analysis of lncRNAs in cardiovascular diseases. This cluster may be exploring newer aspects of how lncRNAs function as biomarkers or contribute to disease mechanisms.

E. Purple Cluster - Genetic and Cellular Research

This cluster seems to focus on genetic and cellular studies related to lncRNAs, possibly involving animal models and gene expression experiments. Researchers like Chunyu Zeng **and** Yujia Wang may be investigating how lncRNAs interact with various cellular processes in the context of cardiovascular conditions.

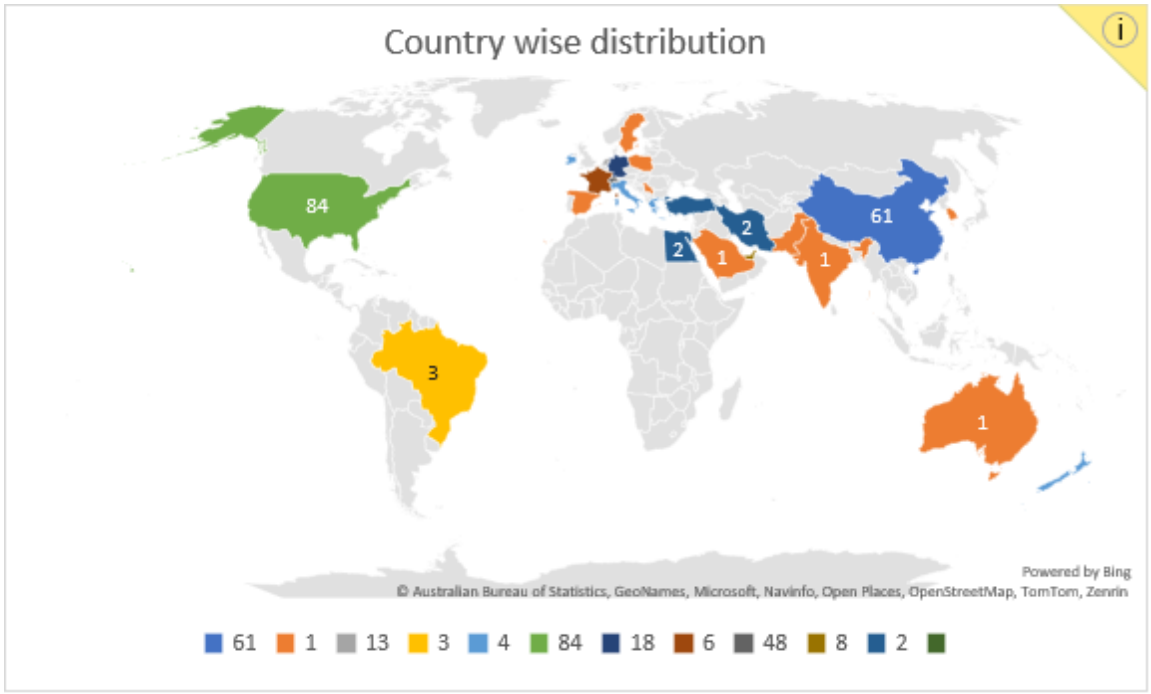


Figure 4. Analysis of Publications by Country

Co-Authorship Network of Organizations

Each domain identified in the network visualization represents a group of organizations that collaborate closely in the study of long non-coding RNAs (lncRNAs) and their role as biomarkers in cardiovascular disease.(Figure 5B) Below is a detailed explanation of how these organizations contribute to this specific area of research.

Cluster 1 - Leading Biomedical and Cardiovascular Research Institutions(Green)

This cluster is composed of some of the world's leading biomedical and cardiovascular research institutions**. Harvard University** and **Massachusetts General Hospital** are at the forefront of clinical and translational research, particularly in developing novel biomarkers for heart disease, including the investigation of lncRNAs. **Chinese institutions** like the **Chinese Academy of Medical Sciences** and **Nanjing Medical University** have rapidly expanded their research capacity, focusing on the molecular mechanisms by which lncRNAs regulate cardiovascular diseases.

Cluster 2 - European Cardiovascular and Genomic Research Leaders(Blue)

This cluster represents European and transatlantic leaders in cardiovascular genomics and translational medicine. **Goethe University Frankfurt, Medizinische Hochschule Hannover,** and the **German Centre for Cardiovascular Research** are major players in studying lncRNAs, with a focus on how these molecules can serve as predictive biomarkers for cardiovascular events, such as myocardial infarction or heart failure. **Imperial College London** and **Karolinska Institutet** contribute by integrating genomics with clinical data to improve personalized medicine approaches for CVD. Their collaborations with **British Heart Foundation** and the **Icahn School of Medicine at Mount Sinai** highlight a strong emphasis on applying findings to clinical practice, particularly in cardiovascular health.

Cluster 3 - Asian Academic Institutions Focused on Molecular Biology and Cardiovascular Health(Yellow)

This cluster includes several of Asia's top academic and medical institutions. **Shanghai Jiao Tong University** and **Sun Yat-sen University** are recognized for their contributions to molecular biology, especially in understanding the regulatory roles of lncRNAs in cardiovascular disease progression. These institutions, along with **Zhejiang University** and **National University of Singapore**, are advancing research on how lncRNAs influence gene expression in cardiovascular health and disease.

Cluster 4 - Emerging Research Organizations in Cardiovascular Science(Purple)

This cluster consists of institutions that are emerging as influential players in cardiovascular research. **The University of Melbourne** and **University of Leicester** are contributing significantly to the field by exploring the genetic and epigenetic roles of lncRNAs in cardiovascular disease. **University Hospital of Basel** and **Centro de Investigación en Red** focus on translational research, bridging the gap between basic molecular findings and their application in clinical environments. **Stanford University** and **Technical University of Munich** add strength in bioinformatics and systems biology, which are critical for analyzing large datasets related to lncRNAs and cardiovascular outcomes, thus facilitating the discovery of novel biomarkers.

Cluster 5 - Specialized Medical and Research Centers(Light green)

This cluster includes specialized medical centers and universities that contribute to niche areas within the broader field of cardiovascular lncRNA research. **Affiliated Hospital of Qingdao University** and **First Affiliated Hospital of Kunming Medical University** focus on integrating clinical observations with molecular research, specifically examining how lncRNAs can be used as non-invasive biomarkers in patient care. **Universidade de Sao Paulo** and **Emory University** contribute to global research efforts by combining epidemiological data with molecular studies to explore the role of lncRNAs in diverse populations.

A .Co-Author analysis

Authorship Network of Organizations

C. *Co-Authorship Network of Countries*

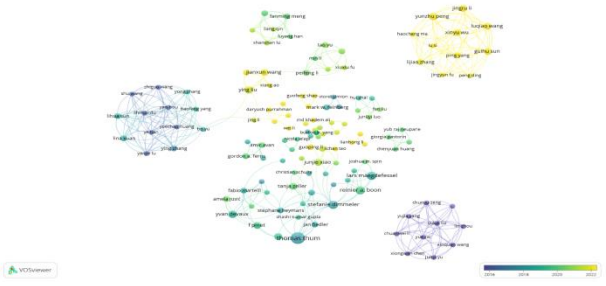


Figure 5. Co- author analysis

Co-Authorship Network of Countries

The image provided is a co-authorship network analysis visualized using VOSviewer, depicting the collaborative relationships between countries within a specific research domain. (Figure 5C)Each node represents a country, and the edges (links) between them represent co-authorship relationships across their research outputs. The size of the nodes typically reflects the volume of research output or the impact of the country in the field, while the thickness and color gradient of the edges indicate the strength of the collaboration and the temporal evolution of these collaborations.

Network Metrics

Degree Centrality:

- **China and the United States** have high degree centrality, reflecting their extensive collaboration networks and central roles in global research. These countries are crucial for fostering international research partnerships.

Cluster Analysis:

- **China-Centered Cluster (Green-Blue):**
  - China has strong connections with countries like the United States, Australia, and several European countries. This indicates that China is a hub for international collaboration, particularly with both Western countries and its regional neighbors.
- **United States-Centered Cluster (Blue-Green):**
  - The United States is connected with numerous countries across Europe, Asia, and the Middle East, highlighting its role as a central node in global research networks. Key collaborators include the United Kingdom, Germany, and Japan.
- **European Cluster (Blue-Purple):**
  - European countries such as **Germany, United Kingdom, Netherlands, and France** form a tightly connected group, with strong collaborative ties within the continent. These countries are pivotal in European research networks and frequently collaborate with other global research leaders like the United States and China.
- **Emerging Collaborations (Yellow-Green Cluster):**
  - Countries like **India, Iran, and Saudi Arabia** show emerging collaborative activities, indicated by the green-yellow color scheme. These nations are increasing their presence in the global research landscape, particularly through collaborations with established countries like the United States and China.

Co-occurrence - keywords

Through a network visualization of keyword co-occurrence, 11 distinct clusters were identified, reflecting various research themes within this domain.(Figure 6)

Green Cluster - Central Focus on Biology and Long Non-Coding RNAs

- **Domain: Biology, Biochemistry, and Molecular Biology**
- **Keywords:** Long non-coding RNA, biology, computational biology, biochemistry, cancer research.
- This cluster represents the largest body of literature focusing on the fundamental biological processes involving lncRNAs. Specifically, lncRNAs are being studied for their roles in gene regulation, cellular mechanisms, and their potential as biomarkers in various diseases, including cardiovascular conditions

Blue Cluster - Cardiovascular Disease and Clinical Implications

- **Domain: Cardiology and Cardiovascular Health**
- **Keywords:** Coronary artery disease, diagnostic accuracy, clinical practice, biomarkers.
- This cluster is primarily focused on cardiovascular disease (CVD), with a significant emphasis on clinical applications. Research within this cluster examines how lncRNAs can be used as biomarkers for diagnosing and predicting the progression of coronary artery disease and other CVDs. The

focus on diagnostic accuracy and clinical practice suggests that lncRNAs are being evaluated for their translational potential in medical settings.

Yellow Cluster - Computational Approaches in LncRNA Research

- **Domain: Genomics and Computational Biology**
- **Keywords:** Computational biology, gene expression profiling, RNA splicing, competing endogenous RNA.
- Computational biology plays a key role in identifying and characterizing lncRNAs. This cluster emphasizes the integration of bioinformatics tools to analyze lncRNA expression profiles and their interactions with other RNA species (e.g., miRNAs, mRNAs). Such approaches are crucial for understanding the regulatory roles of lncRNAs in CVD and their potential as therapeutic targets.

Red Cluster - Heart Failure and LncRNA Pathophysiology

- **Domain: Cardiology and Pathophysiology**
- **Keywords:** Heart failure, ischemia, pathogenesis, fibrosis, mechanical engineering.
- This cluster investigates the role of lncRNAs in the pathogenesis of heart failure and related conditions such as ischemia and fibrosis. Research here explores how specific lncRNAs contribute to the molecular mechanisms underlying heart failure, providing insights into their use as biomarkers for disease progression and therapeutic targets.

Purple Cluster - Molecular Techniques in LncRNA Research

- **Domain: Molecular Biology and Genetics**
- **Keywords:** Apoptosis, gene knockdown, autophagy, molecular biology, reperfusion injury.
- This cluster explores the molecular biology techniques used to study lncRNAs, particularly through gene knockdown experiments and their effects on cellular processes like apoptosis and autophagy. These studies are essential for understanding the functional roles of lncRNAs in cardiovascular diseases and their potential as biomarkers for early detection.

Cyan Cluster - Epidemiology and Public Health Implications of LncRNAs

- **Domain: Public Health and Epidemiology**
- **Keywords:** Population, stroke, public health.
- This cluster highlights the epidemiological aspects of CVD research, particularly in how population studies are being used to identify lncRNA biomarkers for conditions like stroke. The role of lncRNAs in population health research underscores their potential for large-scale diagnostic applications in public health interventions.

Light Green Cluster - LncRNAs in Disease and Inflammatory Pathways

- **Domain: Immunology and Disease Mechanisms**
- **Keywords:** Inflammation, coronary syndrome, disease, biomarkers.
- Inflammation is a critical factor in the pathogenesis of CVD, and this cluster focuses on how lncRNAs are involved in inflammatory processes. The identification of lncRNAs as biomarkers in inflammatory pathways provides valuable insights into their role in the development and progression of CVD, particularly in acute coronary syndromes.

Pink Cluster - Metabolic Health and LncRNA Biomarkers

- **Domain: Endocrinology and Metabolism**
- **Keywords:** Diabetes mellitus, metabolic health, insulin resistance.
- This cluster investigates the intersection between metabolic disorders and CVD, with a focus on how lncRNAs may serve as biomarkers for conditions like diabetes and metabolic syndrome. Given the strong link between metabolic health and cardiovascular risk, this research is critical for developing integrated approaches to managing CVD through the use of lncRNA biomarkers.

Orange Cluster - LncRNAs and Cancer Biology

- **Domain: Cancer Biology**
- **Keywords:** Cancer research, cell biology, gene knockdown, RNA splicing.
- While this cluster focuses on cancer biology, there is significant overlap with cardiovascular research, particularly in how lncRNAs are studied for their regulatory roles in cell biology. The insights gained from cancer research are often applicable to CVD, as many lncRNAs are implicated in both cancer and cardiovascular conditions.

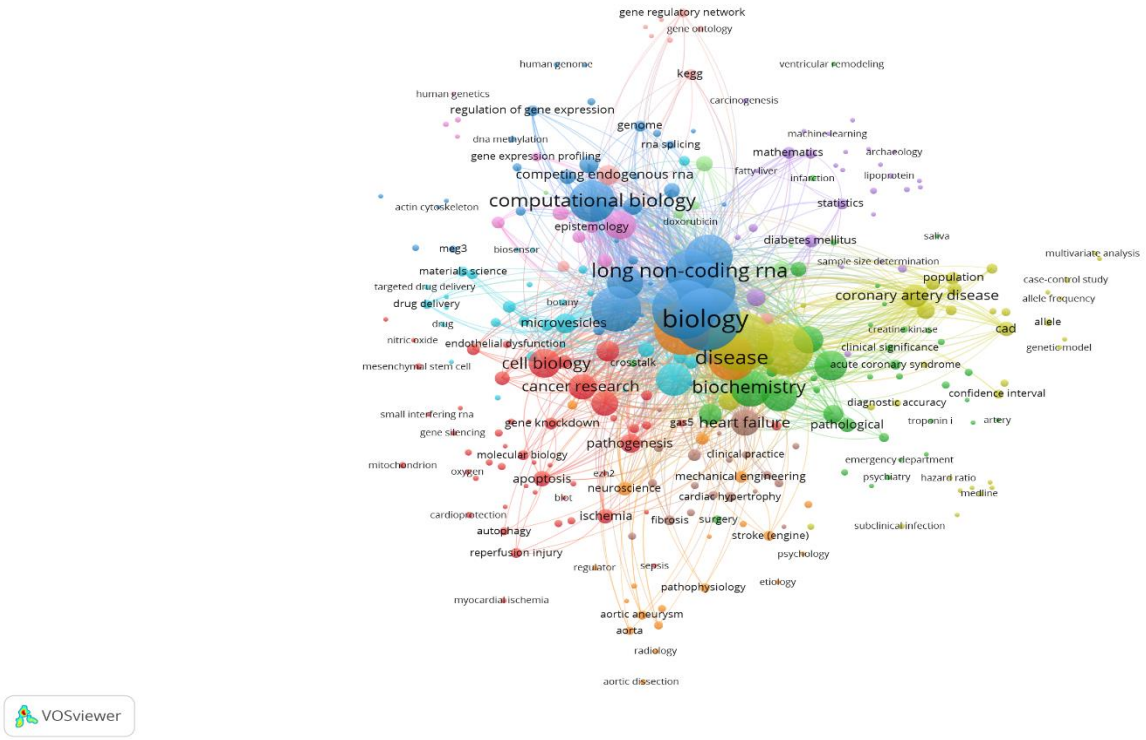
Brown Cluster - Epigenetics and LncRNAs in CVD

- **Domain: Genetics and Epigenetics**
- **Keywords:** Human genetics, epigenomics, DNA methylation.
- Epigenetic modifications such as DNA methylation play a crucial role in the regulation of lncRNAs. This cluster examines how epigenetic changes influence lncRNA expression and function in CVD, offering potential biomarkers for early diagnosis and therapeutic interventions.

Dark Blue Cluster - Biomedical Engineering and LncRNA Applications

- **Domain: Biomedical Engineering and Cardiovascular Devices**
- **Keywords:** Mechanical engineering, cardiovascular devices, clinical significance.
- This cluster explores the application of biomedical engineering in the development of cardiovascular devices, with a focus on how lncRNA research can inform the design of new diagnostic and therapeutic tools. The integration of lncRNA biomarkers with biomedical devices represents an innovative approach to improving patient outcomes in cardiovascular care.

Figure 6.Co-occurrence Results Overview



Citation Network Analysis

Sources

1. The image provided is a citation network visualization of journals and sources.(Figure 7A) The nodes represent individual journals, and the connections (edges) represent citation relationships between these journals. The size of the nodes reflects the influence or impact of a journal within the network, often through the number of citations. The color of the nodes reflects the recency of the publications in the journal, with lighter colors indicating more recent activity.
2. **Central Journals and Influential Sources**: Journals like the International Journal of Molecular Sciences, Journal of Cellular and Molecular Medicine, Cells, and Circulation Research are identified as central hubs, frequently cited in the context of lncRNAs and cardiovascular disease.
3. **Specialization and Clustering**: The network reveals clusters of journals that specialize in specific subfields, such as Frontiers in Cardiovascular Medicine and Journal of Molecular and Cellular Cardiology, indicating focused research on the cardiovascular implications of lncRNAs.
4. **Emerging Journals**: Newer journals, including Non-Coding RNA Research and Molecular Diagnosis & Therapy, are gaining traction in the field, likely publishing cutting-edge research on lncRNAs and their applications as biomarkers.
5. **Multidisciplinary Influence**: Journals like Cells and Scientific Reports exhibit broad influence, suggesting that while they publish on cardiovascular lncRNAs, they also engage with a wider range of molecular biology topics, highlighting the interdisciplinary relevance of lncRNA research.
6. **Citation Flow**: The connections between journals illustrate a flow of knowledge from basic science to clinical applications, indicating that foundational research on lncRNAs is being translated into practical medical insights, particularly in cardiovascular contexts.

Co-authorship network *with a focus on* **author citations**

The provided image represents a **co-authorship network** with a focus on **author citations.** (Figure 7B) Each node represents an author, and the connections between the nodes represent collaborations (co-authorships). The size of the nodes indicates the relative citation impact of the author, while the color gradient reflects the recency of their publications, ranging from older research (darker colors) to more recent contributions (lighter colors).

Highly Cited Authors

- **Thomas Thum** stands out as the most prominent author in the network, indicated by the large size of his node. This suggests that his work has received a significant number of citations, making him a highly influential figure in the field of lncRNAs and cardiovascular disease.
- **Reinier A. Boon** and **Stefanie Dimmeler** also appear as key contributors with large nodes, indicating their high citation impact. These authors likely publish widely-cited work related to the identification and role of lncRNAs in cardiovascular conditions.
- **Yvan Devaux** and **Lars Maegdefessel** are also notable for their citation impact, connecting widely to both central and peripheral nodes, suggesting their work is both influential and collaborative.

Collaboration and Citation Flow

- Authors like **Jan Fiedler, Amelia Jusic**, and **Jingyun Fu** are well-connected to the central figures, implying that their work is frequently co-cited alongside highly influential authors like **Thum, Boon**, and **Dimmeler.** This suggests that they are collaborators on key studies or contribute significantly to the same body of work that is heavily cited.
- The dense interconnections among the central nodes suggest that much of the high-impact research in this area is produced through collaborations among these key authors. These collaborations are likely cited together in studies that explore the role of lncRNAs as biomarkers in heart disease.

Emerging Authors with Growing Citation Impact

- Authors such as **Yunzhu Peng, Ying Liu**, and **Peifeng Li** appear in lighter colors, indicating more recent contributions to the

field. Though their nodes are smaller compared to the central figures, the fact that they are connected to the network shows they are part of ongoing research that is gaining citations.

- Authors like **Tao Yu** and **Xiuxiu Fu**, located on the periphery with smaller nodes, likely represent newer entrants into the field whose work has not yet amassed a large citation count but may grow as the field evolves.

Peripheral Authors and Niche Research Areas

- Authors such as **Tao Yu** and **Xiuxiu Fu** are located in more isolated clusters, suggesting that they may be working on more specialized or emerging subtopics within the broader field. Their smaller node sizes indicate that their work has fewer citations compared to the central figures but may be highly relevant to niche areas of lncRNA research.

Citation Distribution Across the Field

- The overall network shows a clear distribution of citation influence, with a few key authors receiving the majority of citations (as indicated by their larger nodes) and a broader set of collaborators contributing to the overall growth of the field. The color gradient indicates that the field is still active, with recent publications from newer authors continuing to contribute to the ongoing research.

Citation Network of Organizations

The image provided is a citation network analysis visualized using VOSviewer, depicting the relationships between various organizations based on citation links. (Figure 7C) Each node represents an organization (such as universities, research institutes, or hospitals), and the edges (links) between them represent citations from one organization to another. The size of the nodes reflects the number of citations an organization has received, while the thickness and color gradient of the edges indicate the strength of citation connections and the temporal evolution of these citations.

Citation Patterns:

Dense Citation Network:

- The network is densely interconnected, especially around the central organizations, indicating that the research domain is well-established with a high degree of citation interdependency. This suggests a robust and

A. Citation network visualization of journals and sources

B**. Co-authorship network with focus on author citation**

C. Citation Network of Organizations

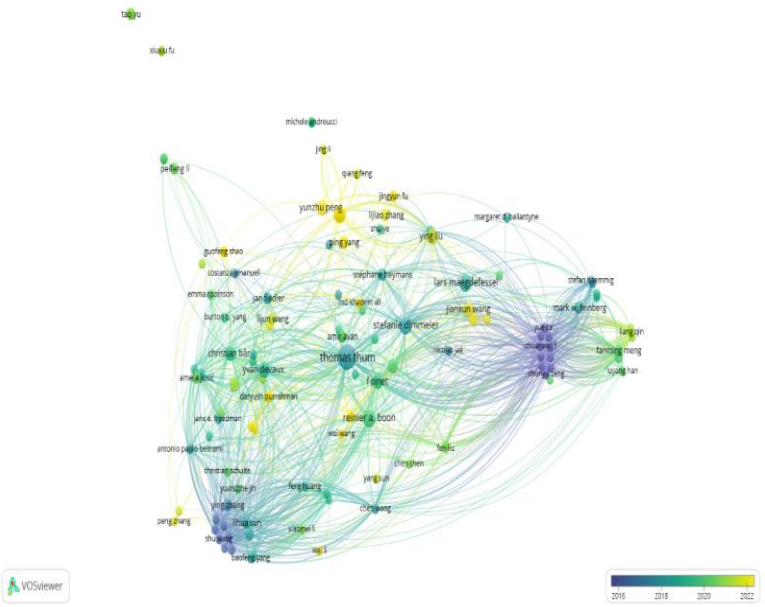


Figure 7. Citation network analysis

collaborative research environment where institutions frequently build on each other's work.

Network Metrics

Degree Centrality

- **Harvard University and Central South University** have high degree centrality, reflecting their broad influence across the research domain. These institutions are central nodes in the citation network, connecting various clusters and serving as major reference points for other organizations.

Cluster Analysis:

- The map reveals distinct clusters that represent different thematic areas or geographical regions:
- **Cluster 1 (Green-Blue):**
  - Harvard University, Central South University, and Massachusetts General Hospital: This cluster is densely connected, indicating strong citation relationships among these institutions. It suggests a core area of research that is highly collaborative and influential.
- **Cluster 2 (Blue-Purple):**
  - Medizinische Hochschule Hannover, German Centre for Cardiovascular Research, and Imperial College London: This cluster is composed of European institutions that are heavily interlinked, indicating strong regional collaboration and citation networks within Europe.
- **Cluster 3 (Green-Yellow):**
  - Zhejiang University, Harbin Medical University, and Sun Yat-Sen University: This cluster represents institutions that are gaining prominence, particularly in Asia. The green-yellow color indicates more recent contributions to the field, highlighting their emerging influence.

Bibliometric coupling - Source Analysis

In the provided bibliometric coupling network, key journals such as the International Journal of Molecular Sciences **and** Frontiers in Cardiovascular Medicine have a strong presence, indicating their influence and interconnectedness with other sources through shared citations. (Figure 8) These journals are crucial in advancing research on long non-coding RNAs (lncRNAs) as biomarkers in cardiovascular diseases. The network also highlights thematic clusters of journals focused on different areas, such as molecular biology and cardiovascular research, which are frequently co-cited, underscoring the interdisciplinary nature of this field. Furthermore, emerging sources like Biomarkers in Medicine and Human Genomics show increasing relevance, reflecting new directions in research. The interconnected structure of the network demonstrates a cohesive research community, where shared foundational literature forms the basis for a broad spectrum of studies. This analysis not only showcases the most influential journals but also provides insights into the evolving research trends within this specialized area of cardiovascular medicine.

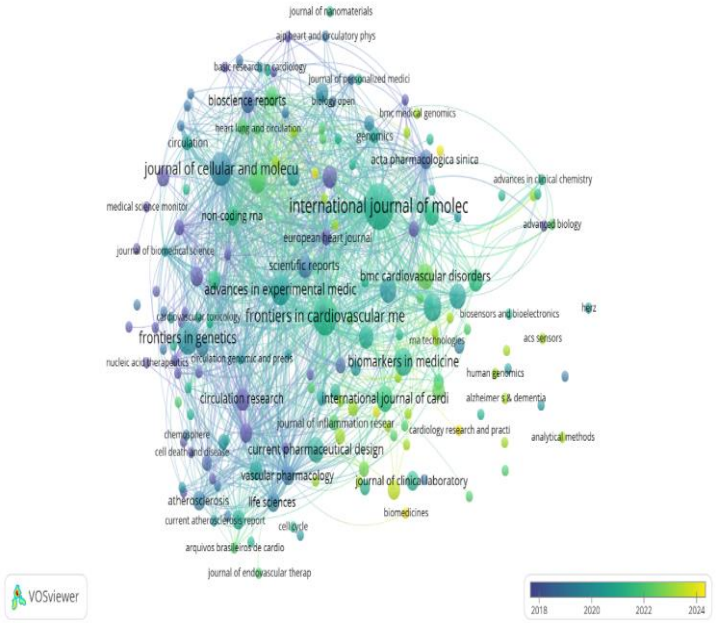


Figure 8. Bibliometric coupling - Source Analysis

DISCUSSION

This bibliometric analysis serves as a critical tool in mapping the trajectory of research activities, illustrating not only the growth of interest in lncRNAs but also the collaborative networks that underpin this field. By synthesizing data from multiple sources, the study provides a comprehensive overview of the current landscape, identifying key contributors and thematic areas that may shape future investigations into the application of lncRNAs in cardiovascular medicine.This study aligns with other bibliometric analyses in the field of cardiovascular biomarkers, such as those focusing on circular RNAs and microRNA. For instance, studies by Zehui et al.[18] and Sun et al.[19] have similarly identified key contributors and trends in cardiovascular research. However, this analysis distinguishes itself through its specific focus on lncRNAs, a relatively underexplored area, and its comprehensive multi-database approach, which enhances the robustness of the findings.

Yearly Trends in Publication Output

The analysis shows a notable increase in research activity on lncRNAs and cardiovascular diseases over the past 15 years, with a sharp rise in publications beginning around 2017 and peaking in 2021.[20–24] This surge can be attributed to the growing recognition of lncRNAs as critical regulators of gene expression and cellular processes involved in cardiovascular pathology. The decline in publications in 2024 likely reflects the incomplete nature of the data for that year and does not necessarily indicate waning interest in the field.

Leading Contributors: Authors, Journals, and Institutions

The analysis identifies several key authors and institutions that have significantly shaped the research landscape. Guo Wei and Thomas Thum are among the most prolific authors, contributing a substantial number of high-impact publications on lncRNAs in cardiovascular contexts.[8,25–27]

Journals such as *Journal of Cardiovascular Research and Frontiers in Cardiovascular Medicine* have emerged as central venues for disseminating research in this area, highlighting the importance of specialized journals in advancing the field.[24,28]

Zhengzhou University stands out as the institution with the highest publication output, reflecting its active role in cardiovascular and molecular biology research. [29]The prominence of Chinese and American institutions underscores the global nature of this research domain, with these two countries leading in both the volume and impact of publications.

The global distribution of research on lncRNAs as biomarkers in cardiovascular diseases is dominated by the United States and China, which together account for a significant proportion of the publications in this field. The strong presence of these countries in the research landscape is reflective of their advanced biomedical research infrastructures and the substantial funding available for cardiovascular research.

Co-Authorship and collaborative network

The co-authorship network shows that Thomas Thum**,** Reinier A. Boon**,** Stefanie Dimmeler**,** and Yvan Devaux are central and highly cited figures in the field of lncRNAs and cardiovascular disease.[30,31] These authors' work has significantly influenced the direction of research, as seen by their large node sizes and extensive collaborations. The network also reveals the importance of collaboration in driving citation impact, with newer authors like Yunzhu Peng **and** Peifeng Li emerging as contributors to the field. The distribution of citations across both central and peripheral authors demonstrates the dynamic and evolving nature of lncRNA research, with a mix of established leaders and emerging researchers driving forward the exploration of lncRNAs as biomarkers in cardiovascular disease. [32,33]

The co-authorship network analysis of organizations highlights the key institutions contributing to the research within this domain, emphasizing the global and collaborative nature of the work. Prominent institutions like Harvard University, Imperial College London, and Goethe University Frankfurt play central roles in the network, driving forward key research initiatives. Emerging hubs in Asia, such as Zhejiang University and Shanghai Jiao Tong University, indicate a shift towards new and potentially groundbreaking research. Future research efforts could benefit from fostering these emerging collaborations and integrating insights from the established global network.

The co-authorship network analysis reveals well-established collaborative groups, particularly between institutions in the United States, Europe, and China. These collaborations have facilitated the rapid exchange of knowledge and methodologies, enabling the field to advance at a remarkable pace. However, the emergence of research hubs in countries such as the United Arab Emirates and Switzerland suggests that other regions are increasingly investing in cardiovascular research, contributing to a more globally distributed research effort. European countries form a cohesive cluster with strong internal and external links, while emerging research nations like India and Iran are increasingly integrating into the global research landscape. Future research efforts could benefit from fostering these emerging collaborations and leveraging the established networks of leading countries to address global research challenges.

Thematic Developments and Emerging Trends

Keyword co-occurrence analysis has identified several thematic clusters within the literature, each reflecting different aspects of lncRNA research in cardiovascular disease. The largest cluster focuses on the biological roles of lncRNAs in gene regulation and disease mechanisms, emphasizing the importance of fundamental molecular biology

research in this area. Another prominent cluster is centered around the clinical applications of lncRNAs, particularly their potential as non-invasive biomarkers for the diagnosis and prognosis of cardiovascular conditions.[3,34,35]

Emerging research themes include the integration of lncRNAs into precision medicine frameworks and the use of computational biology tools to further explore their regulatory functions.[36,37] These trends suggest that future research will likely focus on developing targeted therapies and diagnostic tools based on lncRNA signatures, potentially transforming the management of cardiovascular diseases.

The common cardiovascular disease and long non-coding RNAs (lncRNAs) expressed can be identified based on the clustering and proximity of keywords. Frequently associated with lncRNAs involved in gene regulation, "coronary artery disease" appears in the network near terms like "biochemistry" and "clinical significance," suggesting that several lncRNAs play a role in this condition.[38–43] LncRNAs related to "heart failure" are prominent and closely linked to "biochemistry" and "pathogenesis." Specific lncRNAs such as H19 and MIAT have been studied extensively in the context of heart failure.[44,45] Terms like "ischemia," "myocardial ischemia," and "reperfusion injury" are associated with lncRNAs. Examples include LncRNA MALAT1, which has been studied for its protective roles in ischemic injury and infarction. Cardiac hypertrophy" and "cardiomyopathy" are linked to various lncRNAs such as LncRNA ANRIL and LncRNA KCNQ1OT1, which have been shown to regulate cardiac remodeling and hypertrophic responses.[46–50] The term "acute coronary syndrome" is linked with lncRNAs involved in plaque stability and inflammation, like LncRNA MIAT and ANRIL.[51,52] Involved in cardiac hypertrophy and heart failure, KCNQ1OT1 influences myocardial remodeling and cellular growth, making it a critical target in hypertrophic heart disease.[53–56]

From the bibliometric analysis and the terms highlighted in the network, it is evident that lncRNAs such as MIAT, H19, MALAT1, ANRIL, and KCNQ1OT1 are commonly expressed and studied in different cardiovascular diseases, including coronary artery disease, heart failure, myocardial infarction, and cardiac hypertrophy.[23,57–59] These lncRNAs are central to the regulation of gene expression and play key roles in the pathophysiology of cardiovascular diseases, making them crucial targets for research and potential clinical applications in diagnostics and therapeutics.

Strength and limitations

While many bibliometric studies rely on a single database, such as Web of Science or Scopus, this analysis utilizes data from three major databases (PubMed, Scopus, and Web of Science). This multi-database approach ensures a more comprehensive coverage of relevant literature. The identification of thematic clusters provides insight into current research focuses and potential future directions in the field of lncRNAs and CVDs. The analysis is limited to published literature, which may overlook significant findings in unpublished studies or those not indexed in the selected databases. The bibliometric approach provides a quantitative overview, but lacks the depth of qualitative analysis that could be obtained through expert interviews or systematic literature reviews.

Future Directions and Implications

The analysis of long non-coding RNAs (lncRNAs) in cardiovascular disease highlights several key future implications for research and clinical applications. First, targeted research should focus on specific lncRNAs, such as MIAT, H19, MALAT1, ANRIL, and KCNQ1OT1, to better understand their roles in disease mechanisms and explore their potential as therapeutic targets. Additionally, the clinical translation of lncRNAs as biomarkers for diagnosis and prognosis should be prioritized, with efforts to validate their utility in clinical settings to improve diagnostic accuracy and inform treatment strategies. Leveraging advancements in genomics and computational biology will be essential for identifying novel lncRNA biomarkers and elucidating their interactions in cardiovascular health. Expanding research to include diverse populations is crucial for understanding variability in lncRNA expression, facilitating personalized medicine approaches. Furthermore, fostering interdisciplinary collaborations among researchers in molecular biology, cardiology, and public health will enhance the understanding of lncRNAs' implications in cardiovascular disease. Lastly, future studies should focus on emerging themes, such as the roles of lncRNAs in inflammation and metabolic health, to provide deeper insights into their multifaceted functions in cardiovascular pathology. Overall, these implications pave the way for innovative research and clinical applications that could significantly improve patient outcomes in cardiovascular disease management

CONCLUSION

This bibliometric analysis provides a comprehensive insight into the research landscape of long non-coding RNAs (lncRNAs) as biomarkers in cardiovascular disease. It highlights the crucial roles of lncRNAs such as MIAT, H19, MALAT1, ANRIL, and KCNQ1OT1 in various cardiovascular conditions, showcasing their involvement in gene regulation and disease mechanisms. The analysis also reveals significant publication trends, influential contributors, and emerging areas of focus in the field. The growing interest in lncRNA research reflects their promising potential in cardiovascular diagnostics and treatment. Moving forward, future research should aim to capitalize on the existing collaborations and explore the identified emerging themes to enhance the translation of these findings into clinical practice, ultimately improving outcomes for patients with cardiovascular diseases.

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Ethics approval

Not applicable as it is a systematic review and meta-analysis.

Informed consent
Not applicable as it is a systematic review and meta-analysis.

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REVIEW ARTICLE

3D SPACE MAINTAINERS IN PEDIATRIC DENTISTRY: SCOPING REVIEW

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ABSTRACT

Background: Conventional space maintainers in pediatric dentistry is a multistep, multi-visit process that involves paramount of patient co-operation and is a difficult especially in young un-cooperative children, and children with gag-reflex and children with special health care needs. Recent multiple case reports successfully describe 3D space maintainers and its effective use in multiple clinical scenarios. The aim of this current scoping review is to discuss the advances in terms of Computer aided design (CAD) and extent of use of 3D space maintainers and its use in pediatric dentistry

Methodolody: PubMed, Scopus, Web of Science databases were evaluated with pre-defined search strategy ((((CAD) OR (CAM)) OR (3d printing))) AND (Space maintainer). Google Scholar and Research Gate were also consulted with the search terms to include all the articles for this current scoping review.

Results: A total of 349 titles were screened rigorously by three independent evaluators and after duplicate exclusion, removal of irrelevant titles, finally led to inclusion of 18 articles for the qualitative synthesis of data.

Conclusion: 3D space maintainers seem to be an effective option for pediatric patients with gag reflex and single visit pediatric dentistry, however the amount of available literature is too sparse, well planned randomized control studies need to be planned to evaluate various parameters such as comfort, longevity and failures etc. 3D Space maintainers can be a game changer in single visit pediatric dentistry practice.

Keywords: Children, 3D space maintainers, CAD-CAM, Gag Reflex

INTRODUCTION

Dental caries is a major public health issue, often leading to premature tooth loss or extraction.[1-3] Primary dentition plays a crucial role in jaw growth, speech, and chewing abilities. Maintaining good oral hygiene promotes overall health of the child.[4,5] Space closure after premature loss of primary teeth is essential, especially in clinical scenarios with smaller jaw dimensions and deficient space. Early loss of primary first or second molars or proximal lesions can lead to various issues, including space closure, crowding of the permanent dentition, loss of arch circumference, drifting of adjacent teeth, impaction of succeeding teeth, midline shift, and impairment of function.[6]

Primary teeth play a critical role as the optimal space maintainers in preventive dentistry. Aside from their aesthetic, communication, and mastication functions, primary teeth serve in directing and aiding the emergence of permanent teeth. However, in clinical situations where early extraction is not an option due to severe cavities and substantial crown destruction, space maintainers are judged necessary.[7-9]

They serve to keep neighboring teeth in appropriate alignment for future orthodontic treatment. Making space maintenance essential in early space loss cases.[10,11] This method supports the maintenance of optimal masticatory and speaking function, while also reducing malocclusion caused by unwanted tooth movement. Various types of space maintainers, including those based on design, fixation, position, and location, are designed to prevent malocclusion in erupting permanent teeth caused by premature tooth loss.[12]

Amongst which conventional space maintainers have been recommended and preferred by practitioners for years. Conventional band and loop are a multistep and multi-visit process that involves adapting and trimming the band, band pinching, impression making, transfer of band material onto impression, stabilizing the band onto impression, duplicating cast, and soldering the loop, and then transferring the final band and loop intraorally in the second visit.[7,13-15] Studies have identified challenges faced by traditional space maintainers, including nickel sensitivity, poor construction quality, increased risk of dislodgment and ulceration, tissue irritation, increased gagging, increased occlusal load, and increased risk of root resorption.[16-19]

Advanced technologies and newer materials are preferable to traditional space maintainers due to their limitations. Some studies recommend utilizing technique-sensitive fiber-reinforced composite resin (FRCR)-based space maintainers, which may include components like polyethylene fibers, pre-impregnated salinized E-glass fibers with Bis-GMA, with or without PMMA, and CAD/CAM-aided ceramic- and polymer-based space maintainers, to address these challenges.[20-23]

Digitalization in dentistry is progressing with the development of 3D printing technology, which generates 3D objects from digital files. This technology is revolutionizing a variety of sectors, including medicine and dentistry, and it holds great promise in pediatric dentistry by offering innovative solutions for dental treatments. Digital space maintainers are individuals who use these types of technologies. Three-dimensional (3D) printing of space maintainers is a manufacturing process that employs computer-aided design and CAD/CAM technology to construct custom 3D space maintainers on a layer-by-layer basis.[24-26]

These space maintainers are also beneficial for patients who prefer a more aesthetically pleasing option compared to traditional metal appliances. Additionally, they can help reduce the risk of processing errors and the need for frequent appointments, leading to a more efficient treatment process. There is a gap in the literature for comprehensive studies that investigate the production of space maintainers through digital workflows, highlighting the necessity for further research in this area. This research aims to fill that gap by conducting a thorough analysis of space maintainers created using digital tools and techniques, contributing to the understanding of digital workflow in dental treatments. This scoping review is intended to offer an overview of the current literature on 3D space maintainers.

Methodology

Search strategy is depicted in Table 1. Electronic searches were performed in the databases: PubMed, Scopus, Web of Science, Google scholar. The search was conducted from inception to 1 march 2024. A broader search strategy was used to prevent missing articles.

Table 1. Search strategy

Search terms	PubMed	((((CAD) OR (CAM)) OR (3d printing))) AND (Space maintainer)
	Scopus	3d AND space AND maintainer
	Web of Science	3D and space maintainer
	Google Scholar and ResearchGate	3D Space maintainer
Search Dates	Inception to 1 March 2024 Last search was performed on 1 April 2024.	
Selection criteria	Inclusion	Clinical studies, Case reports, In-Vitro Studies,
	Exclusion	Space maintainers and other appliances in adults and narrative and systematic reviews

The search was performed using broad terminology ((((CAD) OR (CAM)) OR (3d printing))) AND (Space maintainer) in PubMed, 3d AND space AND maintainer in Scopus-, 3D and space maintainer in Web of Science. The review was conducted according to PRISMA guidelines using PICO criteria.

P (Population: Children below 16 years);
I (Intervention: 3D printed space maintainers);
C (Control: conventional space maintainers);
O (Outcome: clinical parameters such as fit, retention and longevity).

Apart from these databases Google scholar first 200 articles were screened with the same search strategy. Research gate was also consulted and cross references were also consulted on this topic to extract all the available literature. Any clinical trial, case report, technical note, invitro study on the topic in children is included for qualitative data synthesis. Exclusion criteria is adult studies and narrative and systematic reviews.

RESULTS

Extensive literature search was carried out using pre-defined search strategy was carried out. A total of 349 titles were screened rigorously by three independent evaluators and after duplicate exclusion, removal of irrelevant titles, 19 articles were included for full text analysis out of which one article was excluded due to unclear description. Figure 1. PRISMA flow chart 18 articles were included for final data synthesis.[27-44]
The characteristics of included studies are presented in Table 2.

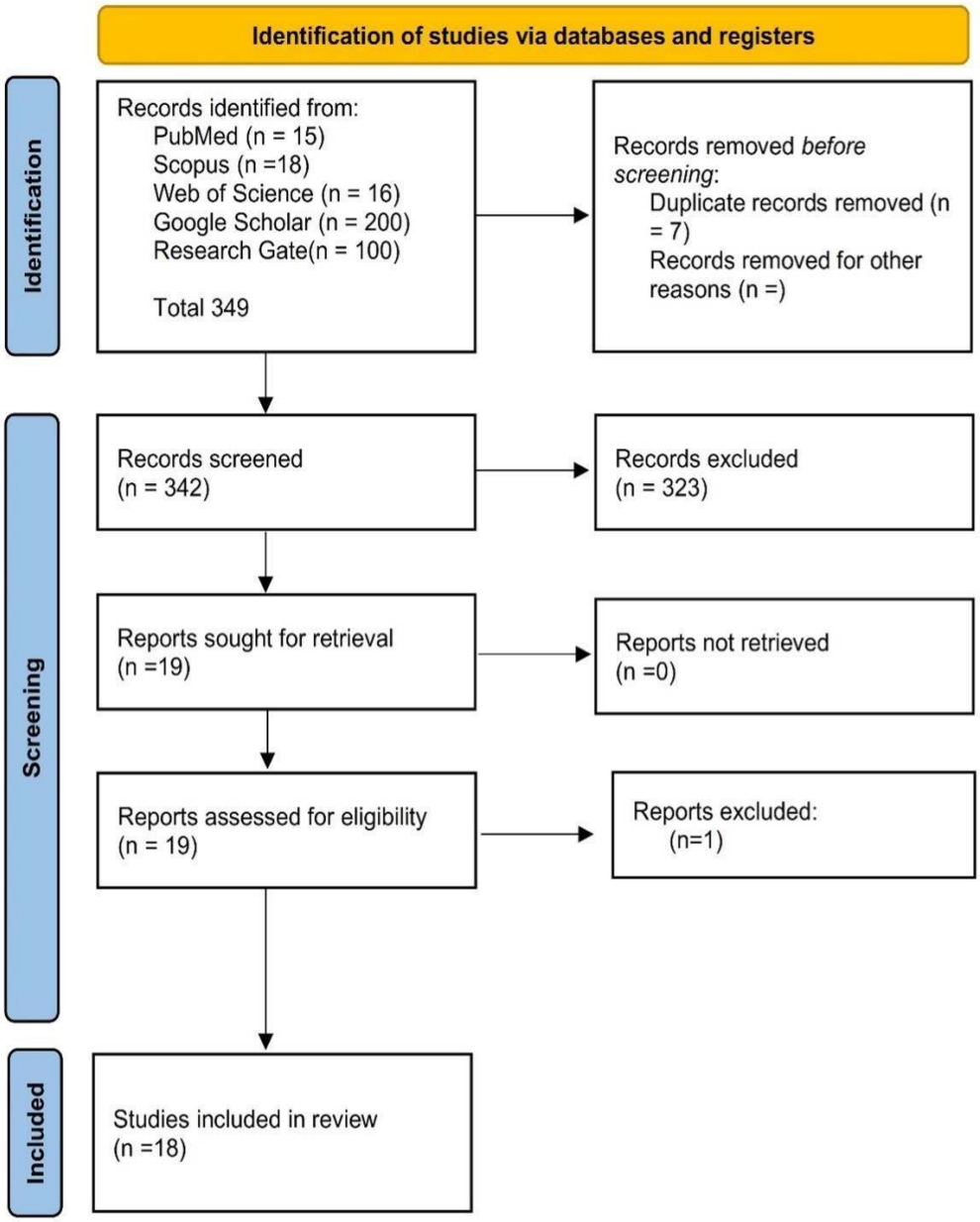


Figure 1. PRISMA Flow Chart

Table 2. The characteristics of included studies

S no	Author-year	Type of study	Age of Subjects	Type of Space maintainer	Scanning	Tooth preparation if any	Designing	Material used	Comparison	Methods to obtain fit and retention	Evaluated Outcomes	Conclusion
1	**Cengiz and Karayilmaz 2024** (1)	RCT	Seventy children (mean age: 6.99 ± 1.18) were divided into two groups	Band and Loop space maintainer	Direct intraoral Scanning 3Shape TRIOS® 3 MOVE (Copenhagen, Denmark).	Not mentioned	The design of SMs was carried out on the Dental CAD 2.2 Valletta (Exocad GmbH, Darmstadt, Germany) program	3D printing using Metal laser sintering of titanium alloy.	Traditional band and loop space maintainer.	Not mentioned Cementation was performed with glass-ionomer luting cement (Nova Glass-L, Imicryl, Konya, Turkey).	Survival of Space maintainers was compared between the groups	Failure rates are higher in 3D printed group and most of the failures are due to decementation. Sixteen (38%) SMs from the Traditional group and 25 (66%) SMs from the 3D printed laser sintered group were recorded as failed due to retention loss.
2	**Rathi et al 2023** (2)	Case report – 2 Cases.	5-6 years old Attention Deficiency Hyperactive Disorder (ADHD)	Band and loop Esthetic fixed space maintainer	Direct intraoral Scanning (Medit T500, Medit Corp., Seongbuk-gu, South Korea)	No crown preparation was performed	Dental CAD 2.2 Valletta (Exo-CAD GmbH, Darmstadt, Germany)	3D printing using Metal laser sintering of titanium and followed by adding opacifier and firing for aesthetics.	No comparison	Cementation was performed with glass ionomer cement. (Type 1, GC, Fuji, Tokyo, Japan)	Survival	6 months follow-up was uneventful
3	**Yangdol et al 2023** (3)	Case report	7 years old child with Autism Spectrum Disorder (ASD)	Band and Loop space maintainer	Indirect scanning was performed (The cast was scanned using a 3D shape digital lab scanner.	Not mentioned	3-Shape dental system software	3D printing using Metal laser sintering of cobalt-chromium alloy.	No comparison	Not mentioned	Not mentioned	Not mentioned
4	**Barakat et al 2023** (4)	In-Vitro Study	Not applicable	Band and loop space maintainers	Direct scanning of extracted 3-shape E2 scanner.	Not mentioned	3-Shape dental system software	Not mentioned	Conventional band and loop.	Phosphoric acid etchant (Universal Etchant Gel, 3M ESPE, USA) was spread over the enamel surface for 30 s and washed for 30 s. Excess water was eliminated using a spongy pellet, to get a moist enamel surface. A layer of the universal bonding agent (3M ESPE St Paul, MN, USA) was added to the etched enamel surface using a brush and cured for 20 seconds by using a light-curing device. Resin cement (G-CEM™ Capsule GC corporation, Tokyo, Japan) was spread to the surface of the tooth and cured for 40 seconds.	Bond strength was compared between conventional and 3D printed band and loop using Universal testing machine.	Bond strength is higher in 3d printed space maintainer group as opposed to conventional band and loop space maintainer group.
5	**Abdelshahed et al 2023** (5)	Clinical study	36 children with age range of 4-7 years.	Esthetic non-functional band and loop (PEEK Ridge, Ridge off,) versus conventional band and loop.	Indirect scanning was performed (The cast was scanned using an optical scanner (name not mentioned)	Not mentioned	Exocad	Milling of PEEK	Conventional band and loop.	In two PEEK groups, bands of PEEK were treated with sandblasting using aluminum oxide particles (110 µm) under pressure of 2 to3 bars to increase the surface area for cementation and by using Viso-link adhesive system made micro mechanical retention between luting cement and PEEK surface.	Survival	9 months follow-up PEEK group had lower failures as compared to the conventional band and loop group.
6	**Tamburrino et al 2023** (6)	Invitro	Not applicable	Esthetic Band and Loop space maintainer			Autocad	DLP Technique using Anycubic Photon Mono SE 3D printing machine and the adopted	-			Flexural strength exceeding 110 MPa, an elastic modulus ranging from 2 to 3 GPa, and a tensile

								resin, "OD-Clear MF Bio monomer free", supplied by 3Dresyns				strength exceeding 50 MPa
7	**Beretta et al 2022** (7)	Technique	3-5 years	Anterior fixed functional esthetic space maintainer	Intraoral scan (Trios, 3Shape, Denmark).	No preparation was performed	3-Shape, Denmark	3d printing of PEEK	No comparison	Etching and adhesive cementation, using preferably a self-adhesive or light-curing bioactive cement (Activa Bioactive Cement, Pulpdent, USA; Transbond Plus, 3M, USA) or other materials (Transbond LR, 3M, USA; Relyx, 3M, USA) to bond the PEEK structure to teeth	Survival	12 month follow up uneventful.
8	**Aboulazam et al 2022** (8)	RCT	20 children aged 5-7 years having unilateral extracted mandibular primary first molars	Posterior band and loop esthetic non-functional space maintainers.	Intraoral scanner "Primescan" (DentsplySirona. Germany)	No preparation was performed	Inlab 19 Software to Removable Partial denture (RPD) module	Milling according to BioHPP PEEK (Bredent. Germany) MCX5 milling machine (DentsplySirona. Germany).	Conventional band and loop space maintainer.	Internal surface was microsandblasted The abutment tooth was acid etched under partial isolation and cemented with the resin cement "Multilink R Speed" (Ivoclar Vivadent AG. Liechtenstein) and cured for 20 seconds	Comparison of comfort	6 month follow up uneventful. Patient satisfaction was more with PEEK space maintainers
9	**Rodrigues et al 2022** (9)	Technical report	Not mentioned	Esthetic Fixed space maintainer	Direct intraoral scan TRIOS 3, 3Shape, Copenhagen, Denmark	Not mentioned	Dental CAD, Exocad GmbH, Darmstadt, Germany	PrograMill CAM 4.1, Ivoclar; 5-axis milling device	No comparison	Not mentioned	-	Not mentioned
10	**Tocuk and Yilmaz 2022** (10)	In-Vitro Study	Not applicable	Band and Loop fixed space maintainer	-	-	-	-	CAD based band and loop is compared to conventional band and loop space maintainer	-	Fit and retention was evaluated between conventional and CAD CAM based space maintainer.	No difference in the fit of both the CAD based and conventional space maintainers.
11	**Gupta et al 2022** (11)	Case report	7 -8 years old	Distal Shoe space maintainer.	Direct intraoral scanning using CEREC Omniscan	Not mentioned	Not mentioned	Not mentioned	No comparison	Not mentioned	Survival	2 months follow up uneventful
12	**Khanna et al 2021** (12)	Case report.	6 years	Band and Loop Fixed Space maintainer.	Indirect scanning was performed (The cast was scanned using a 3D digital dental scanner (Medit T500, Medit Corp., Seongbuk-gu, South Korea)	Not mentioned	Dental CAD 2.2 Valletta (Exo-CAD GmbH, Darmstadt, Germany)	3D printing using Metal laser sintering of titanium	Conventional space maintainer.	Cementation was performed with glass ionomer cement. (Type 1, GC, Fuji, Tokyo, Japan)	-	Follow-up uneventful 6 months.
13	**Lee 2021** (13)	Case report	8 years	Fixed Esthetic reverse Band and Loop.	Direct intra-oral scanning using TRIOS Color Cart; 3Shape, Copenhagen, Denmark)	Conservative crown preparation on the First primary molar	3-Shape Software for designing	The SM was milled out of a zirconia block (1100 Enamel; DENTALMAX, Seoul, Korea).	-	Crown preparation on the primary molar and creating a rest on the permanent first molar. Air abrasion of the inner surface followed by cementation using a self-etching primer (ED PRIMER II; Kuraray Noritake Dental, Okayama, Japan) was applied to the primary first molar.33 The customized band and rest were luted	Longevity and survival	Follow up of 22 months without any complications

										with adhesive resin cement (Panavia F 2.0; Kuraray Noritake Dental) that included the functional monomer 10-methacryloyloxy-decyl-dihydrogen- phosphate (10-MDP)		
14	**Essawy et al 2021** (14)	Clinical study	13 children with unilateral loss of primary first molar	Fixed functional space maintainer	Indirect scanning of cast (InEos x5 Dentsply sirona	Not mentioned	Exocad Program (In lab dentsply sirona, Germany).	CAM milling of PEEK blocks.	No comparison	The abutment tooth was isolated, etched, and air dried after trimming, finishing, and polishing the space maintainer. The bridge was cemented using luting Rely X Unicem resin cement (3M ESPE Dental products, St. Paul, USA)	Survival	12 month Follow up uneventful
15	**Guo et al 2020** (15)	Original research- In Vitro Study	Not applicable as study was on standard models	Removable space maintainers (RSM)	Indirect scanning was performed (Scanning of Dental Casts) using D800, 3Shape, Copenhagen, Denmark	-	3-Shape Software for designing of Removable space maintainers.	Polymethyl methacrylate (PMMA)	Comparison was done with conventional removable space maintainer	Not mentioned	Fit was the outcome that was evaluated. The spacing between tissue surface of RSM and the cast was measured and compared.	Digital was better as it had less spacing.
16	**Pawar 2019** (16)	Case report.	7 years	Band and Loop Fixed Space maintainer.	Indirect scanning was performed (The cast was scanned using a 3D digital dental scanner (Medit T500, Medit Corp., Seongbuk-gu, South Korea)	Not mentioned	Dental CAD 2.2 Valletta (Exo-CAD GmbH, Darmstadt, Germany)	3D printing using Metal laser sintering of titanium	No comparison	Not mentioned	-	Follow up was done after 3 months.
17	**Ierado et al 2017** (17)	Case report of 3 cases.	8-10 years	Lingual arch, band and loop and one Removable space maintainer.	Indirect scanning was performed (Scanning of Dental Casts) (D810, 3Shape, Denmark).	Not mentioned	3Shape Dental Design software	Roland DWX-50 features 5-axis-milling of PEEK (poly ethyl ether ketone) Block	No comparison	Not mentioned	-	-
18	**Harleen Kaur Soni 2016**(18)	Case report	6 years	Fixed Esthetic Band and Loop	Indirect Scanning	No information	No information	Milling of Bruxzir Zirconia	No comparison	The gingival one-third of the distal surface of canine and gingival one-third of the mesial surface of the primary second molar etched with phosphoric acid for 15 seconds. After water rinse and drying, the hybridization was achieved by using two steps etch and rinse adhesive system (Adper Single Bond 2, 3M/ESPE). The adhesive layer was polymerized during 15 seconds. The inner surface of the space maintainer was cemented with the help of resin luting cement (RelyX ARC – self adhering flowable composite, 3M/ESPE)	Survival	Reported follow up of 6 months

DISCUSSION

Types of 3D space maintainers

Fixed/Removable; Esthetic/Non-esthetic; Functional/ Non-Functional type of 3D space maintainers were reported in literature pertaining to pediatric dentistry. Removable esthetic 3D space maintainers were described in the study by Guo et al 2020.[30] Multiple studies reported the use of 3D Fixed space maintainers.

Esthetic 3D fixed space maintainers: Esthetic 3D fixed space maintainers can be inherently esthetic (PEEK, Zirconia) or masked (metal masked with opacifier).

Esthetic 3D fixed space maintainers: PEEK material based- 3D posterior fixed space maintainers were reported in the studies by Abdelshahed et al 2023,[40] Aboulazam et al 2022,[37] Essawy et al 2021,[31] PEEK material based- 3D Lingual arch has been reported in the study by Ierado et al 2017.[28] PEEK material based- 3D anterior fixed space maintainers are reported in the study by Beretta et al 2022.[38] Zirconia based milled 3D fixed space maintainers were reported in the studies by Harleen Kaur Soni 2016,[27] Lee 2021.[32] Metal fused opacifier added masked 3D fixed band and loop was reported in the study by Rathi et al 2023.[43]

Non-Esthetic 3D fixed space maintainers: Metal based non esthetic 3D fixed space maintainers were reported in the studies by Cengiz and Karayilmaz 2024 ,[44] Yangdol et al 2023,[42] Khanna et al 2021,[33] Pawar 2019.[29]

Method of Scanning

Two types of scanning are performed in relation to fabrication of 3D space maintainers**.** *Direct scanning* and Indirect scanning. Direct scanning involves the use of intra-oral scanners to scan directly in the patient. Indirect scanning utilizes lab scanners to scan a cast that is made after taking impression from the patient.

Direct scanning is utilized in the studies by Cengiz and Karayilmaz 2024,[44] Rathi et al 2023,[43] Beretta et al 2022,[38] Aboulazam et al 2022,[37] Rodrigues et al 2022,[36] Gupta et al 2022,[34] Lee 2021.[32]

3Shape TRIOS, Medit T500, CEREC Omniscan, Primescan Dentsply were the most commonly used commercial intra-oral scanners available for direct scanning Cengiz and Karayilmaz 2024,[44] Rathi et al 2023,[43] Beretta et al 2022,[38] Aboulazam et al 2022,[37] Rodrigues et al 2022,[36] Gupta et al 2022,[34] Lee 2021.[32] Indirect scanning was utilized in the studies by Harleen Kaur Soni 2016,[27] Ierado et al 2017,[28] Pawar 2019,[29] Guo et al 2020,[30] Essawy et al 2021,[31] Khanna et al 2021,[33] Abdelshahed et al 2023,[40] Yangdol et al 2023.[42] 3Shape E2 Series, D800, D810, InEos X5 DentSply Sirona were the lab scanners used for indirect scanning of casts after impression in the included studies.

Computer Assisted Design for 3D space maintainers:

After the scanning is achieved (Direct or Indirect) the scan is further processed in STL format to be uploaded in designing software. 3 Shape, ExoCAD, were the software's that was utilized for designing the space maintainers in the all the included studies.

Materials used for 3D space maintainer fabrication

PEEK, PMMA, Zirconia, Titanium alloy powder, Cobalt-Chromium alloy powder. PEEK material is esthetic it can be utilized for 3D printing as well as milling purposes. Zirconia blocks can be used for milling purposes. Titanium alloy powder, Cobalt- Chromium alloy powder can be used for 3D printing through laser sintering.

Processing techniques used for 3D space maintainer fabrication

The digital 3D space maintainers can be fabricated using additive or subtraction technology.

Additive technol*ogy*: Additive technology includes different types of techniques such as; Stereolithography, Digital Light Processing, Fused Filament Fabrication/Fused Deposition Modelling, Selective Laser Sintering/Melting and Electron Beam Melting, Digital Light Processing. Stereolithography (SLA) is a 3D printing process that uses a UV laser and liquid resin to create parts layer by layer. The resin is light-reactive and thermosets, meaning that when exposed to certain wavelengths of light, the resin's short molecular chains join together to create rigid or flexible geometries. The UV light cures the resin layer by layer until the final object is complete. Digital light processing (DLP) is a 3D printing technology that uses a digital projector to cure a layer of photopolymer resin at once, eliminating the need for path-planning procedures. DLP is a type of stereolithography (SLA), but uses a more conventional light source, such as an arc lamp, rather than a UV light. Fused Filament Fabrication/Fused Deposition Modelling. Fused deposition modeling (FDM), also known as fused filament fabrication (FFF), is a 3D printing technique that uses a continuous filament of thermoplastic material to create a 3D object. The filament is fed through a moving extruder head and deposited onto a substrate in layers, where it solidifies to form the object. FDM is a filament-based technique that uses thermoplastic materials, mainly polymer and its composites.

Selective laser sintering (SLS) is a 3D printing technology that uses a laser to fuse powdered material into a solid structure. The laser automatically targets points in space defined by a 3D model, binding the material together to create a solid structure. SLS is a powder-based technology that uses an even layer of powder, which is then sintered, and the process is repeated until the part is complete. *Subtraction technology:* Subtraction technology involves process such as Computer aided milling (CAM) of solid blocks of materials such as PMMA, PEEK and Zirconia, Metal blocks. 3D printing was utilized in the study by Beretta et al 2022.[38] Milling was utilized in the studies by Harleen Kaur Soni 2016,[27] Ierado et al 2017,[28] Essawy et al 2021,[31] Lee 2021,[32] Rodrigues et al 2022,[36] Aboulazam et al 2022,[37] Abdelshahed et al 2023.[40] SLS was utilized in the studies by Yangdol et al 2023 and Cengiz and Karayilmaz 2024.[42,44] DLP was utilized in the study by Tamburrino et al 2023.[39]

Tooth preparation for 3D space maintainer

Most of the included studies did not mention any tooth preparation for receiving of space maintainer. Few studies mentioned minimal tooth preparation before the intraoral scanning step.[32]

Methods to obtain retention of 3D space maintainers

Methods such as surface roughening using sandblasting air abrasion is employed in few studies to improve the bond strength of the fabricated 3D space maintainer Lee 2021,[32] Aboulazam et al 2022,[37] Abdelshahed et al 2023.[40] Other measures such as etching the enamel surface of abutment tooth is carried out in the studies by Barakat et al 2023,[41] Beretta et al 2022,[38] Lee 2021,[32] Essawy et al 2021.[31] Cementation of metal powder sintered 3D space maintainers was accomplished using glass ionomer luting cement Cengiz and Karayilmaz 2024,[44] Rathi et al 2023,[43] Khanna et al 2021,[33] Pawar 2019.[29] PEEK, Zirconia, PMMA based space maintainers were cemented using composite resin based luting cements such as Transbond Plus, 3M, Rely-X, Multilink R Speed (Ivoclar Vivadent AG), Panavia F 2.0; Kuraray Noritake Dental, Viso-link adhesive system. Harleen Kaur Soni 2016,[27] Essawy et al 2021,[31] Aboulazam et al 2022,[37] Beretta et al 2022,[38] Abdelshahed et al 2023.[40]

In-Vitro studies

In-vitro studies evaluated different parameters related to 3D printed space maintainers. The study by Barakat et al 2023,[41] evaluated and compared the bond strength between conventional and 3D printed band and loop using Universal testing machine. The authors have reported that bond strength is higher in 3D printed space maintainer group as opposed to conventional band and loop space maintainer group. The study by Tocuk and Yilmaz 2022, [35] evaluated the fit and retention between conventional and 3D space maintainer and results of the study reported that there is no significant difference between both the groups. The study by Guo et al 2020, [30] evaluated the fit. The spacing between tissue surface of space maintainer and the cast was measured and compared. The authors reported that 3D space maintainers were better as it had less spacing. The study by Tamburrino et al 2023 has first reported DLP technology for 3D printing the band and loop space maintainer by using resin (OD-Clear MF Bio monomer free", 3Dresyns) which in their finite element analysis study reported excellent mechanical properties such as Flexural strength exceeding 110 MPa, an elastic modulus ranging from 2 to 3 GPa, and a tensile strength exceeding 50 MPa.[39]

Conventional versus 3D Space maintainers Clinical Studies

Only few studies were clinical trials comparing conventional space maintainers with 3D Space maintainers. Most of the studies reported better patient satisfaction ad better survival rate in 3D space maintainers. Aboulazam et al 2022,[37] Essawy et al 2021,[31] Abdelshahed et al 2023.[40] Except the study by Cengiz and Karayilmaz 2024,[44] which reported higher failure rates in 3D space maintainers.

Limitations and Directions for Future Research: Most of the studies reported in the literature are isolated case reports, without a proper follow-up, a well-planned randomized control trial can be planned comparing parameters such as clinical survival between 3D space maintainer and conventional space maintainers in clinical scenarios with a minimum follow up of 24 months.

CONCLUSION

3D printed space maintainers have both advantages and disadvantages and certain limitations. Advantages include ease of fabrication, improved customization, chair side fabrication and delivery, reduced discomfort due to decrease in the number of steps, possibility of customization with various materials disadvantages include increased bulk, and difficulty in tight interproximal contact situations. More Randomized controlled trials are required to evaluate the clinical success rate of 3D space maintainers in comparison to conventional space maintainers.

DECLARATIONS

Ethics approval and consent to participate

Not Applicable

Consent for publication

Not Applicable.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Conceptualization ST, LA, GM; methodology SS, GM; software, MMM; validation, MC, GM, MMM; Formal analysis, MMM, ST; Writing—original draft preparation, GM, MMM; Writing—review and editing, GM, MDB, GC; Visualization, GC, MMM; supervision, GM, GC; project administration, MDB. All authors have read and agreed to the published version of the manuscript.

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RESEARCH ARTICLE

COMPARATIVE ASSESSMENT OF T-PRF ALONE AND T-PRF INJECTED WITH ANTIBIOTIC GELS, HERBAL PRODUCT SEPARATELY: A HISTOLOGICAL STUDY

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ABSTRACT

Back ground: Antibiotics and herbal product incorporation into T-PRF are scant. Hence present study aimed to assess the fibrin network pattern, cellularity, cell distribution for T-PRF injected with metronidazole gel (MTZ), amoxicillin+ clavuanic acid (Amox-clav) gel and Neem (N) gel with T-PRF alone histologically through light microscope (LM) and scanning electron microscope (SEM).

Materials and Method: Present histological study utilizes 24 healthy volunteers where 20 ml of blood was drawn; transferred to titanium tubes; centrifuged; T-PRF clots prepared; MTZ, Amox-clav and Neem gels were incorporated, compared with T-PRF alone by preparing histological slides and observed under LM and SEM. Data was subjected to statistical analysis using one-way anova, fishcer test, chi-square test and frequency distribution analysis for all the group comparisons.

Results: all the groups in the present study showed non-significant results. For fibrin network pattern MTZ and Amox-clav group had shown greater percentages of thick and dense but values were non-significant (p=0.555#), cell distribution shown wide range pattern, presence of cells, all showed non-significance. Regarding mean score of cell distribution there was a range of 26-50% (score 1-2) with non-significance (p=0.386#). Values were non-significant for SEM examination for all groups (p>0.05)

Conclusion Thus within limitations T-PRF can be a sustained a drug delivery carrier system by incorporating antibiotics or herbal extract because of its thicker fibrin meshwork and greater cellular entrapment. This opens a gateway to give a new treatment protocol for treating a periodontal disease.

Keywords: Histology, Microscopy, Neem, Periodontitis, Platelet Rich Fibrin, Titanium

INTRODUCTION

Periodontitis is a painless multi-factorial disease that challenges a dental surgeon or periodontist with its attachment loss and bone loss.[1] To treat this periodontal disease, surgical and non-surgical periodontal therapies have been employed. Biomaterials like bone grafts, guided tissue regeneration materials (resorbable or non-resorbable) have been utilized to treat the periodontal soft & hard tissue defects to achieve promising results.[2] Platelet concentrates (PC's) usage was started in 1970's and has become a boon to dentistry. Because of their autologous nature and rich presence of growth factors (GF) they became a cost effective treatment strategy. First generation PC's like Platelet Rich Plasma (PRP), Leukocyte Platelet Rich Fibrin (L-PRF), Advanced Platelet Rich Fibrin (A-PRF) were used back and forth in all the fields of dentistry and medicine.[3] Various therapies like intra bony defect (IBD), gingival recession, sinus lift, socket preservation treatments have advocated these PC and achieved adequate results. But because of their shorter resorption time, rapid growth factor releases and addition of anti-thrombin (for PRP) lead to the search for better biomaterials of autologous nature. During this quest, titanium has attracted the researchers.[4] Because of its hemo-compatibility, non-corrosive nature and regular use of this metal in implant dentistry & medical surgical field led to the formulation of Titanium- Platelet Rich Fibrin (T-PRF).[5] To this support, O' Connell[6] also stated the possible contamination of silica particles in silica coated plastic tubes. Breakage of glass tubes within or outside centrifuge also increased the usage of titanium. In order to prepare T-PRF clots, Grade IV titanium tubes were utilized, by Tunali M et al.,[7] 2013. Various authors like Bhattacharya HS et al.,[8, 9] 2020, 2022 and Gummaluri SS et al.,[10] 2020 utilized medical grade titanium tubes for the preparation of T-PRF clots and achieved good treatment outcomes. In all these quoted studies, there was thicker fibrin meshwork, with higher cellular entrapment and packeting pattern of T-PRF was recorded than that of L-PRF when checked for light & scanning electron microscopy and immunohistochemical analysis. Chatterjee A et al.,[11] 2017 and Mitra DK et al.,[12] 2019 also stated that T-PRF had a better meshwork pattern than L-PRF in their histological studies.

T-PRF was used as a biomaterial to treat IBD and achieved a decrease in probing pocket depth (PPD), gain in clinical attachment level (CAL) and bone fill when compared from baseline to 9 months post-operative.[13] Since ages, antibiotics usage was recorded in literature for both surgical and non-surgical periodontal therapies as a purpose of control of post-operative infection and pain. But because of adverse systemic effects of antibiotics, researchers started their search for a better delivery of drugs.[14] Materials like carbopol polymer, collagen membranes, polyvinyl pyrrolidone, carboxy methyl cellulose etc. were also tried as carrier systems but their efficiency of positivity on the body mainly depends upon the interaction of immune system of body and the material used.[15] Tihan et al.,[16] 2019 utilized collagen sponge (CS) incorporated with doxycycline and oxytetracycline as a local drug delivery system and concluded that oxytetracycline loaded CS had a greater sustained release than Doxycycline loaded CS. They also concluded that oxytetracycline and 0.5% glutaraldehyde will help in tissue regeneration by preventing infections at application site.[16] Various drugs like Tetracycline, Doxycycline, Minocycline, Metronidazole, Azithromycin etc. have been used as Local drug delivery (LDD) in periodontitis patients in the form of fibers, gels, chips, microspheres, films, nano particles and so on.[17] Esra ercan et al.,[18] 2022 conducted a study incorporating doxycycline hyclate liquid in T-PRF & Collagen sponge was incorporated with Doxycycline was used as control group and concluded that T-PRF+Doxy group had a greater sustained drug release than Collagen sponge, thus indicating T-PRF as a sustained drug delivery system.

In both non-surgical and surgical treatments, several other antibiotics, including clindamycin, minocycline, lincomycin, and neem gels, were utilized as LDD or as topical applicants and produced good drug concentration levels in the pocket.[19] But to the authors knowledge no study was performed utilizing Amoxicillin+clavulanate (Amox-clav) gel, metronidazole (Metz) gel and neem (N) gel incorporating in the T-PRF membrane separately. Assessment of membrane structure and cellularity histologically is an indirect way of predicting the ability of restoration of soft and hard tissues. It is not possible to perform a surgical re-entry to assess the amount of regeneration in humans because of their ethical concerns. Hence present study aimed to evaluate the fibrin network, meshwork pattern, cellularity and cell distribution among T-PRF incorporated with amox+clav, metz and N gels

separately and compared with plain T-PRF using light microscopy and SEM for surface morphology.

Materials and Method

Sample Size Estimation
A sample of 20 per group was estimated with 80% power and 5% alpha error and effect size of 0.25, hence 80 samples (4 groups= 20 samples per group) were considered. G Power software version 3.0 was used for estimation of sample.

Design, Enrolment and Ethics
In this present histological analysis, 24 healthy volunteers (12 males (mean age of 25.2 years) and 12 females (mean age of 24.6 years)) were considered after taking proper informed consent. Initially 30 healthy volunteers were recruited but 6 people were not interested and declined to participate in the study. All protocols were clarified to them and only attentive people were recruited. Prior to the start of study ethical clearance was obtained with protocol number 71086061123 from institutional ethics. Study was performed at Department of Periodontics, GITAM Dental College and Hospital where healthy volunteers were recruited. Slides preparation & assessment for Light microscopy (LM) were done in Department of Oral pathology & Microbiology GITAM Dental College and Hospital, Visakhapatnam and Scanning electron microscopy (SEM) at Saveetha Dental College and Hospital, Chennai. Study had followed the Helsinki declaration 1975 that was modified in 2013 in the study.

Inclusion and Exclusion Criteria
Volunteers who were >18 years of age, systemically healthy, having > 2,00,000 lakhs per cubic millimetres platelet count, who were not under any sought of medications that would alter the systemic health, not having any sought of inflammatory diseases etc. were included. Whereas volunteers who were not ready to participate, not fascinated in the study, pregnant and lactating females, smoking, having any sought of systemic health complications were excluded.

T-PRF clots and antibiotic gels preparations
Twenty millilitres of blood was drawn and transferred to sterile medical grade titanium tubes (Supra Alloys Company, Camarillo USA), using modified Tunali M et al.,[20] 2014 and Bhattacharya HS et al.,[8] 2020 protocol (3500rpm for 15 minutes). T-PRF clots were prepared in a centrifuge (Remi R 8C, New Delhi, India). Further, using sterile tweezers, clots were carefully retrieved from the tubes, placed in a sterile area and injected with respected Amoxiclav/ MTZ and Neem gels respectively. Twenty grams of neem leaves were boiled in one litre of water to produce ten grams of liquid azadirachta indica extract. At 25µg/ml, the minimum inhibitory concentration must be achieved. To make a basic gel, 20g of carbopol 934P was soaked in one liter of water. To prepare the corresponding gel, additional calcium chloride (0.02%) and sodium chloride (NaCl) (0.9%) were added. In order to get a final mixture with a 2.5% potency, 2.5% neem extract (N) was added to this mixture.[21] Metronidazole gel (MTZ gel) of 15% concentration was prepared by combining poloxamer 123 30% w/w, propylene glycol 5% w/w, methyl-isothiazolinone 0.15% w/w, metronidazole 15% w/w and adding distilled 100%.[22] Amoxicillin + clavulanate acid gel (Amox-clav gel) was prepared by procuring the powders with a weight ratio of 7:3. To this 3 parts of sodium carboxy methylcellulose and 33% of magnesium stearate was added. All the materials were blended into a uniform single mixture. Further 0.5% polycarbophil powder and glycerine were added to form a 25% concentration.[23] All the gels that were prepared were properly sterilized and stability tested. They were procured from Periobiologics^{TM} lab, Hyderabad, India.

Drug injections into T-PRF clots
Based on Esra Ercan et al.,[18] 2022 protocol, all the prepared the clots that were retrieved, placed on sterile kidney tray separately, filled with 5 units of respective antibiotic gels and herbal extract. individually Later these were transferred to 10% formaldehyde solution and subjected to microscopic slide preparation and 2.5% glutaraldehyde for surface morphological analysis.

Light Microscopy method and slides preparation
Light microscopy and slides preparation were done based on Bancroft's manual steps.[24] After the obtainment of clots from respective groups, they were subjected to fixation in 10% formaldehyde solution for at least 24 hours. Later they were washed and subjected to different concentrations of alcohol such as 25%, 50%, 75% and 100% to remove the excess water. Later these clots were embedded individually into wax blocks using paraffinization technique. Then the cytology blocks that were prepared are sectioned into small sections which were transferred to slides, excessive paraffin was eliminated by subjecting the slides to heat at 55^{o}C and under xylene to remove remnants of paraffin. Then these fixed slide sections were stained with

haematoxylin and eosin stains to observe under the penta-head microscope. Slides examination was done by an experienced oral pathologist who has a previous experience of visualizing the slides and images of T-PRF. A total of 80 slides (20 per each group) were prepared and to avoid confusion slides were numbered according to patients and groups (Figure 1 and 2).

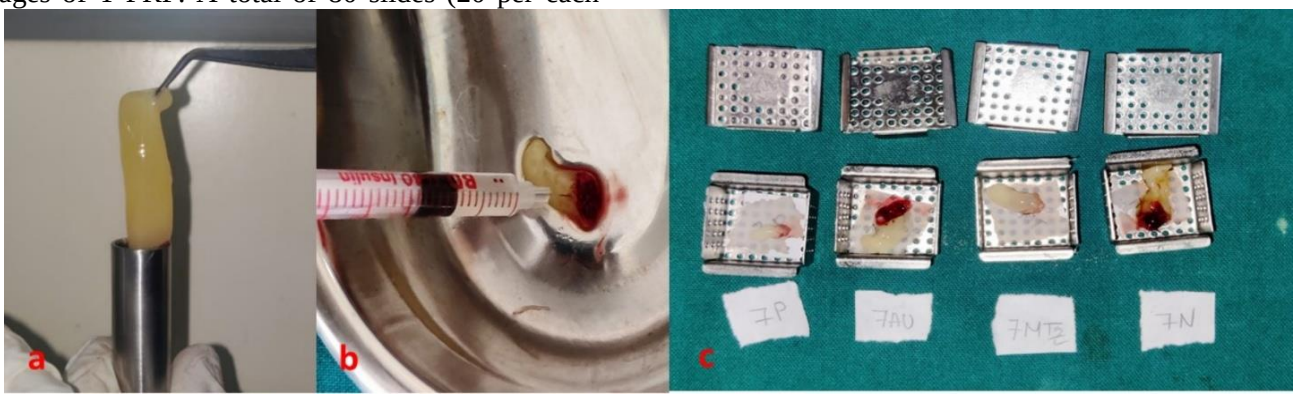


Figure 1 (a) depict the retrieval of T-PRF clot from titanium tubes, (b) depict the method of injecting Amox- clav/ Metronidazole (MTZ)/ Neem gels and (c) depict the paraffinization of samples of all the groups

Procedure for Scanning Electron Microscopy

T-PRF clots were prepared from additional three volunteers by centrifuging and injected with antibiotic gels or herbal extract. These 12 samples were transferred to 2.5% glutaraldehyde solution for tissue fixation. Further, samples were subjected to various graded concentration of alcohol (25%, 50%, 75%, 90% and 100%) for about 20 seconds. Later, alcohol treated T-PRF injected clots were transferred to a desiccation chamber and desiccated using calcium chloride ($CaCl_2$). For SEM examination platinum sputtering done and checked the surface morphology of injected T-PRF clots under scanning electron microscope (model JSM-IT800 ultra high-resolution field emission microscope, JOEL, USA) (Figure 3).[9] For checking the semi quantification of elemental composition of T-PRF, Energy Dispersive X-ray (EDX) (Figure 4) was performed for all the groups at the mid membrane region using the same SEM machine which contains additional. This would help in identification of chemical elements.

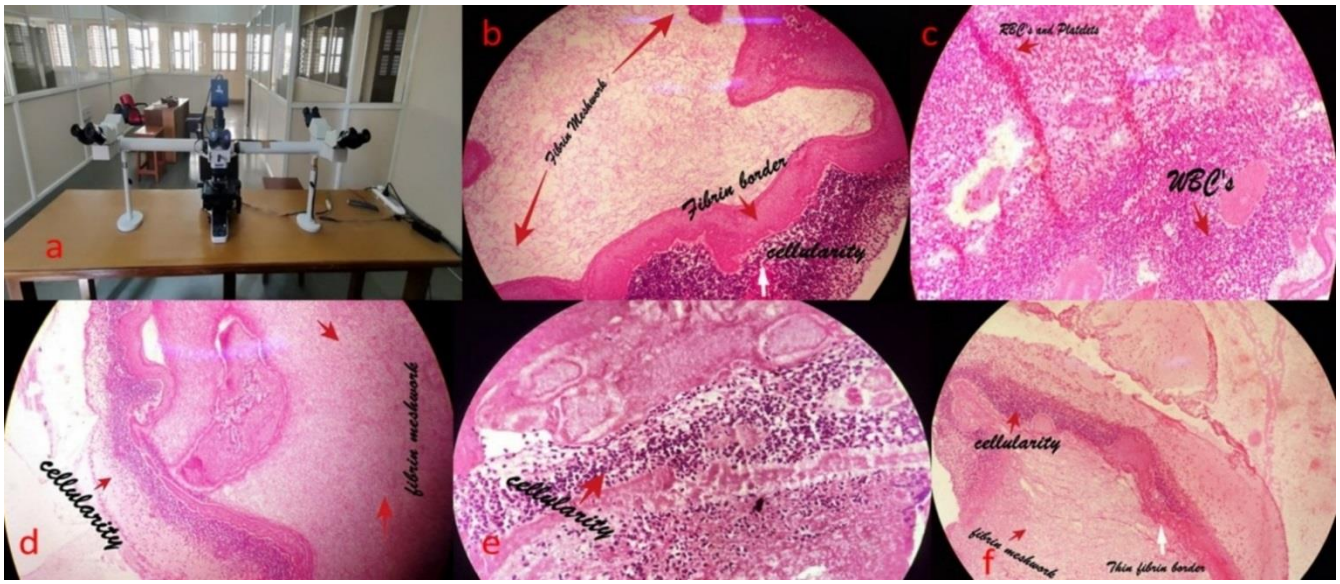


Figure 2 (a) depict the image of a penta head microscope, (b, c) depict the image of plain T-PRF at 10x and 20x magnification where fibrin meshwork, cellularity, border are shown; (d) shows the histological image of T-PRF Metronidazole (MTZ) group; (e) depict the image of T-PRF Amox+ clav gel; (f) depict the histology of T-PRF Neem gel.

Parameters Assessed

Parameters such as Fibrin network pattern- thick and dense or thin and loose; meshwork whether packeting, layered or scattered; cellularity whether present or absent; cell distribution whether narrow or wide; presence or absence of red & white blood cells (RBC, WBC) and platelets. Mean cells percentage scores that ranged from 1-4 (score 1 <25%, score 2 26-50%, score 3 51-75%, score 4 >75% of cells distribution).[9] For SEM, fibrin thickness, fibrin structure, cellular entrapment and presence of RBC,WBC and platelets were assessed at 1000x and 5000x magnification. All the parameters were assessed by experienced oral pathologist and came for conclusion along with a periodontist who has previous experience of assessing the slide sections of T and L-PRF clots.[9]

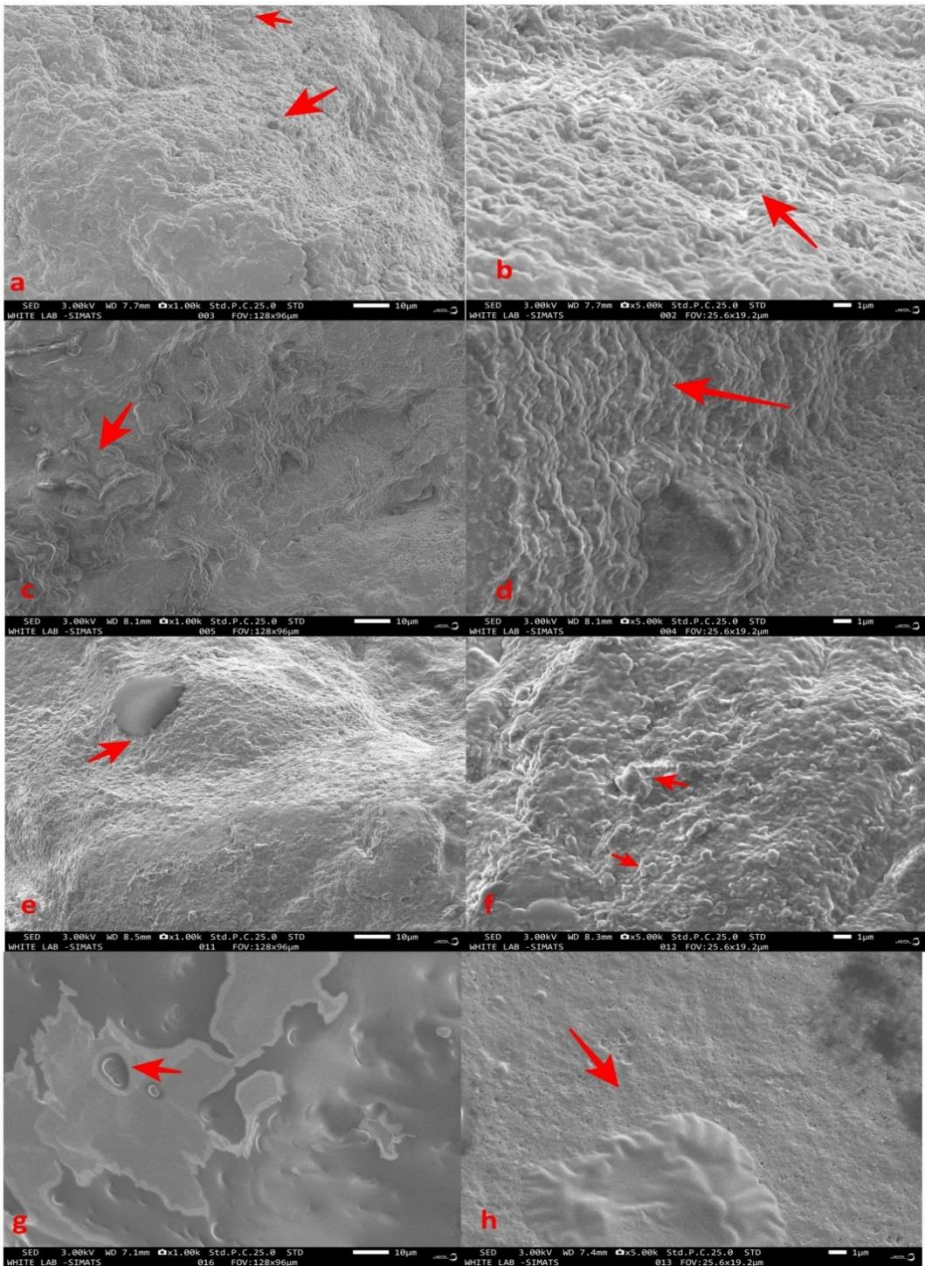


Figure 3 depicts the scanning electron microscopic images of T-PRF plain (a, b), T-PRF +Amox+clav gel (c, d), T-PRF+ MTZ gel (d, e), T-PRF+ Neem gel (f, g) at 1000x and 5000x magnifications. Red arrow depicts the presence of thicker fibrin mesh work and cellular entrapment for 3a to 3d), Red arrows in 3e and 3f report the presence of red blood cells (RBC) and white blood cells (WBC) with haziness showing the coating of drug on the

surface. Red arrows on 3g depict the RBC & fibrin coated with neem and 3h depict the surface porosity present indicating a looser fibrin meshwork in neem group.

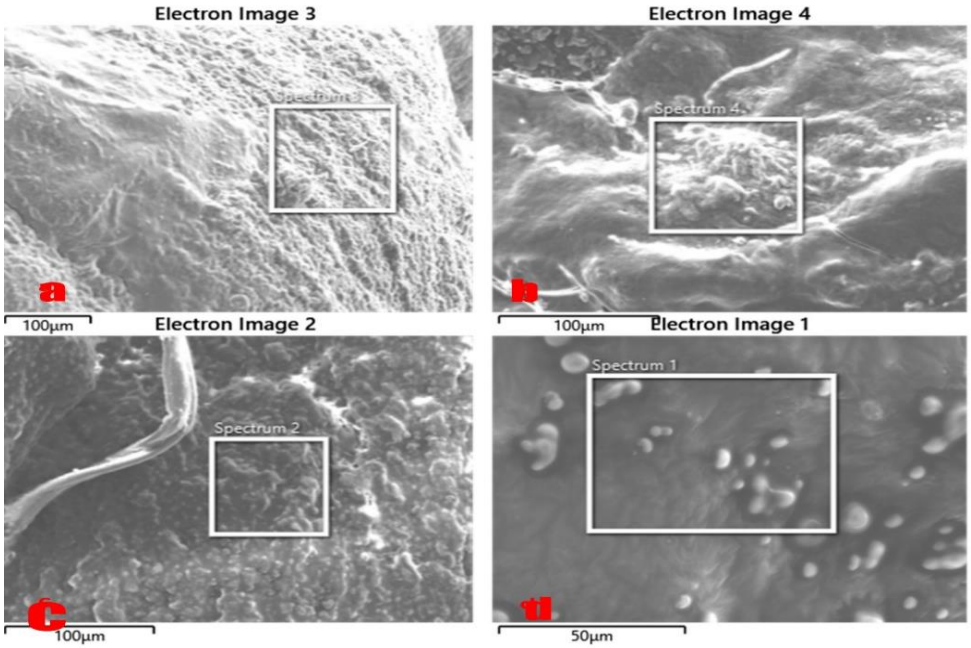


Figure 4 depict the image of energy dispersive X-Ray (EDX) of Plain T-PRF (a), T-PRF+ Amox+ clav gel (b), T-PRF + MTZ gel (c) and T-PRF+ Neem gel (d)

Statistical analysis

Entire data was transferred into Microsoft excel spread sheet and analysed using statistical package for social sciences (SPSS) version 23.0, IBM Pvt Ltd, Chicago, Illinois, USA. Frequency distribution analysis was done to qualitative data, mean and standard deviation was expressed for amount of cellularity across the clot. Fisher exact test was employed to find association in different groups, one Way ANOVA test was employed to find significant difference in score in different groups. P value <0.05 was considered statistically significant. Peason's chi-square test was performed to check the parameters of all the groups in SEM examination

RESULTS

Regarding fibrin network patterns in all the groups, T-PRF plain had an equal distribution of 50% of both Thick & Dense and Thin & loose. In case of T-PRF+ MTZ gel group 80% was thick & dense fibrin network pattern while 20% was thin & loose. For T-PRF+ neem gel group there was 60% of thin & loose fibrin network pattern and 40% was thick & dense pattern. Further T-PRF + Amoxiclav gel group showed 70% thick& dense and 30% showed thin & loose pattern. All the values were statistically non-significant (p=0.555#) (Table 1).

Table 1. The significant comparisons of fibrin network patterns among all the study groups

FIBRIN NETWORK	T-PRF Plain N (%)	T-PRF MTZ N (%)	T-PRF Neem N (%)	T-PRF Amox clav N (%)	P-Value
Thick and Dense	10 (50)	16 (80)	8(40)	14 (70)	0.555#
Thin and Loose	10(50)	4 (20)	12 (60)	6 (30)	

Statistically not significant, N- Number, %- percentages, Amox clav- Amoxicillin+Clavulanic acid gel, p value <0.05 was considered statistically significant, MTZ- Metronidazole, T-PRF- Titanium Platelet Rich Fibrin

Table 2. The significant comparisons of all the groups regarding fibrin pattern, cell distribution, presence of red blood, white blood cells and platelets

PARAMETERS	T-PRF Plain N (%)	T-PRF MTZ N (%)	T-PRF Neem N (%)	T-PRF Amox clav N (%)	P-Value
Fibrin network- pattern					
PACKETING	12 (60%)	8(40%)	8(40%)	18(90%)	0.221#
LAYERED	0(0%)	6(30%)	6(30%)	0(0%)	0.371#
SCATTERED	8(40%)	6(30%)	6(30%)	2 (10%)	0.436#
Cell Distribution					
NARROW	6(30%)	6(30%)	8(40%)	6(30%)	0.990#
WIDE	14(70%)	14(70%)	12(60%)	14(70%)	
Cellularity					
PRESENT	20 (100%)	20 (100%)	20 (100%)	20 (100%)	1.000
RBC'S					
PRESENT	20(100%)	20(100%)	20 (100%)	20(100%)	1.000
PLATELETS AND WBC'S					
PRESENT	20(100%)	20(100%)	20(100%)	20(100%)	1.000

#Statistically not significant, N- Number, %- percentages, Amox clav- Amoxicillin+Clavulanic acid gel, p value <0.05 was considered statistically significant, MTZ- Metronidazole gel, T-PRF- Titanium Platelet Rich Fibrin

Regarding fibrin network pattern, for T-PRF plain group 60% showed packeting pattern and 40% showed scattered pattern. For T-PRF MTZ group 40% showed packeting and 30% layered and remaining three slides couldn't be assessed. Further T-PRF+ Neem group showed similar pattern to that of metronidazole group with 30% percent scattered pattern additionally. In case of T-PRF + Amoxiclav group reported 90% packeting pattern and 10% showed scattered pattern. When all the groups were statistically compared, they reported a statistical non-significance. While coming to cell distribution all the groups showed non-significant (p=0.990#) 70 % of wide distribution except for T-PRF+ Neem group where it recorded 60% and 30% of all groups showed narrow distribution. Further regarding presence of cells, all the test and control groups reported non-significant (p=1.00#) presence of cells including platelets, RB & WB cells (Table 2). Regarding the mean score of cellularity, all the groups reported a statistical non-significance (p=0.386#). In this, T-PRF+ MTZ and T-PRF Amoxiclav group reported a mean of 1.2±0.78, 1.2±1.03; while T-PRF plain reported 0.9±0.9 and T-PRF+ Neem gel group reported a mean of 0.6 ± 0.69 (Table 3). Regarding SEM images all the groups showed similarity regarding fibrin thickness, fibrin structure, cell entrapment, red blood cells, white blood cells, platelets with no statistical significance (p>0.05). Thus, indicating injecting drugs or herbal extract didn't affect the morphology of T-PRF (Table 4).

Table 3. The mean significant scores of all the groups for light microscopy.

GROUPS	N	Mean	Std. Deviation	p-Value
T-PRF Plain	20	0.900	0.99	0.386#
T-PRF MTZ	20	1.200	0.78	
T-PRF Neem	20	0.600	0.69	
T-PRF Amox clav	20	1.200	1.03	

#statistically not significant, N- Number, p value< 0.05 was considered statistically significant, MTZ- Metronidazole, Amox clav- Amoxicillin +Clavulanic acid, Std- Standard

In case of semi quantification of EDX, highest carbon (C) % was reported in T-PRF+ Neem followed by T-PRF+ MTZ group then T-PRF+Amoxiclav group and T-PRF plain. Highest Oxygen (O) % was reported in T-PRF Plain followed by T-PRF+Amoxiclav gel then T-PRF+MTZ and T-PRF+ Neem. Sulphur (S) was also reported in all the groups in traces. Silicon (Si) was reported only in T-PRF+Amoxiclav gel sample (Table 5).

Table 4. Depict the frequency distribution percentages of scanning electron microscopy of T-PRF clots injected with Augmentin or Metronidazole or Neem gels with T-PRF alone

GROUPS		T-PRF Amoxi-Clav gel	T-PRF MTZ gel	T-PRF NEEM gel	T-PRF PLAIN	P value
		Count (%)	Count (%)	Count (%)	Count (%)	
Fibrin thickness	Thick	2 (50%)	3 (75%)	2 (50%)	3 (75%)	0.785#
	Thin	2 (50%)	1 (25%)	2 (50%)	1 (50%)	
Fibrin Structure	Organized	4 (100%)	4 (100%)	2 (50%)	3 (75%)	0.211#
	Disorganized	0	0	2 (50%)	1 (25%)	
Cell Entrapment	Present	4(100%)	4(100%)	4(100%)	4(100%)	-
	Absent	0	0	0	0	
Red Blood Cells	Present	2(50%)	2(50%)	2(50%)	3 (75%)	0.859#
	Absent	2(50%)	2(50%)	2(50%)	1 (25%)	
White Blood Cells	Present	4(100%)	4(100%)	3 (75%)	4(100%)	0.362#
	Absent	0 (0%)	0 (0%)	1 (25%)	0 (0%)	
Platelets	Present	3 (75%)	2 (50%)	2(50%)	4(100%)	0.362#
	Absent	1 (25%)	2(50%)	2(50%)	0 (0%)	

%- Percentages, # non-significant, P >0.05 was considered statistical non-significance, Amoxi-Clav- Amoxicillin+ Clavulanic acid, MTZ- metronidazole

Table 5. Depict the mean values of Energy Dispersive X-ray (EDX) of Titanium Platelet Rich Fibrin (T-PRF) plain and incorporated with Amoxiclav gel/ Metronidazole gel/ Neem gel

Elements	T-PRF Plain (%)	T-PRF+ Amoxclav gel (%)	T-PRF+ MTZ gel (%)	T-PRF+ Neem gel (%)
C	68.6 ± 0.4	71.40± 0.31	77.5± 0.4	87± 0.4
O	30.9 ± 0.4	26.65± 0.30	22.1± 0.4	12.6± 0.4
S	0.5 ± 0.0	0.84± 0.03	0.4± 0.0	0.4± 0.0
Si	-	1.11± 0.03	-	-

C-Carbon, O- Oxygen, S- Sulphur, Si- Silica, Amoxi-Clav- Amoxicillin+ Clavulanic acid, MTZ- metronidazole, %- Percentages

DISCUSSION

Mixing antibiotics to the L-PRF was done by Bernardo F et al.,[25] 2023 where they concluded that drugs mixed with L-PRF in the oral surgery treatment helped in reduced post-operative pain and enhanced therapeutic effect of systemic antibiotics. This mixing of antibiotics in PRF preparation didn't affect the structural properties of PRF. Many antibiotics like metronidazole, amoxicillin, clindamycin, alendronate, metformin etc. in gel forms were used as local drug delivery systems in surgical and non-surgical treatments. To our

knowledge, no study was performed till now by injecting these antibiotics and herbal extract gels in T-PRF and assessed for histological analysis. All the participants in the study were voluntarily willing and participated in the study. No complications and pain were reported at the blood drawing site. Due to scarce comparisons, present study results will be compared with the existent literature of any platelet concentrate.

Various drugs like vancomycin, ampicillin, diclofenac sodium have been incorporated into PRF and reported that there was no loss in structure, had greater anti-microbial efficacy for antibiotic studies while lower post-operative pain was recorded as diclofenac sodium study concerned in oral surgery 3rd molar extraction.[26, 27] But injecting antibiotics prior to centrifugation is always a issue of concern as they might disrupt the centrifugation process and coagulation process might hampered. Apart from this, Polak D et al.,[28] in their study injected different concentrations of antibiotics and concluded that injecting more than 0.5 ml of antibiotic into PRF might alter the structure, make it fragile and interfere with PRF maturation.[28] In the present study we had used T-PRF, injected antibiotics and herbal extract after centrifugation which had thicker fibrin meshwork, carried an inherent property of swelling and holding of antibiotics within itself.

Recently Ercan et al.,[18] 2022 used T-PRF for sustained drug delivery system (SDDS) where they have incorporated doxycycline hyclate liquid within T-PRF and observed that T-PRF acted as a SDDS and kinetically released the drug for longer time duration than doxycycline incorporated collagen sponge. Hence, present study was conducted to check whether incorporation of N/Amoxiclav/MTZ in T-PRF led to any change in fibrin meshwork, cell pattern, cell distribution etc. and check histologically under light microscope.

Regarding fibrin network though there was no significant difference among the groups thick and dense pattern was reported with MTZ and Amox-clav group than Neem gel group. This is in accordance for MTZ and Amox-clav groups and contrast to Neem gel group with studies conducted by Tunali M et al.,[7] 2013 and Chatterjee A et al.,[11] 2017 where they stated a thick and dense fibrin network pattern. Present study results for fibrin network was similar to Bhattacharya HS et al.,[9] 2022 specified that T-and L-PRF shared a like arrangement with non-significance and thicker network found in middle membrane region with statistical significance. This thick and dense pattern that was recorded in the present study might be due to inherent thick membrane that was formed during centrifugation.

Regarding fibrin pattern all the groups showed greater percentages of packeting pattern which is in harmony with Tunali M et al.,[20] 2014 and Chatterjee A et al.,[11] 2017 where they stated a packeting pattern with thicker fibrin and contrast with Bhattacharya HS et al.,[9] 2022 where they described a scattered pattern. This might be due to stimulation of platelets by the passivated titanium tube (inner titanium dioxide layer) lead to thicker meshwork with packeting pattern.

While coming to cell distribution, all the groups showed wider distribution of cells over the length of the clot. This is in harmony with Chatterjee A et al.,[11] 2017, Tunali M et al.,[7] 2013, Bhattacharya HS et al.,[9] 2022 and 2020[8] where they also reported cells had a wider distribution. This shows that incorporation of antibiotics or herbal extract didn't alter the distribution of cells over the clot.

All T-PRF clots incorporated with antibiotics or herbal extract showed the presence of RBC's, WBC's and Platelets. While coming to mean scores of presence of cells, all the groups showed non-significant 26-50% cells distribution. These parameters are in harmony with Bhattacharya HS et al.,[9] 2022; Tunali M et al.,[7] 2013 & Chattarjee A et al.,[11] 2017 where they also conveyed the wider distribution of cells. This may be due to greater centrifugation time of 15 minutes and higher rotations of 3500 rpm which had helped in entrapment of higher percentage with wider distribution over the clot. Hence present study results shared similarity with that of Esra ercan et al.,[18] 2022 where T-PRF holded Doxycycline hyclate whereas in the present study T-PRF holded MTZ, Amox-clav and Neem gels separately without disturbing the cell distribution, fibrin network and distribution pattern. This might be due to inherent swelling property; soft nature of the clots makes it a viable treatment modality for making a ray of hope in utilizing this T-PRF as SDDS.

While checking for SEM comparison present study results were in accordance with Ercan E et al.,[18] 2022; Bhattacharya HS et al.,[9] 2022 and Mitra DK et al.,[12] 2019 where they have concluded that T-PRF had greater cellular entrapment with thicker fibrin

structure and presence of RBC, WBC and platelets. Because of T-PRF clot inherent property injecting drugs didn't alter the morphology and under SEM surface haziness (whitish or dark) was noted in all the test groups indicating the adherence and presence of drug gels within and outside the clots. Further it can be understood that T-PRF had a greater holding capacity within itself. Present study had a greater fibrin thickness and organized pattern even after injecting drugs or herbal extract and were much

better than Ravi S et al.,[29] 2020 for SEM while accordant results were reported for light microscopy, where they stated that T-PRF had looser fibrin pattern that help in greater angiogenesis and also concluded that T-PRF had 0.1% titanium that got incorporated when checked under energy dispersive X-ray spectroscopy (EDX).

Current study results of semi-quantitative elemental analysis showed similarity regarding the elements of C, O, and S with that of Carey and Giuliano[30] 2016 and Dias F J et al.[31] 2020 where they recorded additional elements like Nitrogen (N), Chlorine (Cl), Fluorine (F) Phosphorus (P) and Sodium (Na) apart from C, O and S. This might be due to the presence of only fibrin structure without red cell component in the present sample. Presence of S in the samples depict the amino acid composition, N, Cl, P and Na were absent in the current study samples and this variation might be due to the absence of red part in the T-PRF structure, influence of added drugs on the clot and EDX was performed at mid membrane region. In T-PRF +Amoxclav gel sample there was a detection of silica (Si) and this might be due to the minor contamination of sample or some sought of artifacts.

Present study histological results were also in accordance with a recent study done by Gummaluri SS et al., [32] 2024 for neem gel incorporation in T-PRF where they shared a loose thin layered pattern of T-PRF+ neem gel clot. This finding may indicate that T-PRF had a proper space to incorporate antibiotic gels or herbal extract to become a standard SDDS.

Limitations of present study might be smaller sample size, performing drug kinetics (using spectrophotometric analysis), antimicrobial efficacy, animal trial or a human randomized clinical trial might be better way of assessing the clinical outcome of this newer treatment strategy making T-PRF as a SDDS. Titanium tubes are costly but it is a sought of one-time investment, non-breakable and re-sterilisable without cross contamination. This article opens up a path way to newer surgical treatment protocol for treating periodontal disease.

CONCLUSION

Within limitations, present study concluded that T-PRF incorporated with MTZ, Amox-clav and Neem gels separately had maintained an intact fibrin network structure and pattern to most extent without disturbing the cell distribution. SEM showed that incorporation of antibiotic gels or herbal extract didn't alter the surface structure and also able to identify the coating of drug on to the clots. Hence present study opened a way that T-PRF can be used as a SDDS by incorporating antibiotics or herbal extract for treating periodontal disease.

DECLARATIONS

Conflicts of interest and financial disclosures

The author declares that he has no conflict percent and there was no external source of funding for the research in question.

Conflict of interest and Financial Disclosures

The authors declare no conflicts of interest related to this study and no source of external funding.

Ethical Approval

This study was approved by the Institutional Ethical Committee of GITAM Dental College and Hospital with protocol number 71086061123. All participants signed an informed consent form.

Informed Consent

All participants signed an informed consent form. Informed consent in regional language Telugu or English was received from the patients included in the study and a patient information sheet in regional language Telugu or English explaining about the study was also given to the patients. After receiving the informed consent from the patients, the samples were collected

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RESEARCH ARTICLE

A NEW APPROACH FOR PAIN IN TEMPOROMANDIBULAR DISORDERS ADDRESSING THE INTERACTION OF DIET, EATING HABITS AND MEDICATIONS

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ABSTRACT

Background: Temporo-mandibular disorders (TMDs) is an umbrella term to describe a condition with a multifactorial etiology characterized by various symptoms including:limitations on opening the mouth, clicking or grinding, orofacial pain, referred muscular pain or headaches.Pain has many origins, such as muscular, neuropathic, or vascular. It is the most frequent symptom, and often limits the patient's daily habits. In the presence of orofacial pain in temporomandibular disorders some functions such as eating, talking, or singing are limited and there are foods that can improve or worsen the symptoms. The patient, in the presence of pain, often has to resort to medications. Even today, this pathology, although quite widespread, does not have precise guidelines for therapy. In addition, the literature is still not clear about the most appropriate types of therapy as muscle pain and neuropathic pain are usually co-present with TMD. Therapy depends upon correctly identifying the etiology, for the treatment or the management of symptoms to be successful and to improve quality of life.
Aim: The aim of this study was to evaluate, through a survey, the correlation between lifestyle (diet, oral habits, medications, pasorthodontic treatments) and temporomandibular disorders (TMDs) and how these two variables interact, in order to counsel the patient with orofacial/TMJ pain on how to best modify their diet/eating habits to reduce the symptoms and modify, if needed, any pharmacological intervention.
Materials and Methods*:*Two hundred and nine (209) patients completed an online survey including questions about their TMD symptoms, their diet, frequency of meals, previous and current orthodontic treatments and most used medications to reduce pain. The questionaries, available in two languages (Italian and English) and composed mostly of yes/no questions, were shared among patients affected by TMDs who were being treated and or had been treated for temporo-mandibular disorders.
Results: Our study confirms what we found in the literature, which is an association between frequency of eating and TMJ/orofacial pain. The consistency or texture of frequently consumed foods was also relevant in the severity of pain as patients reported an association between the consumption of hard foods and pain. Among the respondents, NSAIDs and myorelaxants were significantly more effective than supplements and corticosteroids. No statistical correlation was found between orthodontic therapy and a decrease in mouth opening.
Conclusions: Food and eating habits seem to have the capacity to affect TMJ/orofacial pain. The role of daily eating habits, should be further investigated. In fact they could represent a new frontier in the management of joint pain as they can be managed directly by the patients, and complement other pain therapy options such as medications and oral devices.

Keywords: Temporomandibular disorders, eating, pain, daily habits, medications

INTRODUCTION

Temporomandibular disorders (TMDs) are pathological conditions including intra-articular abnormalities of the position and/or of the structure of the disc, known as intra capsular disorders, as well as muscles dysfunctions that are known as extra capsular disorders[1] or more commonly referred to as orofacial pain disorders (OPD), when encompassing both types of disorders and functional disability.

TMJ/orofacial pain disorders are quite common in people, even more now than in the past as they are currently much more understood and studied determining a higher possibility to get the right diagnosis.[2]

The main reason for which people inquire about therapy is the pain experienced, which is primarily of muscular origin and this is the reason why, currently in the US, the standard of care is to refer to these problems not as TMD but as orofacial pain disorders with or without TMJ involvement. TMJ/OPDs affect females more than males, because of various biological aspects and, in particular, likely due to hormonal influence[3]In general, TMJ/OPDs have an annual incidence of 4% and peak in individuals between 20 and 40 years of age[4]The patients with juvenile idiopathic arthritis they may be at risk.[5,6,7]

Regarding diagnosis, the standard system introduced by Dworkin is the most used. It is based on diagnostic criteria for TMD and it has been accepted by the scientific community all over the world [9]

Instrumental examination can help in the diagnosis. The best instrumental examination to visualize and analyze the position of the disk is magnetic resonance imaging (MRI), but to evaluate hard tissues of the joint, such as the mandibular condyles and the temporal fossae, a CT scan is very useful, in particular the cone beam computerized tomography (CBCT), which is an imaging technique with a very low dose of radiation.[8,9]

Due to occlusal factors, which are considered as a possible etiology of some symptoms of TMDs, oral splints are used for symptomatic treatment. These oral devices can reduce muscle spasms and stiffness thus relaxing the orofacial muscles. Moreover, the oral devices can even alleviate the joints symptoms by changing the position of the jaw, the condyle and the disk. In some patients, after an initial successful oral splint therapy, the result might need to be stabilized through orthodontic therapy.[7]

Depending on the etiology of the orofacial pain (intra-capsular disorders, muscle pain, neuralgia, trauma or malocclusion), clinicians may decide accordingly to treat occlusion or muscle pain or TMJ disorders, or to refer the patient to a neurologist, or to a chronic pain specialist, or to a physiotherapist for treatment of the overall postural problems affecting the orofacial muscles. In fact, laboratory research and clinical experience have led to the observation of the connections between postural adaptations, anatomical conditions and TMJ/OPD.[3] The posture of the jaw is determined by a complex feedback-feedforward system in which the central nervous system integrates the input signals from various receptors localized in the oral and peri oral musculature, tongue, periodontal ligament and many other tissues, integrating them and processing a signal that determines the postural tonic system.[10]

Another important aspect in the management of TMJ/OPD, is the pharmacological therapy which can be very helpful in the acute phase of the disorder. Since this disorder can have an inflammatory aspect, like every skeletal-muscle condition, the use of nonsteroidal anti-inflammatory drugs (NSAIDs), can be helpful. Furthermore, although corticosteroids have an anti-inflammatory effect, the clinicians should prescribe them judiciously due to the various systemic side effects[11].

In addition, in the extracapsular forms, muscle relaxants are often used because they can reduce muscular pain, soreness and spasm, in particular in patients affected by bruxism and clenching. However, without addressing the cause or etiology of bruxism and clenching, the effect of the muscle relaxant is going to be temporary. TMJ/OPDs are quite common in patients affected by clenching or bracing, and these parafunctions can be linked to psychological stress for which psychotherapy can be effective Moreover, current literature shows a correlation between orofacial and TMJ pain and sleep disorders, in particular sleep bruxism that is usually caused by poor nocturnal breathing and especially obstructive sleep apnea. Several studies demonstrated that bruxism can be an etiological factor in articular pain and functional limitations.[12,13]

Sabina Saccomanno, S Saran, A Messore, A Pirino, R Fontanella, G Bruno, F Scoppa A New Approach for Pain in Temporomandibular Disorders : Addressing the Interaction of Diet,Eating Habits and Medications.Bulletin of Stomatology and Maxillofacial Surgery. 2025;21(3).165-177. doi: 10.58240/1829006X-2025.3-165

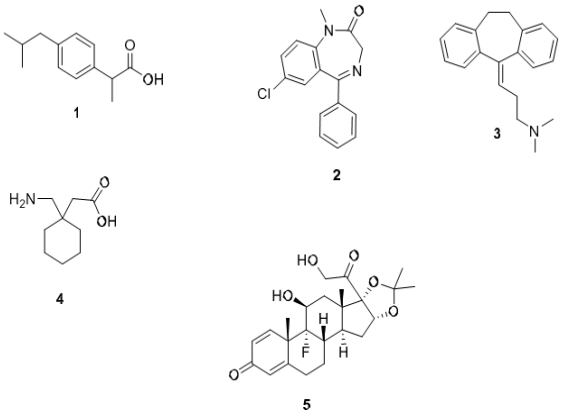


Figure 1. Chemical structure of ibuprofen (**1**), diazepam (**2**), amitriptyline (**3**), gabapentin (**4**) and triamcinolone acetonide (**5**).

Certainly, bruxism subjects the TMJ and the orofacial musculature to considerable stress that can cause pain.TMJ/OPD can affect the capability to chew and eat. The kind of food and its consistency can affect the activity of the TMJ and in particular the function of the oral-facial muscles, as it is reported in the literature and in clinical experience that patients with orofacial pain try to avoid hard foods that trigger or increase that pain. Nowadays people are used to eating more frequently than in the past and that could be a factor that exacerbate the jaw's muscle activity and spasms, especially if it is hard-to-chew foods or gum.[13,15,16]

A correlation between chewing and TMJ/OPDs was found by Tabrizi et al. as they observed that the prevalence of pain disorders increases in patients who routinely chewed gum.[15]

TMJ disorders seemed to have increased in recent years, probably due to some heavily processed foods which, according to many studies, favor inflammatory diseases that contribute to chronic pain. It can be surmised that changing daily eating habits, eating frequency and the types of food consumed can be important tools to reduce the level of pain caused by TMJ/OPDs. Furthermore, there is a relationship between diet and respiratory problems which can favor oral respiration. In particular it is known that some foods can trigger nasal congestion, like those containing tyramine, which facilitates the production of histamine. The subsequent oral breathing ~~can~~ changes the rest position of the mandible and the function of the oral muscles, thus creating or supporting TMJ dysfunctions. Since the stomatognathic system is influenced by diet and by eating modalities and timing, from a medical perspective, many studies suggest that intermittent fasting can help decrease the activity and severity of a few diseases and conditions, in particular those characterized by inflammation which could also directly affect the TMJ. Adawi et al., in a multi-center study, reported that intermittent fasting, like during Ramadan, could reduce the inflammation index and the disease's activity in many types of arthritis [16,17] and, when appropriate, patients with TMJ/OPDs should be referred to their regular physician to assess the need for nutritional counseling. Some clinicians recommend natural supplements, such as Glucosamine and Vitamin C, to help patients with this chronic condition, but there is not enough literature to support this kind of therapy.[18,19]

The concept of fasting as a component of health was already known at the time of Buddha who fasted regularly. We now live in a society in which calorically rich foods are so widespread and readily available that people eat them even when they are not hungry, out of boredom or to soothe themselves (*comfort food*). In the past, having rich meals was a symbol of wealth and wellness, but today there is a need for a more balanced diet to achieve and maintain proper health, while avoiding obesity, diabetes and other inflammatory diseases. Medical literature indicates that a balanced diet can help in preventing many diseases, and in particular inflammatory conditions. [20,21]

Finally, great importance is now given to microbiota, which is influenced by diet. Microbiota is linked to many aspects of health, in particular to brain function and to the integrity of the immune system. It is well documented that an altered microbiota can produce cytokines which can create low grade. inflammation affecting all the joints, including the TMJ, and promoting chronic fatigue syndrome, which sometimes is a co-morbidity of people suffering from chronic orofacial pain. Furthermore, an altered microbiota cannot only be just the consequence of psychological disfunction, but also, by changing the equilibrium of serotonin, microbiota may promote the chronicity of this dysfunction.[22] It is remarkable that a dysfunctional microbiota seems to be implicated in the development of depression as well, which is a condition that can increase the risk of TMJ/OPDs and their chronicity, therefore depression is considered a co-morbidity.[23]

Recent studies have also highlighted that an unhealthy diet can indeed alter the microbiota, which can affect changes in neurotransmitters linked to

cognitive impairments, and in return, and according to the biopsychosocial theory, cognitive impairment itself creates social problems that can exacerbate or promote chronicity of TMJ/OPD.[24]

This study aims to analyze the correlation between diet (specifically types of food and eating practices) and TMJ/orofacial pain disorders, by way of a questionnaire, and to see if these disorders can be connected to the frequency of eating and the type of diet. This study wants to investigate if there are kinds of foods that can exacerbate painful symptoms and if limited times of fasting, or increasing the time between meals, can reduce the level of pain. It tries to find out if there are other medical and behavioral approaches that can be helpful in mitigating orofacial pain. Furthermore, it tries to understand the relationship between gender, level of stress, frequencies of eating and the level of pain. At the same time, it studies which pharmacological tool seems to be more effective in managing TMJ/orofacial pain considering the use of natural supplements as well. This study aims at analyzing the level of pain and the patients' request for therapies and instrumental examinations, and finally, it explores the connection of pain, the level of mouth opening in patients and whether they underwent orthodontic or splint therapy.

MATERIALS AND METHODS

We enrolled in our research 209 patients, with an age range between 20 and 60 and with a slight majority of subjects being between 40 and 60 years of age. The exclusion criteria were people without temporo-mandibular disorders and patients who did not accept to answer the questionnaire. The only inclusion criteria was for patients to be suffering from TMD and willing to participate in the questionnaire. All subjects gave their informed consent for inclusion before they participated in the study .The study was conducted in accordance with the Declaration of Helsinky. The questionarrie was anonymous. All participants provided an informed consent and accepted the privacy policy for the protection of personal data before compiling the survey.

The questionnaire contained 16 questions, some in a yes/no format and others in a multiple choice or on a scale format, disseminated through an online ~~form~~ service (Google Form service, Google LLC, Mountain View, California, U.S.). The questionaries were administered to all patients bilingual form (Italian- English). No personal information that identifies the individuals was collected and the data was analyzed only in aggregate form. All responses were collected and anonymized using the Google Form service. A pre-pilot quantitative questionnaire was distributed to some patients of the authors and to identify any errors that it may have contained, which were subsequently corrected. The authors to this regard found no need to per- form a pilot study.

The questions asked to patients are the following:

1. Gender
2. Age
3. Level of education
4. Did you ever have an orthodontic therapy? Yes/No
5. Did you ever use an oral appliance (bite)? Yea/No
6. Did you ever have an instrumental examination for the diagnosis? If yes, which one?
7. Do you have limitations in opening your mouth? Yes/No
8. Do you notice that when you are under stress, you eat more and your TMJ pain increases? If yes, how much? (1-10)
9. Did you ever fast for few hours/days? If yes, how much did it reduce your TMJ pain? (1-10)
10. Do you feel the more times you eat during the day the more TMJ pain increase?
11. Did you ever notice that a particular food influence your TMJ pain causing worsening? If yes, which one?
12. Do anti inflammatory drugs (NSAIDs) reduce your TMJ pain? (1-10)
13. Do muscle relaxants reduce TMJ pain? If yes, how much? (1-10)
14. Did you ever take corticosteroids? If yes, how much did they help? (1-10)
15. Did you ever take any supplement with the anti-inflammatory features? If yes, how much did it help? (1-10)
16. Did you ever have an orthodontic therapy? Do you have limitations in opening your mouth? Yes/No

Statistical Analysis

The descriptive statistics were reported as a mean and standard deviation or median and interquartile range. The frequencies were summarized as percentages.

The pain score represents our primary outcome, and it consists of a continuous variable. Painkillers were the most used response to pain. This variable was divided into four categories: NSAIDs, corticosteroids, muscle relaxants and supplements. Since the variable pain score did not follow a normal distribution (Shapiro-Wilk W test, P = 0.01), we ran the Kruskal-Wallis test and, after that, the Wilcoxon rank-sum for the pairwise comparisons. At first the α-level was fixed at 0.05 and then reduced to 0.008, when we performed pairwise comparisons, taking into account 6 tests.

All data was analyzed with the statistical software IBM SPSS Statistics v25. This is a study carried out on subjects who, in freedom and autonomy, filled out a survey questionnaire, it was not possible to calculate the sample size in advance through power analysis. A calculation of the margin of error was therefore carried out on the basis of the respondents actually obtained. The collected sample was of 209 subjects. Considering that the reference population has approximately 5.4 million patients and considering a confidence level of 95%, an estimated margin of error is obtained for the parameters of interest equal to at 7%. The formula used for the calculation is the following:

Table 1. The sample analyzed consists of 209 individuals and 199 are women.

RESULTS

Descriptive univariate statistics of analyzed variables are firstly presented. Frequency tables are shown for qualitative variables while centrality and variability indexes are presented for quantitative variables.

Table 1. The sample analyzed consists of 209 individuals and 199 are women.

Gender

		Frequency	Percent	Valid Percent
Valid	Female	199	95.2	95.7
	Male	9	4.3	4.3
	Total	208	99.5	100.0
Missing		1	.5	
Total		209	100.0	

$$\text{Margin of error} = z \times \frac{\sigma}{\sqrt{n}}$$

Table 2. The majority of the sample is between 30 and 60 years old with a slight majority between 40 and 60 years.

Age

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	10-20	9	4.3	4.3	4.3
	20-30	52	24.9	25.0	29.3
	30-40	65	31.1	31.3	60.6
	40-60	69	33.0	33.2	93.8
	other	13	6.2	6.3	100.0
	Total	208	99.5	100.0	
Missing		1	.5		
Total		209	100.0		

Table 3. The level of education most observed is college with the 65.7% of the units.

Level of education

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Middle school	2	1.0	1.0	1.0
	High school	45	21.5	22.1	23.0
	College	134	64.1	65.7	88.7
	PhD	23	11.0	11.3	100.0
	Total	204	97.6	100.0	
Missing		5	2.4		
Total		209	100.0		

Table 4. About 57% of the interviewees have received an orthodontic therapy.

Did you ever have an orthodontic therapy?

		Frequency	Percent	Valid Percent
Valid	No	88	42.1	42.5
	Yes	119	56.9	57.5
	Total	207	99.0	100.0
Missing		2	1.0	
Total		209	100.0	

Table 5. 58.7% of the respondents have used an oral appliance.

		Frequency	Percent	Valid Percent
Valid	No	86	41.1	41.3
	Yes	122	58.4	58.7
	Total	208	99.5	100.0
Missing		1	.5	
Total		209	100.0	

Table 6. The majority of those who ~~did~~ had instrumental examination for the diagnosis had MRI and CT, while 54.1% of the interviewees have never done imaging.

Did you ever have an instrumental examination for the diagnosis? if yes. which one?

Frequency			Percent	Valid Percent
Valid	Both	39	18.7	18.8
	CT	31	14.8	15.0
	MRI	25	12.0	12.1
	none	112	53.6	54.1
	Total	207	99.0	100.0
Missing		2	1.0	
Total		209	100.0	

Table 7. The majority (61.5%) has a limitation in opening their mouth.

Did you ever notice that a particular food influence your TMJ pain causing worsening? If yes, which one?

		Frequency	Percent	Valid Percent
Valid	None	38	18.2	18.2
	Hard food	106	50.7	50.7
	Chewing food	7	3.3	3.3
	Solid food	27	12.9	12.9
	Sticky food	23	11.0	11.0
	Irritating F&B	8	3.8	3.8
	Total	209	100.0	100.0

Table 8. On a scale from 1 to 10 the mean value, given to "Do you notice that when you are under stress you eat more and your TMJ pain increases?", is 5.68

Statistics

		Do you notice that when you are under stress, you eat more and your TMJ pain increases? if yes, how much? (1 is minimum; 10 is maximum)	Do you feel the more times you eat during the day the more TMJ pain increase? (1 is minimum; 10 is maximum)-	Did you ever fast for few hours/days? if yes, how much did it reduce your TMJ pain? Please answer just if you ever fasted (1 is minimum; 10 is maximum)
N	Valid	197	198	142
	Missing	12	11	67
Mean		5.68	5.62	4.55
Median		6.00	6.00	5.00
Std. Deviation		2.908	3.006	2.800
Percentiles	25	3.00	3.00	2.00
	50	6.00	6.00	5.00
	75	8.00	8.00	6.25

142 people have ever fasted for a few hours/days. On a scale from 1 to 10 the average value given to reducing of the TMJ pain, thanks to the fasting, is 4.55.We also got 5.62 as the average value for "Do you feel the more times you eat during the day the more TMJ pain increase?"

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Table 9. Of all the respondents,-81.8% have experienced increasing of the TMJ pain after eating some food. In particular, 50.7% say that the pain was mostly caused by hard food.

Do you have limitations in opening your mouth?

		Frequency	Percent	Valid Percent
Valid	no	80	38.3	38.5
	yes	128	61.2	61.5
	Total	208	99.5	100.0
Missing		1	.5	
Total		209	100.0	

Table 10. On a scale from 1 to 10, the pharmaceutical treatment for ~~the~~ TMJ pain with the highest average point given by the interviewees is for muscle relaxants (4.98) and the one with the lowest is corticosteroids (3.45).

Statistics

		Do anti inflammatory drugs (NSAIDs) reduce your TMJ pain? (1 is minimum; 10 is maximum)	Do muscle relaxants reduce your TMJ pain? if yes, how much? (1 is minimum; 10 is maximum)	Did you ever take corticosteroids? if yes, how much did they help?(1 is minimum; 10 is maximum)	Did you ever take any supplement with anti inflammatory features? if yes, how much did it help? (1 is minimum; 10 is maximum)
N	Valid	178	140	96	128
	Missing	31	69	113	81
Mean		4.72	4.98	3.45	3.99
Median		5.00	5.00	2.00	4.00
Std. Deviation		2.651	2.932	2.894	2.841
Percentiles	25	2.00	2.00	1.00	1.00
	50	5.00	5.00	2.00	4.00
	75	7.00	7.00	5.75	6.00

To evaluate if pain under stress, meals frequency and benefit of fasting are significantly different in subgroups determined by the food that generates the most discomfort, an ANOVA test was used, comparing the average score attributed to the three scales according to the group.

Table 11. First of all, it emerges that the average scores are different and ~~how~~ the patients who do not have particular hassles associated with food and drink are also those who benefit less from the diet or less annoyance from the high frequency of meals.

Descriptives by group

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
						Lower Bound	Upper Bound
Do you notice that when you are under stress, you eat more and your TMJ pain increases?	None	36	4.69	3.097	.516	3.65	5.74
	Hard food	100	6.16	2.681	.268	5.63	6.69
	Chewing food	7	5.14	3.078	1.164	2.30	7.99
	Solid food	24	6.08	3.243	.662	4.71	7.45
	Sticky food	22	4.36	2.718	.579	3.16	5.57
	Irritating F&B	8	6.88	2.475	.875	4.81	8.94
	Total	197	5.68	2.908	.207	5.27	6.08
Do you feel the more times you eat during the day the more TMJ pain increase?	None	36	3.78	2.789	.465	2.83	4.72
	Hard food	103	6.30	2.879	.284	5.74	6.86
	Chewing food	7	6.43	3.207	1.212	3.46	9.39
	Solid food	26	5.38	3.213	.630	4.09	6.68
	Sticky food	18	5.11	2.610	.615	3.81	6.41
	Irritating F&B	8	6.38	2.446	.865	4.33	8.42
	Total	198	5.62	3.006	.214	5.20	6.04
Did you ever fast for few hours/days? if yes, how much did it reduce your TMJ pain?	None	24	2.29	1.574	.321	1.63	2.96
	Hard food	70	5.21	2.637	.315	4.59	5.84
	Chewing food	5	4.40	2.608	1.166	1.16	7.64
	Solid food	20	4.65	2.870	.642	3.31	5.99
	Sticky food	16	4.56	3.366	.841	2.77	6.36
	Irritating F&B	7	5.43	3.047	1.152	2.61	8.25
	Total	142	4.55	2.800	.235	4.08	5.01

Table 12. For all three variables, the ANOVA test allows us to reject the null hypothesis of equality of the mean scores in the subgroups (p<.05), allowing us to state that the differences in pain under stress, pain linked to meal frequency and the benefit of fasting are significantly different depending on the food.

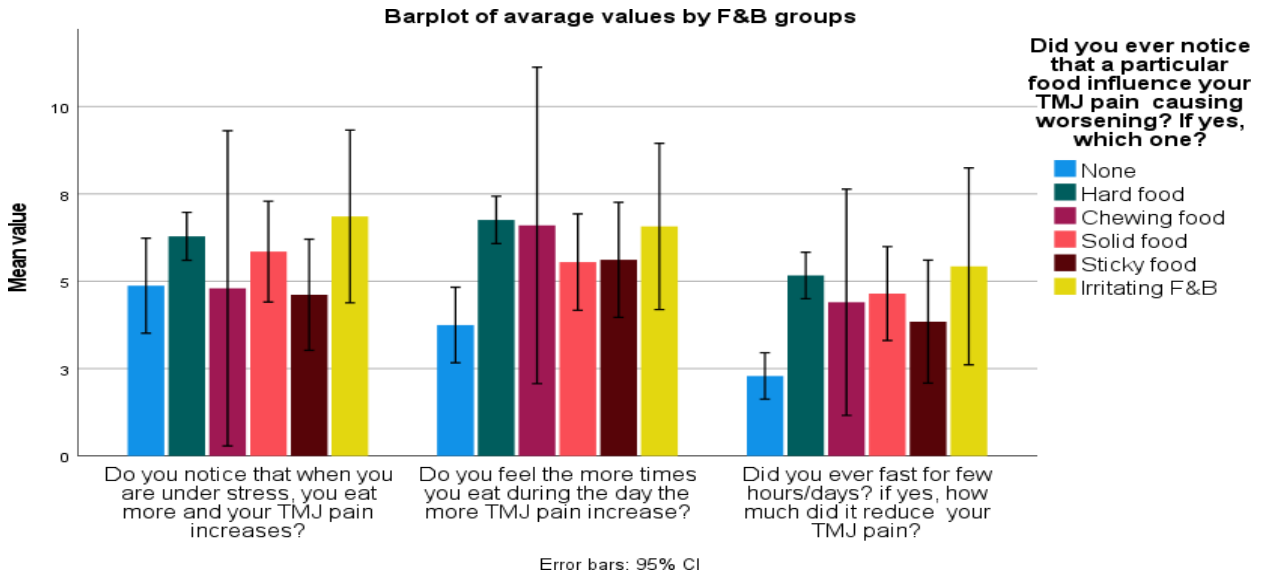
ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Do you notice that when you are under stress, you eat more and your TMJ pain increases?	Between Groups	113.473	5	22.695	2.808	.018
	Within Groups	1543.735	191	8.082		
	Total	1657.208	196			
Do you feel the more times you eat during the day the more TMJ pain increase?	Between Groups	185.178	5	37.036	4.457	<.001
	Within Groups	1595.413	192	8.309		
	Total	1780.591	197			
Did you ever fast for few hours/days? if yes, how much did it reduce your TMJ pain?	Between Groups	159.009	5	31.802	4.571	<.001
	Within Groups	946.146	136	6.957		
	Total	1105.155	141			

In particular, for “Do you notice that when you are under stress, you eat more and your TMJ pain increases?”, the group suffering ~~for~~ with hard food presents significantly higher scores, at level 0.10, of both None group (p=.090) and Sticky food group (p=.083). Instead, for “Do you feel the more times you eat during the day the

more TMJ pain increases?", the group suffering ~~for~~ with hard food has significantly higher scores, at level 0.001, than the None group (p<.001).
Finally, for "Did you ever fast for few hours/days? if yes, how much did it reduce your TMJ pain?", the group that has no particular ~~annoyances~~ problems with food and drinks, has significantly lower scores than almost all other groups, particularly lower than Hard Food (p<.001), than Solid Food (p<.042), than Sticky Food (p=.089) and than Irritating F&B (p=.069), the latter two comparisons having ~~with~~ a level of significance at 0.10.

Table 13. To process data we divided 6 food groups: none, hard foot, chewing food, solid food, sticky food and irritating F&B.



DISCUSSION

Temporo-mandibular disorders (TMDs) are conditions capable of compromising the quality of life of the affected patients. In fact, their typical clinical signs are muscle pain, TMJ clicking and grinding noises, deflection and deviation of the jaw in opening and restriction in mouth opening. These signs can make it difficult to perform everyday activities such as chewing and speaking, contributing to frustration, sleep disturbances and anxiety.[25]

TMD/orofacial symptoms are very common, especially among women and they are considered the most common orofacial pain conditions of nondental origin [5]. Our study confirmed this aspect, as 95.2% of the sample, who chose to answer the questionnaire, were women.

61.5% of the patients reported a limited mouth opening, one of the typical signs of TMDs. A decreased range of mouth opening was found to be very common,. A limited mouth opening could be caused by disc displacement or muscle contraction therefore a correct diagnosis is essential for a correct treatment. The results of this study did not find a statistical correlation between the decreased mouth opening and orthodontic therapy. In fact, in addition to oral health, a correct orthodontic therapy aims to preserve or restore the proper functioning of the joint and muscles. When properly conducted, orthodontic therapy allows the joint to work in the most physiological way.

Regarding the diagnosis of TMDs, the largest portion of the sample (54.1%) never had any instrumental examination. Reportedly, a MRI was used to diagnose the disorder in 12% of the sample, a CT in 15% of the sample and both imaging techniques in 18.8%. So, both a MRI and a CT, although with different potentials, seemed effective in diagnosing temporo-mandibular disorders.

A MRI has the advantage to visualize soft tissues clearly, and MRI findings are useful for the visualization of disc position and configuration, condylar morphology and joint effusion. A CT is useful to visualize only hard tissues, such as condylar and temporal morphology. Matsubara et al. showed that a MRI examination could be the principal instrumental examination in patient with

TMD symptoms, as a CT and a CBCT, which mainly reveal hard tissues, may not be as useful for evaluating the anatomical and physiological complexity of TMJs.[27]

In this study, a high correlation was found between daily eating habits and the severity of symptoms and as both the frequency and the type of food subject the joints to different levels of effort and pain. 142 people in this study had tried various degrees of fasting and it was reported as being quite effective in reducing TMJ, since chewing is not performed and the joint is not subjected to stress.[28]

Conversely, the patients reported that increasing the number of meals or eating certain foods also increased joint pain. Regarding the type of food consumed, a correlation was found between TMJ pain (reported by 85.2 % of the patients) and hard food, which seems to increase joint pain. During chewing of particularly hard foods, the muscle force exerted by the stomatognathic system is considerable and it's usually asymmetric (according to chewing dominance, preferring right or left side) and this can cause damaging stress to the temporo-mandibular joint.[29]

These findings should be investigated with further studies. to evaluate a targeted therapeutic protocol involving a specific diet and chewing strategies; to evaluate possible associations between foods and parafunctions such as bruxing, muscle bracing, clenching and biting, which were not addressed in this study; to compare the effectiveness of addressing parafunctions such as muscle bracing, excessive muscle recruitment, pencil biting, or leaning on a hand ~~etc~~. to reduce the orofacial/TMJ pain; or to evaluate the possibility to prevent orofacial/TMJ pain by reducing predisposing factors such as a soft diet since infancy (which is not promoting proper muscle function) or poor nasal breathing (which sets up a cascade of physiological changes). [30]Unfortunately there are some variables that cannot be controlled easily, such as the hormonal impact of being females, which directly affects the TMJ function and therefore affects the development of pain.[31]

Regarding pharmacological treatment, muscle relaxants were found to be the most effective in reducing TMJ pain, probably because the largest percentage of the patients were affected by orofacial muscular symptoms. However, unexpectedly, the interviewees found corticosteroids not very effective despite inflammation being one of the main mechanisms underlying TMDs symptoms.

Limitations of the study are the fact that it is an anonymous questionnaire and many questions may not be well understood , and that the sample could be larger .Future studies should investigate these correlations on larger samples.[32]

CONCLUSION

The study supports the findings in literature suggesting that daily eating habits affect the severity of TMDs symptoms. These findings could help in the management of joint pain by suggesting the patient follow a specific diet, among other strategies. In fact, it would seem that consuming softer foods and reducing the number of meals, also reduces pain being that of muscular or joint origin, thus improving the patients' quality of life, although long term consumption of softer foods has other epigenetic impacts on the stability of occlusion and the orofacial skeletal complex. Furthermore, it would be interesting to investigate if different types of food can also be a trigger in the development of temporo- mandibular disorders.

In addition, the patients reported that the most common drugs they used to manage pain consisted in FANS, myorelaxant and supplements.

Our study confirmed that a limited mouth opening is a very common sign of TMDs, in fact it is present in the majority (60%) of the sample, and the results of the study show that there is no statistical correlation between orthodontic therapy and a limited mouth opening.

However, further studies need to be conducted to confirm a more specific role of food and eating practices in TMD/orofacial pain, its interaction with other contributing factors (sleep disorders, orthotics, early traumas or medications) and to evaluate new therapeutic approaches for patients affected by orofacial pain.

DECLARATIONS

Conflicts of interest and financial disclosures

The authors declare no conflict of interest

Funding

This research received no external funding.

Ethical Approval

Institutional Review Board Statement: The study was conducted at one clinical center in conformity with the Good Clinical Practice Guidelines, following the recommendations of the World

Medical Association Declaration of Helsinki ethical principles for medical research involving human subjects, as revised in Fortaleza (2013). The patient included in this work was treated in a private dental practice with written consensus.

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CASE REPORT

THE IMPORTANCE OF A MULTIDISCIPLINARY APPROACH TO METABOLIC SYNDROMES IN CHILDREN WITH OSAS. A CASE REPORT

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ABSTRACT

Objective: OSAS is currently a relatively frequent multifactorial sleep disorder often associated with metabolic dysfunction. This case study highlights the importance of creating a collaboration team of different specialists such as a pediatrician, a dentist, a sleep specialist, an otolaryngologist and a speech therapist, which, in this case, was essential to promote an early and comprehensive diagnosis, not only to limit and to prevent complications, but for a better therapeutic management of the symptoms.

Case review: A 10-year-old girl who came in for orthodontic treatment was found to have signs and symptoms of metabolic disorders including obesity, obstructive sleep apnea, breathing issues, transmissive hypoacusis and orofacial dysfunctions. She was previously treated orthodontically without resolution of any symptoms. At that point, instead of a "serial" approach, the girl was evaluated and then treated by a team of professionals who addressed together her various metabolic, functional and structural issues.

Results: The integrated program of palatal expansion and myofunctional therapy, in particular, made it possible to intervene on the skeletal structure and on the muscular function of this patient by promoting nasal respiration, a balanced mastication, adequate mobility and muscle tone of the orofacial complex and of the oropharynx. The improvement of the metabolic status was associated with better breathing and important weight loss.

Conclusion: A multidisciplinary approach seems to be the best strategy for solving cases of MetS (metabolic syndromes) and concomitant OSAS in children. A key role is played by the orthodontist and the pediatrician, by being able to intercept cases of OSAS in children and in adolescent and by being able to intervene directly on some components such as palatal contraction.

Keywords: metabolic syndrome, OSAS, rapid expander, myofunctional therapy, approach multidisciplinary

INTRODUCTION

Children can be impacted by a wide range of sleep disordered breathing (SDB), from upper airways respiratory syndrome (UARS) to obstructive sleep apnea syndrome (OSAS).[1] Results from recent studies have shown that obstructive sleep apnea syndrome (OSAS) is significantly associated with metabolic complications such as obesity, increased insulin values, increased cholesterol and triglycerides in the blood as well as an increased risk of developing cardiovascular disease.[2-4]

Pediatric OSAS is defined as a multifactorial disorder that occurs during sleep, characterized by a prolonged and/or intermittent partial obstruction

of the upper airways that alters the normal ventilation, oxygen saturation and sleep architecture.[2] The severity of the condition is defined by the number of apneas and hypopneas per hour of sleep (Apnea - Hypopnea Index: AHI).
Complications of OSAS in pediatric age can be divided into three categories:

1) *Neurocognitive (*excessive daytime sleepiness, reduced quality of life, aggressive behavior, poor school performance, depression, attention deficit, and hyperactivity);
2) *Cardiovascular(*systemic hypertension, absence of decreased blood pressure during sleep, left ventricular dysfunction, pulmonary hypertension, altered heart rate, autonomic nervous system dysfunction)*;*
3) *Metabolic* (increased reactive C protein, insulin resistance, hypercholesterolemia, increased transaminases, reduced insulin-like growth factor, reduced or altered secretion of growth hormones).[3]

Some studies confirm that in individuals with OSAS, the risk of metabolic dysfunction is six to nine times higher than in a subject without OSAS. Although the exact mechanism responsible is still unknown, it seems that the fragmentation of sleep and intermittent hypoxyemia, typical of OSAS[5] can cause pathophysiological alterations that, interacting with those of metabolic syndrome (MetS) [4-7] would be able to cause or perpetuate the progression of metabolic damage.
For this reason, other studies consider the OSAS as a further component of MetS.[8,9]
The *prevalent* phenotypes in children with OSAS are three:

1) The *common phenotype is called adenoids*, characterized by adenotonsillary hypertrophy, dark circles under the eyes, oral respiration, low tongue posture, by dental-skeletal malocclusion and by a skinny body;
2) The *adult phenotype of an obese child*, not so much related to adenotonsillar disease as related to excessive body fat.
3) *The syndromic phenotype*, which includes micro-retrognathia associated with genetic syndromes such as Pierre Robin's, or patients with true macroglossia of the tongue, common in syndromes such as Down's.[10,11]

The incidence of OSAS in pediatric age is higher in children between two and six years, in association with the presence of adenoids and hypertrophic tonsils and in adolescents, with close relation to weight gain.[3] These two periods coincide with those in which the orthodontists intervene on the young patients to carry out first interceptive therapy and then therapy with fixed appliances. The orthodontists are a key figure in the diagnosis and early treatment of OSAS, as they able to identify and intervene directly on some anatomic components involved in the pathophysiology of this respiratory disorder, such as a narrow palate and mandibular retrusion.[12-14]
OSAS are a relatively frequent condition and knowing how to recognize symptoms in addition to encouraging early detection, reduces the risk of , significant complications.[15,17]

Every orthodontist should introduce during the first visit a questionnaire validated for pediatric OSAS, to be filled out by parents, and create a team of experts with whom to interface for the care management of each case, including the evaluation of the lingual frenum.[18,19]

Children suspected of having OSAS should be sent to perform a nocturnal polysomnography (PSG), the gold standard for the diagnosis of this condition.[20]
The therapeutic solutions to pediatric OSAS require a multidisciplinary approach: the main therapeutic tools are represented by pharmacological options, surgery (usually adenotonsillectomy), orthodontics, and myofunctional /speech therapy.
With regards to orthodontic therapy, many studies have reported that use of the rapid palatal expansion (RPE), in growing patients with transverse contraction of the maxilla, leads to an increase in the total volume of the upper airways and therefore, of the nasal airflow.[21]
RPE devices are able to reduce AHI in children with OSAS by providing an appropriate therapeutic alternative.[22]
Although Nasal Continuous Positive Airway Pressure (CPAP) devices can offer good results in subjects with MetS and concurrent OSAS, given its effectiveness in reducing individual pathophysiological alterations of OSAS even in young children, it is not recommended as a first therapeutic choice due to the significant compliance it requires.
The management of metabolic syndromes encourages the reduction of excess weight based on behavioral changes on both eating habits and physical

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activity in order to achieve a balance between caloric consumption and intake, thus reducing fat mass and keeping/increasing muscular mass.

For behavioral therapy to be successful, families must be involved and be supportive as much as possible. In the event of unsuccessful outcomes of the behavioral approach, in children or adolescents with severe obesity and associated co-morbidity, pharmacological solutions or bariatric surgery should be considered.[23-25]

The following Case Report highlighted that, in the case of this particular child suffering from OSAS, the improvement of the respiratory system facilitated the resolution of most of the concurrent metabolic alterations, suggesting a possible causal and reciprocal interconnection between the two pathologies.

In this regard, there are many studies that suggest the relationship between non optimal respiration and consequent adaptations of metabolic processes, such as those involving secretion of insulin and the regulation of glucose.[26-27]

REPORT OF A CASE

Information about the patient A.P. she was born on July 2008 by preterm birth at 32 + 4 weeks. Her weight at birth was 1.9 kg (4.2 lbs) and since birth she presented high bilirubin, muscular hypotonia, difficulty in sucking and feeding, poor reactivity to stimuli and the absence of crying ("silent newborn").

Around the first month of life, she started to exhibit respiratory difficulties due to bronchiolitis, bronchospasm, rhinitis with fever, airways obstructions caused by milk and mucus requiring pediatric suctioning. A.P. received antihistamines, cortisone, antibiotics and bronchodilators. Her initial nutrition and hydration involved the use of a syringe, receiving donkey's milk and fruit compote during her second years of life to facilitate swallowing and weight gain. However, her growth in the first year was still below the reference percentiles. (Saccomanno & Paskay, 2021) She endured numerous hospitalizations and ER visits due to viral and bacterial infections, fever, breathing and eating problems. She continued to exhibit a widespread developmental delay and slow growth throughout her second year of life. A polysomnography revealed that she was suffering from a mild- moderate OSA as well. She would get tired easily while chewing so she ate only soft foods.

Between age 3 to 4, A.P. increased her weight and improved her respiratory pattern but by then she presented with an ogival palate and a persistent facial dysmorphism. She was evaluated by an endocrinologist, a cardiologist, a hematologist, an orthopedist, an ENT and a neurologist. The findings confirmed neuromuscular and fine motor control delay; a chronic adenotonsillar hypertrophy and inflammation requiring tonsillectomy/ adenoidectomy (TA) and remodeling of the lower turbinate at age 4; a high-positioned diaphragm that restricted deep breathing; metabolic syndrome with dyslipidemia, increased level of platelets and hypothyroidism; a distended, globular abdomen; marked valgus knees ("knock-knees") and postural lordosis.

At age 5, A.P. was diagnosed with bilateral transmissive hearing loss, with bilateral endotympanic effusion. At age 6, her polysomnography indicated oxygen desaturation within normal limits but she suffered breathing crisis nevertheless. Her first palatal expansion did not significantly improve her breathing nor her metabolic situation.

At age 7 she was fitted with hearing aids. However, her transmissive hearing loss exceeded 60% and the hearing aids did not provide a meaningful audiological gain. She continued to have significant breathing issues for which treatments were only symptomatic. Her body weight and BMI increased above normal range for her age, even on a 1000 kcal/day diet. Her sleep apnea episodes increased with the increasing of her weight, with sleep frequently interrupted by apnea episodes, without relief from nebulizers, cortisone sprays and bronchodilators. Myofunctional therapy was attempted for one month, but suspended as her respiratory and audiological status became critical.

By age 8, A.P. was experiencing headaches, exhaustion, chronic fatigue, light sensitivity, poor academic performance, overall apathy and intolerance for crowded or noisy places. However, she received a medical advice inviting her to play sports or physical activities and she chose Latin American dance attending the activity biweekly.

By age 10 her breathing issues were unmanageable, the dysfunction at the level of the middle ear was markedly worse with recurrent infections, she presented an atypical swallowing, a class II malocclusion in mixed dentition, an upper maxillary contraction and an open bite.

She was 10 years of age when she was enrolled in a multidisciplinary plan, with RPE and myofunctional therapy twice a week.



Figure 1. Extraoral photos of the face and the profile at age 8.

Part 1. Summary of A.P.'s medical history and main complaints or reasons for consultation

At the time of the first visit in our office, (Sept 2016), the patient was in treatment with an ENT for respiratory problems and wore hearing aids due to bilateral transmissive hypoacusia.

—The *symptoms reported by parents were:* Night snoring, chronic fatigue, apathy, headache, poor school performance, hypersensitivity to light, noise and crowded environments.

—The *OSAS symptoms complained by the patient were:* Sleep interrupted and daytime fatigue.

—A *subjective clinical evaluation* (questionnaire) was filled in by A.P.'s parents, the result of which confirmed the pathological findings of her sleep disorders.

Part 2. Summary of relevant and recent medical history

- *Drug therapy:* The patient has been receiving therapy with cortisone, bronchodilators and broad-spectrum antibiotics for problems such as bronchiolitis, bronchospasm and upper airway infections, occurring frequently since her fifteenth day of life.
- *Comorbidities:* The patient has other coexisting conditions such as: dyslipidemia, childhood obesity and transmissive deafness.
- *Predisposing factors:* Prematurity (32 + 4 weeks); an ogival palate; mouth breathing; Being overweight, with a BMI (Body Mass Index) of 26.74.
- *Further screening and diagnosis.* Polysomnographic findings indicated obstructive sleep apnoea (OSAS) with mild AHI index for pediatric age. Apneas and hypopneas associated with phasic hemoglobin desaturations were noted. Some central apnoea episodes were also reported.

Diagnostic Evaluation before treatment

Table 1. AHI: Apnea/hypopnea index (No. events / hour), with oxygen saturation well below average.

Diagnostic parameters	Values
AHI	4,6 events/h
AHI supine	4,1 events/h
ODI	14,0
Nadir O2	80%
Medium SaO2	66%
CT 95	0.30%

Diagnostic Evaluation after treatment

Table 2. AHI: Apnea/hypopnea index (No. events / hour), with oxygen saturation well below the average.

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- Clinical evaluation - anatomical, functional and aesthetic findings
- Type of respiration: mostly oral;
- Type of swallowing: dysfunctional with anterior lingual thrust;
- Face and profile analysis: oval - shaped face and short neck-chin distance;
- The lip seal is present but the perioral muscles are hypofunctioning;
- The palatine vault: It has a transverse restriction "ogival" morphology;
- Tongue: mildly hypotonic within a reduced intraoral space
- Tonsils and adenoids: surgically removed (in 2010 and 2014)
- Mild overall muscular hypotonia

Radiographic evaluation - dental and orthodontic
Orthopantomography. Permanent teething. Presence of all third molar gems (1.8-2.8-3.8-4.8). Second molars present (1.7-2.7-3.7-4.7) but not yet erupted in the arches.



Figure 2. Orthopantomography

-ray latero-lateral findings. Skeletal class II.. Hypodevelopment of the mandible and of the maxillary complex. Hyperdivergent skeletal growth. Restricted airway, low lingual posture.



Figure 3. Lateral X-ray teleradiography and cephalometric analysis



Figure 4. Lateral X-ray cephalometric analysis

Objective clinical examination of the malocclusion. The observation of the upper arch confirmed the transversal contraction of the palatine vault, the presence of an interincisive diastema, the persistence of a deciduous right canine (5.3) while the presence of crowding was noticed in the lower arch. The assessment of the occlusal arches identified a normal overjet (2 mm), a normal overbite (1 mm), the presence of a class II dental ratio and a bilateral open bite, possibly facilitated on the right by the lack of exfoliation of 5.3 and on the left side by an atypical swallowing.

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Treatment plan

The treatment plan comprised several stages:

- Rapid expansion of the palate associated with myofunctional therapy to expand the palate: a RPE device was used and followed this protocol: one activation per day (¼ turn/day) for one month (0.2 mm each activation, 30 days). At the same time, A.P. began myofunctional therapy, lasting 4 months.
- Myofunctional therapy associated with fixed orthodontic therapy, following the expansion of the palate. The myofunctional therapy was reprogrammed to adapt to the new intraoral environment with daily exercises to home and once a week with a speech therapist.
- Behavioral therapy. Changes in lifestyle were implemented, interesting both eating habits and physical activity and emotional support.

Clinical Procedures

*Instructions on device and complication management: t*he parents were informed about the difficulty of swallowing in the first days of application, difficulty in speech articulation, feeling of tension at the level of the teeth, possible headache, nasal discharge (rhinorrhagia), and interincisal diastema.

Instructions on precautions to be taken: parents were told how to activate the device; how to perform an accurate daily hygiene using pipe cleaners and hydropulsator Waterpick®); brushing her teeth after every meal; foods to avoid (filamentous or excessively crunchy foods).

Orthodontic follow-up: a checkup every week for a month, then once monthly.

Parents' report on symptoms: after fifteen days of RPE activation, the parents reported that their girl was more rested and did not snore anymore.

Girl's report on her symptoms: at each activation she reported having leaked cerumen from the ears, nasal discharge and bruises under the nose and on the cheeks.

As mentioned, the first 2 weeks of therapy were characterized by reported dental pain and at the level of the base of the nose, initial difficulty of chewing and opening of the interincisive diastema. However, after the fifteen days of activation, she reported improvement in chewing and swallowing, with transition from a liquid and semi-solid diet to integration of all food textures.

At the end of the palatal expansion and after a cycle of myofunctional therapy, the following objectives were achieved: reaching a correct, physiological morphology of the palate and habitual nasal breathing, bilateral alternating chewing and the integration of all food consistencies into the diet, including foods of solid consistency.

Follow-up of *PSG/PG with diagnosis from a sleep doctor.* Snoring phases and sporadic breathing breaks were observed within normal limits.

DISCUSSION

Recent data show that OSA is associated with a pattern of metabolic changes, similar to that of obesity, due to the production of oxygen free radicals (Reactive Oxygen Species - ROS), caused by hypoxemia. That would increase the deleterious effects of systemic inflammation on adipose tissue and metabolic components associated with vascular disease and diabetes, thus exacerbating the metabolic complications related to obesity.

Children with OSAS have higher levels of blood pressure, reactive C protein, insulinemia, as well as a higher prevalence of left ventricle hypertrophy, demonstrating that OSAS considerably increase the risk of developing severe chronic cardiovascular and metabolic complications. In pediatric patients with OSAS and concomitant MetS, early diagnosis and intervention ensure that these serious consequences are limited.

The following case study wanted to demonstrate the key role played by the recovery of the respiratory, swallowing and chewing functions to restore the proper metabolic balance in a growing patient.

To summarize the therapeutic plan, it was carried out in several stages and included the participation of numerous professional figures. During the first phase, therapy included both the expansion of the palate thanks to the use of a RPE device that allowed to obtain a significant) transverse increase of 6 mm, and the myofunctional re-education in order to restore the neurophysiological nasal respiration, encourage bilateral alternating chewing, and the intake of regular solid foods, thus replacing the girl's exclusive nutrition with liquid and semi-solid foods consistency. As a result of the expansion of the palate, the patient was able to remove hearing aids, confirming the findings of some studies which indicate that restoring the correct size of the upper jaw allows an

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improvement of transmissive hearing loss in patients.During the second phase of the therapy plan, the girl continued myofunctional re-education to eliminate dysfunctional swallowing and then began fixed orthodontic therapy to correct her class II.

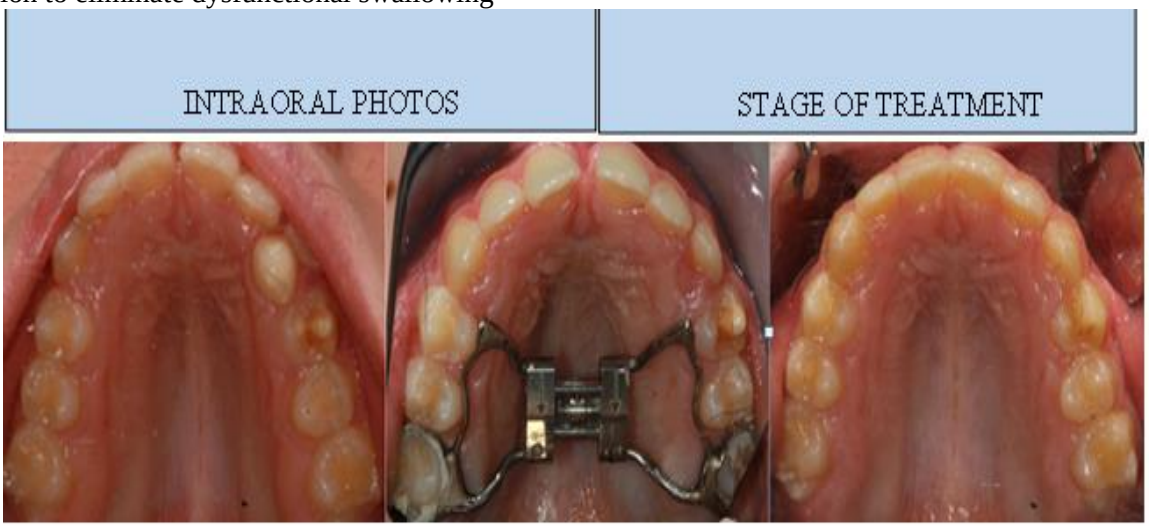


Figure 5a Before the treatment Intraoral occlusal photo of the upper arch prior to palatal expansion,note the presence of ogival type palate (2018)

Figure 5b During treatment Occlusal intraoral photo of the upper arch with R.E.P mounted

Figure 5c After treatment Occlusal intraoral photo of the upper arch following the palatal expansion

The other important therapeutic strategy was the increase in energy expenditure: a regular and aerobic physical activity favored the reduction of insulin resistance and fat mass, in particular abdominal fat, as well as decreasing pressure values.
However, further studies are needed to define the real impact of the correction of chronic hypoxemia on metabolic parameters.



Figure 6a,b. Extraoral and intraoral photos (2016), after failed palatal expansion and surgical therapy.



Figure 7 a,b,c,d. Extraoral and intraoral frontal photographies. Evolution over time from 2018 to 2019.



Figure 8a,b. Extraoral and intraoral photos (2019*),* post expansion and myofunctional therapy

FOLLOW-UP AND RESULTS

The benefits of multidisciplinary treatment gradually emerged in the treatment of A.P. Her respiratory appearance and efficiency improved; blood count stabilized (the hematocrit went from 43.3 to 41.9; the platelets from 508 to 426; the white blood cells from 25.73 to 7.23; the neutrophil granulocytes from 21.81 to 4.48; and the monocytes from 1.34 to 0.55). (Table 3).

Her weight decreased by 6 kg, her waist circumference decreased by 7 cm, and, according to her mother, she grew considerably in height. Her hearing aids were removed following the last audiological evaluation in which her

auditory threshold was optimal.
The quality of her sleep improved and a polysomnography performed after treatment documented the resolution of sleep apneas.
The girl was able to stop taking the medications related to the upper airways issues right after the first phase of palatal expansion and myofunctional therapy.
Fixed orthodontics continued as the third phase of therapy.

Table 3. Parameters

Parameters	Pre-treatment	Post-treatment
Body weight	• Overweight (BMI increased: 26,74)	• Loss of 6 kg • Reduction of waist circumference of 17 cm • Reported Considerable growth in height
Audiometric examination	• Bilateral medium-severe transmissive hypoacusia (requiring hearing aids)	• Auditory threshold within normal limits (removal of hearing aids)
Polysomnography (PSG)	• Obstructive sleep apnea (OSAs) with mild AHI for pediatric age	• Improvement of sleep quality reportedly
Blood tests	• Altered blood values • hematocrit 43.3 • platelets 508 • white blood cells 25.73 neutrophil granulocytes 21.81 • monocytes 1.34	• Normal blood values • hematocrit 41.9 • platelets 426 • white blood cells 7.23 • neutrophil granulocytes 4.48 • monocytes 0.55
Psychophysical ability	• Chronic asthenia, apathy, cefalea, poor school performance, • hypersensitivity to light, noise and crowded environments.	• Improvement of mood, school performance and physical activities. Reportedly more sociable.
Palatal transverse width	• Ogival palate	• Transverse increase of the palate 6 mm
Respiration	• Prevalent oral respiration	• Exclusive nasal respiration
Swallowing and chewing	• Dysfunctional swallowing with anterior lingual thrust associated with masticatory difficulty (prevalent intake of liquid or semi-liquid food)	• Correct and functional swallowing and chewing (food intake of any consistency)

CONCLUSIONS

The following case study wanted to demonstrate the key role played by the recovery of the respiratory, swallowing and chewing functions, along with the structural changes brought by orthodontics, to restore the proper metabolic balance in a growing patient. The integrated multidisciplinary treatment produced health benefits for A.P., who in the past already underwent a palatal expansion in two stages without significant results, as the altered orofacial functions were not corrected, along with the structural changes. Thanks to this multidisciplinary treatment, the girl re-learned to breathe exclusively with her nose and to chew her foods with all textures, allowing an improvement and optimization in orofacial and oropharyngeal muscle tone and mobility.

The new integrated program of palatal expansion and myofunctional therapy, building on top of the intervention of other professionals (neurologist, orthopedist, internist etc.) and encouraging a meaningful physical activity (in the case of this patient she learned to love dancing), made it possible to act synergistically on both orofacial structures and functions as well as positively impacting the whole body.[28-31]

DECLARATIONS

Funding: This research received no external funding.

Ethical Approval

Institutional Review Board Statement: The study was conducted at one clinical center in conformity with the Good Clinical Practice Guidelines, following the recommendations of the World Medical Association Declaration of Helsinki ethical principles for medical research involving human subjects, as revised in Fortaleza (2013). The patient included in this work was treated in a private dental practice with written consensus.

Conflicts of Interest: The authors declare no conflicts of interest.

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RESEARCH ARTICLE

EFFECT OF BRUSHING SIMULATION ON THE COLOUR STABILITY OF PROVISIONAL FIXED PROSTHODONTIC MATERIALS – AN IN VITRO STUDY

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ABSTRACT

Background: In dentistry, provisional fixed prosthodontic materials are essential because they act as temporary restorations that preserve prepared teeth, retain their aesthetic appeal, and ensure their proper function until the final prosthesis is made. Colour stability refers to the ability of a material to maintain its original colour over time, despite exposure to various environmental factors. The aim of this study is to assess how brushing simulation affects the colour stability of two types of provisional fixed prosthodontic materials.

Materials and Methods: Ten disc-shaped specimens (10 mm diameter, 2 mm height) from Ivoclar and AVUE T Crown (five each) were prepared to evaluate colour stability. Using a brushing simulator (ZM3.8 SD Mechatronik), samples underwent 10,000 brushing cycles (2 hours) with Dabar Red Herbal toothpaste. Colour measurements were taken before and after brushing with a Konica Minolta CM5 spectrophotometer, and Delta E values were calculated. Paired t-tests in SPSS version 23 compared pre- and post-brushing colour stability, with results tabulated.

Results: The study evaluated the colour stability (ΔE) of Ivoclar and AVUE T Crown provisional materials under simulated brushing. Ivoclar samples had minimal colour change (average ΔE 0.716), indicating good stability. AVUE samples showed significant colour shifts (average ΔE 6.498). The statistically significant p-value of 0.04 highlights the impact of brushing on colour stability, stressing the need for durable materials in routine oral hygiene.
Conclusion: This study demonstrates that Ivoclar materials exhibit superior colour stability compared to AVUE materials, maintaining clinically acceptable ΔE values under simulated brushing conditions. These findings underscore the importance of selecting materials that resist colour change, ensuring aesthetic integrity and patient satisfaction in temporary dental restorations.

Keywords: Provisional Fixed Prosthodontics, Colour Stability, Brushing Simulator, Spectrophotometer, Aesthetic Dental Applications

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INTRODUCTION

One of the primary objectives of dental care is to replicate teeth and create smiles that are as aesthetically pleasing and natural-looking as possible, while also meeting each patient's unique demands. Over the past ten years, there have been substantial advancements in new and targeted treatment modalities, consistently improved and more aesthetically pleasing dental materials, and innovative methods and technologies.[1,2] Esthetics play a major role in the social and mental well-being of individuals. Dental esthetics can be in any form, such as replacing missing anterior tooth/teeth, correcting the space between the teeth, correcting dental protrusion or retrusion, smile correction, and bleaching discoloured tooth/teeth.[3]

In restorative dentistry, provisional fixed prosthodontic materials are essential because they act as temporary restorations that preserve prepared teeth, retain their aesthetic appeal, and ensure their proper function until the final prosthesis is made. These materials, which are usually used to create crowns or bridges, must match patients' aesthetic needs in terms of strength, biocompatibility, and most importantly colour stability. Provisional restorations play a critical role in the success of restorative treatment.[4,5] Provisional restorations have three main purposes: to protect the pulp and periodontal tissues from mechanical, chemical, and thermal stresses; to ease the patient's discomfort; and to facilitate speech and mastication. These materials need to have sufficient mechanical and physical qualities to endure the functional requirements of the oral environment, considering their transient nature. But the colour stability of the temporary restoration is a crucial factor that affects both the patient's satisfaction and its effectiveness.[4]

Colour stability refers to the ability of a material to maintain its original colour over time, despite exposure to various environmental factors. When it comes to provisional restorations, colour stability is essential for patient confidence and pleasure, particularly when these materials are utilised in parts of the mouth that are visible.[6] Long-term treatment-related discolouration of temporary crowns and bridges may cause patient discontent and increase replacement costs.Therefore, color stability is quite important when selecting temporary materials, especially in the creative fields. The degree of discolouration may be influenced by a variety of factors, including inadequate polymerization, water sorption, chemical reactivity, the restoration's surface roughness, diet, and dental care.[7]

Tooth brushing, a regular oral hygiene practice, is a key factor affecting the colour stability of provisional materials. The mechanical impact and chemical exposure from toothpaste during tooth brushing can affect the surface characteristics and colour of dental materials.[8] Comprehending the impact of simulated tooth brushing on the colour stability of provisional materials is crucial for forecasting their real-world performance and helping choose the best materials for temporary restorations. While several components of provisional materials have been studied in the past, more thorough research is still needed to determine how tooth brushing affects the materials' capacity to maintain colour stability.[9]

A toothbrush simulator is a mechanical simulator capable of running a programmable three-dimensional brushing pattern.[10] The effectiveness of a toothbrush, the flexibility, abrasion resistance, and colour stability of prosthetics and restorative materials can all be tested with it.[11] This toothbrushing simulator aids in the investigation of several characteristics, such as the substrate's assistance in the operation, the effects of various movement types, and the motions of various toothbrush types.[12,13] With the help of this brushing simulator equipment, one may also study the effects of varying pressure and overbrushing. Since the bristles are always in contact with the tooth surface, this simulated toothbrush approach, helps to assess the gloss, roughness, and even colour change of the teeth.[13]

This study will utilise standardised colour measurement techniques and brushing simulation protocols to assess changes in colour stability. The findings will provide valuable insights into the durability and aesthetic longevity of these materials, guiding clinical practices and material development for enhanced patient outcomes.[14] By addressing the effects of brushing on provisional fixed prosthodontic materials, this research seeks to contribute to the broader understanding of material performance in dynamic oral environments, ultimately supporting the advancement of dental restorative procedures and patient care.[15,16] The aim of this study is to assess how brushing simulation affects the colour stability of two types of provisional fixed prosthodontic materials. Dental practitioners can choose materials for temporary restorations more intelligently and provide patients with better-looking

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results by knowing which materials show the least colour change under simulated brushing conditions.

MATERIALS AND METHODS

Sample preparation: Fixed prosthodontic materials from two brands, Ivoclar and AVUE T Crown, were evaluated. A total of 10 samples were assessed, with each brand providing five disc-shaped specimens. These specimens measured 10 mm in diameter and 2 mm in height. The assessment aimed to compare the colour stability of the materials from the two brands under standardised conditions.

Brushing Simulator: Ten-disc shaped samples were placed in a brushing simulator (ZM3.8 SD Mechatronik). The Samples are subjected to 2 hours of brushing with Dabar Red Herbal toothpaste which is equal to around one year of brushing and to around 10000 cycles in total among which 5000 cycles were performed in the linear X axis, 5000 cycles in the linear Y axis. For long term evaluation of variations in colour stability of Ivoclar and AVUE T Crown provisional fixed Prosthodontics materials the brushing simulation was done for 10000 cycles (Figure 1 & 2).



Figure 1 and 2. Samples are positioned in the brushing simulator

Colour Stability Assessment: Using a Konica Minolta CM5 spectrophotometer (Figure 3), the colour stability was assessed. It was carried out twice, once before and once after brushing simulation. Prior to assessing the colour stability, distilled water was used to rinse the discs. The pre and post data for L, A, and B values came from the spectrophotometer. Lastly, the spectrometer's software was used to compute Delta E values.



Figure 3. demonstrates how to measure colour stability values with a spectrophotometer.

Statistical Analysis: The colour stability values were measured both before and after the brushing simulation was run. The values were tabulated, and using the tabulated values, a descriptive analysis known as the "paired t test" was run using the statistical program "SPSS version 23." The analysis outcome was presented in tabular form.

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RESULTS

The study assesses the effect of brushing simulation on the colour stability of two brands of provisional fixed prosthodontic materials, Ivoclar and AVUE, by comparing their colour parameters (L*, a*, b*) before and after the simulation. For Ivoclar (Samples 1-5), the changes in L* (lightness), a* (red-green), and b* (yellow-blue) values before and after the brushing simulation were minimal. For instance, Sample 1 showed a slight increase in L* from 54.20 to 55.04, a small decrease in a* from 1.15 to 1.10, and a minor shift in b* from -0.45 to -0.32, resulting in a ΔE of 0.85. Similarly, other Ivoclar samples exhibited small shifts in these values, leading to ΔE values all below 1.43.

This indicates that Ivoclar materials maintain their colour well under brushing conditions, demonstrating high colour stability. In contrast, AVUE (Samples 6-10) showed more significant changes in L*, a*, and b* values. Sample 6, for example, had a notable decrease in L* from 82.56 to 73.89, an increase in a* from 0.14 to 2.37, and a significant change in b* from 3.22 to 6.64, resulting in a high ΔE of 9.58. Similar substantial changes were observed in Samples 7 and 8, with ΔE values of 10.40 and 8.26, respectively. These large shifts in L*, a*, and b* values indicate considerable colour change and poor stability under brushing simulation for AVUE materials (Table 1).

Table 1: L*, a*, and b* of two brands of temporary fixed prosthodontic materials—AVUE and Ivoclar—as well as ΔE values are noted

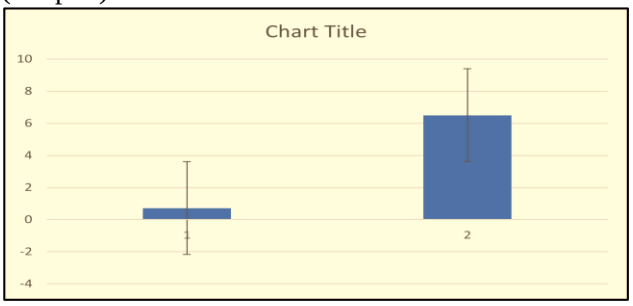
Sample	Pre			Post			ΔE
	L*	a*	b*	L*	a*	b*	
1	54.20	1.15	-0.45	55.04	1.10	-0.32	0.85
2	54.14	1.10	-0.34	54.30	1.05	-0.40	0.17
3	59.47	1.18	0.53	58.99	1.20	0.66	0.49
4	54.04	1.14	-0.40	54.67	1.05	-0.36	0.64
5	62.30	1.41	1.89	63.72	1.22	2.01	1.43
6	82.56	0.14	3.22	73.89	2.37	6.64	9.58
7	82.72	-0.40	2.48	74.29	2.80	7.66	10.40
8	76.42	0.23	2.59	68.22	0.79	1.78	8.26
9	74.02	0.30	2.37	74.72	0.29	2.25	0.71
10	68.23	1.03	1.89	71.70	0.36	2.07	3.54

Overall, Ivoclar samples exhibited minor variations in colour parameters, resulting in lower ΔE values and better colour stability (Table 2).

Table 2. Colour Stability (ΔE) of Ivoclar and AVUE Materials

Sample	Ivoclar	AVUE
1	0.85	9.58
2	0.17	10.4
3	0.49	8.26
4	0.64	0.71
5	1.43	3.54
Average	0.716	6.498
Standard deviation	0.469	4.185

In contrast, AVUE samples showed larger changes in L*, a*, and b* values, leading to higher ΔE values and indicating less color stability. This suggests that Ivoclar materials are more reliable for maintaining color integrity in dental applications compared to AVUE materials. A thorough analysis of the colour changes (ΔE) seen in samples from each brand is shown in the table and graph that are displayed. The ΔE values for five samples from each brand are listed in the table. The ΔE values for Ivoclar are 0.85, 0.17, 0.49, 0.64, and 1.43, with a standard deviation of 0.469 and an average of 0.716. These are relatively low values. According to these findings, Ivoclar materials show good colour stability and few colour changes following brushing simulation. On the other hand, the AVUE samples, with an average of 6.498 and a standard deviation of 4.185, display much larger ΔE values: 9.58, 10.4, 8.26, 0.71, and 3.54. These numbers point to poor colour constancy in the majority of AVUE samples, suggesting a significant colour shift. In contrast, Sample 9 of AVUE displays a ΔE of 0.71, which is comparable to the Ivoclar samples; nonetheless, this is an anomaly when compared to the other high ΔE values. The average ΔE values and standard deviations between the two brands are graphically compared in the bar graph (Graph1).



Graph 1: Colour stability is assessed bycomparing the values of ΔE

With a smaller error bar and a substantially lower average ΔE, Ivoclar exhibits more consistent performance with less variability among samples. On the other hand, AVUE exhibits a significantly higher average ΔE along with a bigger error bar, indicating increased variability and less consistent colour stability. The p-value of 0.04 indicates statistical significance, as it is less than the conventional threshold of 0.05. This suggests that the effect of brushing simulation on the colour stability of provisional fixed prosthodontic materials is not due to random chance. The results imply that brushing significantly affects the color stability of these materials, highlighting the importance of selecting materials that can withstand routine oral hygiene practices. This finding is crucial for both clinicians and patients, as it emphasises the need for materials that maintain their aesthetic appearance over time under typical usage conditions.

DISCUSSION

Color stability is important when selecting dental materials, particularly in areas that are visually sensitive.[17] The oral cavity's many environmental conditions can have an impact on the color stability of temporary fixed prosthodontic materials over time. The color stability of these materials can be affected by brushing, a common oral hygiene procedure. A color difference (ΔE) larger than 3.5 is typically regarded as visible to the human eye in the CIE Lab* color space. Maintaining the aesthetic appearance of dental materials is facilitated by a ΔE value below 3.5, which means that any color shift is neither

clinically significant nor visible to the unaided eye.[18] The diverse ways that brushing affects different materials imply that each material's unique makeup and characteristics affect how susceptible it is to color change. Because of their distinct surface qualities, chemical composition, and structural traits, many materials may respond to brushing in different ways.[19,20]

In contrast to other brands, the Ivoclar group had ΔE values that were within the clinically acceptable range of < 3.3, showing superior color stability when exposed to colorant solutions.[21,22]

The varying effects of brushing on different materials suggest that the susceptibility to colour change is influenced by the specific composition and properties of each material. A study also highlights significant colour stability differences among Ivoclar materials due to intrinsic discoloration, extrinsic staining, and material-specific traits.[23]

Crown discoloration can be a serious problem. These the temporary crown materials nevertheless tend to absorb liquids, which can lead to stains and color changes

Patient happiness and the perceived caliber of dental care are impacted by this absorption from food and drink, which also degrades the restoration's appearance. Because temporary restorations are frequently utilized while permanent solutions are being considered, their visual durability is essential. Ongoing research in material science aims to improve colour stability, ensuring that provisional restorations meet the aesthetic and functional demands of both patients and practitioners.[24] Balancing aesthetic requirements with performance remains a key focus in dental material development.[25] Sustainability is therefore an important factor to consider when choosing a temporary material to employ in visually important locations.[23]

A previous study examined the colour stability of twelve provisional materials, including methacrylates and bis-acryl resins. Specimens were stored in artificial saliva or a coffee-saliva solution, with colour measured at baseline, 1, 2, and 4 weeks. Results showed significant colour changes influenced by the storage solution (P<.0001). Coffee caused notable colour change in Luxatemp, Protemp, and Temphase at 4 weeks. Provipont had the greatest colour change (ΔE=9.40 coffee; 8.51 saliva), while Zeta CC had the least (ΔE=0.31 coffee; 0.23 saliva).[26] Another Previous research has demonstrated that differences in colour stability among Ivoclar materials are influenced by intrinsic discoloration, extrinsic staining, and material-specific characteristics. These findings underscore the importance of understanding these factors for clinicians to make informed decisions regarding the selection and longevity of Ivoclar restorative materials in aesthetic dental treatments.[7] Moreover, studies have shown that provisional prosthetic materials exhibit variable colour stability under different oral conditions, with factors such as the illuminant and the environment playing a significant role.[27] In a study evaluating the colour stability of LuxaCrown, Protemp4, and heat-cure PMMA, 40 specimens of each material were divided into four groups and stored in three staining solutions and artificial saliva. Using a spectrophotometer, color measurements were made at baseline and after one day, one week, one month, three months, and six months. Results indicated that heat-cure PMMA maintained the best colour stability across all time intervals compared to Protemp4 and LuxaCrown.[6] In the present study Ivoclar's lower and more consistent ΔE values indicate that its materials maintain their colour better, making them more suitable for applications where aesthetic consistency is crucial. The minimal changes in L*, a*, and b* values for Ivoclar samples suggest high resistance to colour alterations due to brushing, an important factor for dental applications where visual appearance is essential. In contrast, AVUE materials displayed larger changes in colour parameters, resulting in higher ΔE values and indicating lower colour stability. The significant shifts in L*, a*, and b* values for AVUE samples, with ΔE values exceeding the clinically acceptable range, highlight their susceptibility to colour change under brushing conditions.

Furthermore, surface glaze treatments greatly enhanced smoothness and decreased stains, highlighting the function of surface treatments in reducing color deterioration.[28] PMMA demonstrated better polishability than bis-acryl resins, with noticeably reduced roughness values after polishing, according to a study on mechanical polishing procedures.[29] Bis-acryl resins were found to be more prone to discolouration than PMMA in staining solution studies, especially when immersed in coffee.[30] The advantages of surface sealants for color stability were further supported by the decrease in ΔE values caused by the application of glaze coatings.[30] Laboratory settings, however, might not accurately mimic oral situations in the real world.[26]

The study's simulated circumstances could not accurately reflect oral environments in real life since different materials can react differently to brushing. While variations in toothpaste formulas, including abrasives and chemical compositions, may also have an impact on the outcomes, inconsistencies may originate from material variability.[31,32] Moreover, it is possible that the standardized values employed may not fully represent the variety of brushing methods and applied pressures found in the literature.[33,34] Future studies could examine personal brushing behaviors for more individualized insights and improve realism by modeling various environmental elements including humidity, temperature, and food effects. The use of advanced measurement tools to detect color shifts could yield more precise data. Additionally, studying the combined effects of brushing, eating, and other mechanical stressors could provide a comprehensive understanding of the degradation of tooth material. The findings will be applicable if results are confirmed by real clinical trials. By guiding the development of innovative materials with enhanced color stability, this research can ultimately aid in the improvement of dental care products. In conclusion, the results clearly show that Ivoclar materials outperform AVUE materials in terms of colour stability after brushing simulation. Ivoclar's lower and more consistent ΔE values indicate that its materials maintain their colour better, making them more suitable for applications where aesthetics is crucial.

CONCLUSION

The colour stability of provisional fixed prosthodontic materials is crucial for clinical success. This study shows that Ivoclar materials have superior colour stability compared to AVUE materials, maintaining ΔE values within the clinically acceptable range. This indicates their suitability for aesthetic dental applications, where appearance is vital. Understanding the factors that influence colour changes, such as material composition and resistance to staining, is essential for selecting materials that uphold aesthetic integrity over time. In conclusion, selecting materials with proven colour stability can enhance the overall success of dental treatments, meeting both functional and aesthetic demands. Continued research and material advancements are key to achieving these goals.

DECLARATIONS

Conflicts of interest and financial disclosures

The authors declare no conflict of interest and there was no external source of funding

Ethical Approval None

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RESARCH ARTICALE

RARE EARTH ELEMENT-DOPED BIOGLASS FOR PERIODONTAL REGENERATION; AN IN VITRO INVESTIGATION OF BIOCOMPATIBILITY AND REGENERATIVE POTENTIAL

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ABSTRACT

Background: Periodontitis is a chronic inflammatory disease that causes progressive destruction of periodontal structures, including the alveolar bone, cementum, and periodontal ligament. Restoring these tissues remains a key challenge in periodontal therapy. Bioactive glass has shown potential for periodontal regeneration due to its osteoinductive and osteoconductive properties. Recently, ion doping has been explored to enhance its regenerative effects, with erbium-doped bioglass (Er-BG) gaining attention for promoting osteogenic differentiation. This study evaluates the physicochemical properties, biocompatibility, and regenerative potential of Er-BG for periodontal tissue repair.

Material and Methodology: Er-BG was synthesized using the sol-gel method. Structural and compositional characterization was performed using Scanning Electron Microscopy (SEM), Energy Dispersive X-ray Spectroscopy (EDS), X-ray Diffraction (XRD), and Attenuated Total Reflectance Infrared Spectroscopy (ATR-IR). Biocompatibility and osteogenic potential were assessed using human periodontal ligament stem cells (PDLSCs) through MTT, Alkaline Phosphatase (ALP), collagen estimation, and calcium deposition assays. Zebrafish embryo models were used for toxicity evaluation.

Results: SEM revealed a porous surface facilitating cell attachment. EDS confirmed erbium integration, while XRD indicated an amorphous structure. Er-BG enhanced ALP activity (~130%), collagen synthesis (~105%), and calcium deposition (~145%). Zebrafish studies confirmed minimal cytotoxicity.

Conclusion:Er-BG demonstrated excellent biocompatibility, osteogenic potential, and low toxicity, making it a promising candidate for periodontal regeneration. Further in vivo studies are needed.

Keywords: **Erbium-doped bioglass, periodontal regeneration, osteogenesis, biocompatibility, bioactive glass, tissue engineering.**

INTRODUCTION

Periodontitis, a chronic inflammatory disease affecting the supporting structures of the teeth, is a major cause of tooth loss in adults. Effective treatment requires not only controlling infection but also promoting the regeneration of damaged periodontal tissues, including the alveolar bone, cementum, and periodontal ligament.[1]Various biomaterials have been explored for their potential to enhance periodontal regeneration. Among these, bioactive glasses (BGs), particularly those designed to stimulate bone regeneration, have shown great promise due to their ability to induce osteogenesis, enhance tissue regeneration, and support periodontal cell growth.[2]

The principal anatomical consequence of periodontitis is the loss of alveolar bone support. The extent and severity of periodontal osseous lesions are typically assessed through clinical and radiographic methods.[1,2] Periodontal defects are categorized as suprabony (horizontal), infrabony (vertical), and interradicular (furcation) defects. According to Goldman (1958)[2], suprabony defects have a pocket base coronal to the alveolar crest, whereas infrabony defects extend apically beyond the bone crest. Intrabony defects, which affect a single tooth root surface, and crater defects, which involve adjacent root surfaces, are classified based on remaining bony walls as 1-wall, 2-wall, or 3-wall defects.[3-5] Achieving complete regeneration of infrabony defects remains a significant clinical challenge.

Autografts are considered the gold standard for bone and periodontal regeneration due to their osteogenic, osteoconductive, and osteoinductive properties. However, drawbacks such as limited availability, donor site morbidity, and extended operative time have led to the development of bone tissue engineering strategies. Despite advancements, achieving complete periodontal regeneration remains unpredictable.[6-8] Among various biomaterials, BGs have attracted attention for their ability to form a highly reactive carbonate hydroxyapatite layer.[9]

BGs are bioactive materials composed of silicon dioxide, sodium oxide, calcium oxide, and phosphorus pentoxide. First introduced by L. L. Hench in 1969, BGs revolutionized biomaterials by providing bonding interfaces with both bone and soft tissues [9]. The original 45S5 Bioglass formulation (45% SiO2, 24.5% CaO, 24.5% Na2O, and 6% P2O5) was the first synthetic material to bond with bone. A significant effect of BG is the release of biologically active silicon (Si^{4+}) and calcium (Ca^{2+}) ions, which stimulate osteoblast proliferation and bone growth around the implant interface. Additionally, BG 45S5 enhances vascular endothelial growth factor (VEGF) secretion, promoting angiogenesis in vitro and vascularization in vivo.[10,11]

Bioglasses have gained significant attention in tissue engineering due to their osteoinductive and osteoconductive effects, making them suitable for regenerating hard and soft tissues. Different types, including calcium, silicon, borate, and phosphate-based BGs, have been explored for tissue repair.[12] Though brittle, bioglasses facilitate hydroxyapatite formation. Recent research focuses on doping BG with various ions to enhance its surface properties and regenerative potential. Ions such as calcium, strontium, silica, silver, and lithium have been studied for their osteogenic effects.[13]

Erbium-doped bioglass (Er-BG) has recently gained interest for periodontal applications. Erbium ions improve bioactivity through antimicrobial and anti-inflammatory properties, which are beneficial for periodontal treatment.[14] Erbium ions can reduce bacterial colonization, inflammation, and enhance osteoblast proliferation and differentiation,[15] Additionally, Er^{3+} exhibits luminescent properties in the near-infrared (1.54 µm) and visible regions, enabling bioimaging and real-time tracking of biomaterial interactions with tissues, which aids in monitoring osteogenesis.[16] These combined properties make Er-BG a promising candidate for periodontal regeneration.

This study aims to evaluate the efficacy of erbium-doped bioglass as a biomaterial for periodontal regeneration. The material was characterized using SEM, ATR-IR, and XRD analyses. Its regenerative potential was assessed by measuring alkaline phosphatase activity, collagen content, and calcium deposition. The biocompatibility of Er-BG was evaluated through an MTT assay, while toxicity assessments were conducted using zebrafish models to ensure its safety.

MATERIALS AND METHODOLOGY

Material Characterization

- SEM Analysis: Surface morphology and porosity were examined using field emission SEM at varying magnifications (500×–50,000×).
- EDS Analysis: Elemental composition and dopant distribution were assessed to confirm rare-earth incorporation.

- XRD Analysis: Crystalline and amorphous phases were identified to determine structural stability.
- ATR-IR Spectroscopy: Functional groups and bioactive features of the doped bioglass were analyzed.

Biocompatibility and Regenerative Potential

- MTT Assay: Cell viability was assessed on human periodontal ligament stem cells (PDLSCs) cultured with different bioglass concentrations (12.5–100 µg/mL).
- Alkaline Phosphatase (ALP) Activity: Osteogenic differentiation was evaluated through ALP expression.
- Collagen Estimation: Extracellular matrix deposition was analyzed via Sirius Red staining.
- Calcium Content Analysis: Alizarin Red S staining was used to measure mineralization levels.

Toxicity Assessment

- Zebrafish Embryo Assay: The in vitro toxicity of the bioglass was examined by monitoring embryo development over 120 hours. Cell viability, hatching rates, and morphological abnormalities were recorded.

RESULTS

1. Surface Morphology and Structural Analysis

Scanning Electron Microscopy (SEM) Analysis
The SEM images of erbium-doped bioglass (Er-BG) revealed a highly porous, rough, and irregular surface texture with a well-interconnected network. The pore sizes varied between 1 to 5 µm, which facilitates cell attachment, proliferation, and nutrient exchange, essential for bone regeneration. Additionally, small spherical aggregates (1–3 µm) were observed, likely formed due to erbium doping affecting the sintering process. These aggregates contribute to increased surface roughness, which may enhance osteoconductivity, providing a favorable environment for bone cell attachment and differentiation (Figure 1).

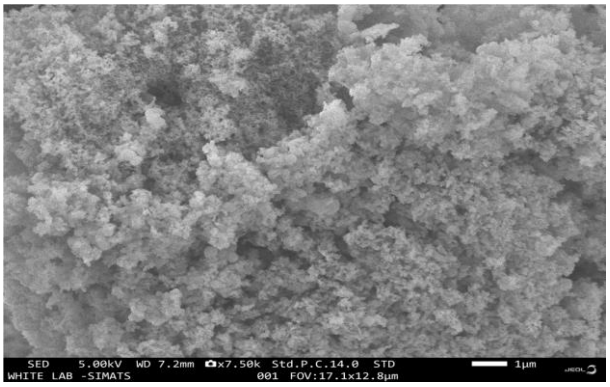


Figure 1. Represents the SEM of prepared erbium doped bioglass

Energy Dispersive X-ray Spectroscopy (EDS) Analysis

The EDS analysis confirmed the successful incorporation of erbium (Er) into the bioglass matrix, along with silicon (Si), calcium (Ca), and phosphorus (P), which are crucial for bioactivity. The homogeneous distribution of erbium ions ensures uniformity within the material, supporting its potential for periodontal and orthopedic applications. The elemental composition analysis further validates that erbium doping did not alter the fundamental bioglass composition, but rather enhanced its bioactivity (Figure 2).

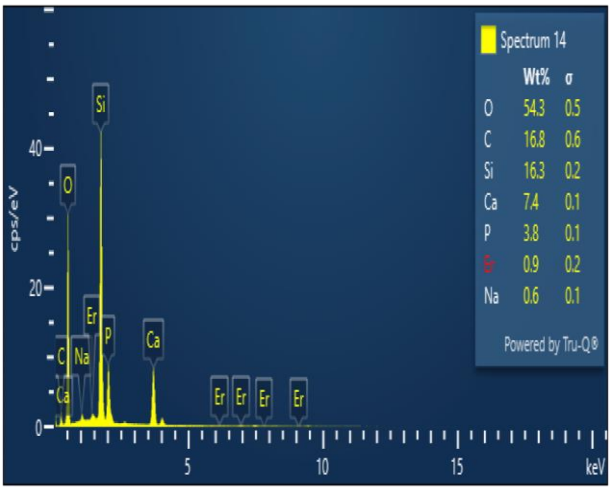


Figure 2. Represents the presence of various elements using the EDS analysis

Attenuated Total Reflectance Infrared Spectroscopy (ATR-IR) Analysis

The ATR-IR spectra confirmed the presence of key functional groups associated with bioglass bioactivity. A strong Si-O-Si stretching vibration was observed at 1035 cm^{-1}, indicating the formation of a stable silicate network. Additionally, phosphate (P-O)

stretching bands at 565.59 cm^{-1} and 603.23 cm^{-1} confirmed the presence of phosphate groups essential for hydroxyapatite formation. Slight shifts in peak intensities were noted due to erbium incorporation, suggesting that Er-doping may influence the structural properties of the bioglass matrix (Figure 3).

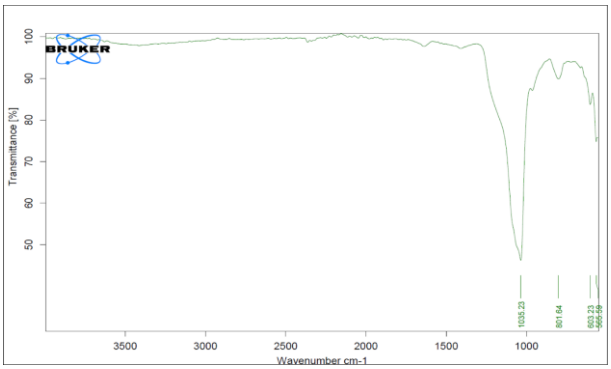


Figure 3. Represents the ATR-IR of prepared erbium doped bioglass

X-ray Diffraction (XRD) Analysis

The XRD pattern exhibited a broad diffraction hump between 20°–35° 2θ, characteristic of an amorphous bioglass structure. However, minor crystalline peaks were observed around 30° and 50°, indicating partial crystallization due to erbium doping. The predominantly amorphous nature of Er-BG ensures excellent bioactivity and ion exchange capability, both of which are critical for bone tissue regeneration (Figure 4).

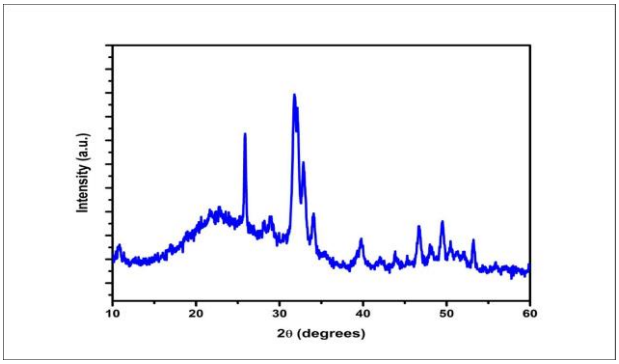


Figure 4. Represents the XRD of prepared erbium doped bioglass

Biocompatibility and Osteogenic Potential

Cell Viability (MTT Assay)

The biocompatibility of Er-BG was assessed through the MTT assay, which demonstrated high cell viability (>90%) across all tested concentrations (12.5–100 µg/mL). The control group consistently exhibited slightly higher viability than the Er-doped bioglass samples, but no significant cytotoxic effects were observed. These results indicate that Er-BG supports cellular function and proliferation, making it a promising biomaterial for bone regeneration (Figure 5).

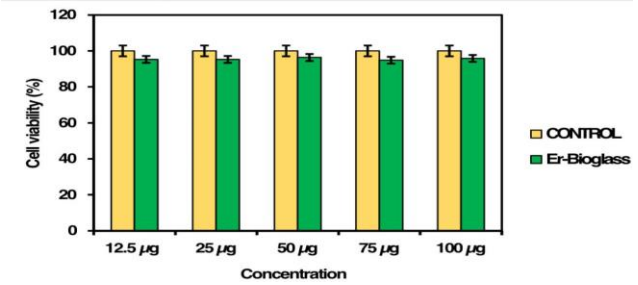


Figure 5. This graph represents the percentage of cell viability in different concentrations of control and Erbium doped bioglass.

Collagen Content Estimation

Collagen synthesis, an essential component of the extracellular matrix and bone maturation, was analyzed in the control and Er-BG-treated groups. The Er-doped bioglass samples exhibited an approximate 105% increase in collagen content compared to the control group, suggesting that erbium incorporation plays a role in promoting collagen production. The presence of an enriched collagen matrix further supports bone formation and tissue regeneration, reinforcing Er-BG's potential as a scaffold material (Figure 6).

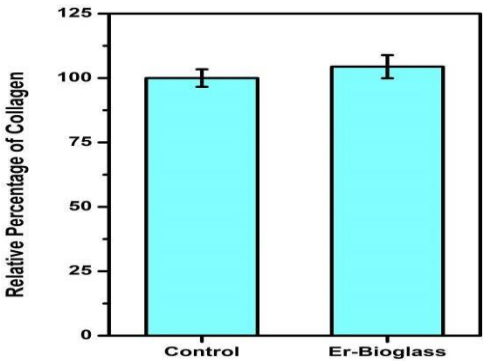


Figure 6. This graph represents the relative percentage of Collagen in control and Erbium doped bioglass

Alkaline Phosphatase (ALP) Activity

Alkaline phosphatase (ALP) is a key osteogenic marker that signifies early osteoblast differentiation. The Er-BG samples demonstrated a 130% increase in ALP activity compared to the control group, confirming its enhanced osteoinductive potential. The increased ALP expression indicates that Er-BG facilitates bone matrix mineralization and osteoblast maturation, which are essential for effective bone regeneration (Figure 7).

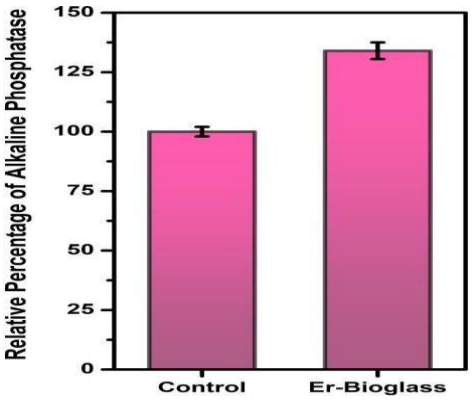


Figure 7. This graph represents the relative percentage of Alkaline Phosphatase in control and Erbium doped bioglass

Calcium Deposition (Mineralization)

Calcium deposition is a crucial indicator of bone tissue mineralization and scaffold integration. The results showed a 145% increase in calcium content in the Er-BG samples, compared to the control. This significant enhancement suggests that Er-doped bioglass promotes greater mineralization, reinforcing its role in bone remodeling and repair (Figure 8).

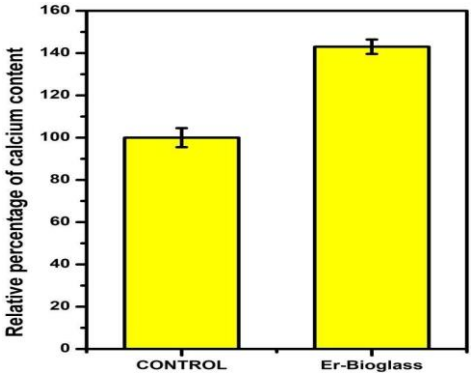


Figure 8. This graph represents the percentage of relative percentage of calcium content in control and Erbium doped bioglass

Cytotoxicity and In Vitro Toxicity Assessment

Zebrafish Embryo Toxicity Test

The potential toxicity of Er-BG was evaluated using zebrafish embryos, which were monitored at different developmental stages (24, 48, 72, 96, and 120 hours post-fertilization). The embryos exposed to Er-doped bioglass exhibited high survival rates, minimal developmental abnormalities, and normal anatomical structure formation. However, a slight delay in hatching was observed at higher concentrations, though no significant increase in mortality was noted. These findings suggest that Er-BG has a strong safety profile and does not exert significant teratogenic effects, supporting its use in biomedical applications (Figure 9).

Cytotoxicity Analysis

Cytotoxicity analysis was conducted in accordance with ISO 10993-5 standards for biomaterials. The assessment showed that cell viability remained above 80% across all tested concentrations, confirming the non-toxic nature of Er-BG. While a slight reduction in viability was noted over time, the values remained well within the non-toxic threshold, further reinforcing its excellent biocompatibility and suitability for tissue engineering applications (Figure 10).

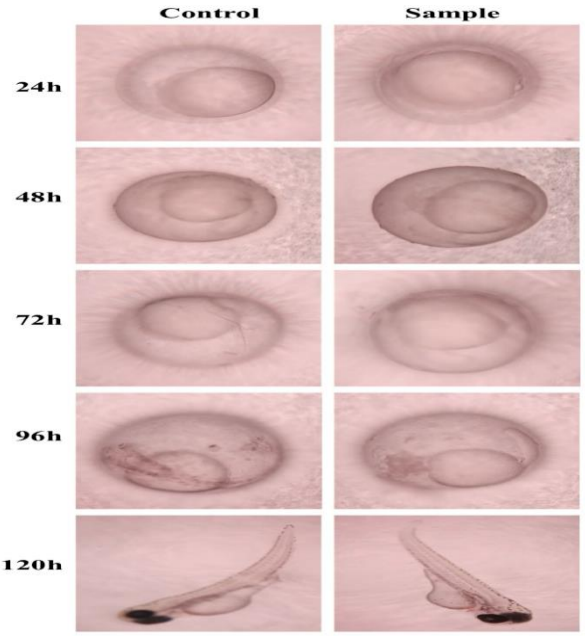


Figure 9. Represent that bioglass doped with erbium exposed eggs and matured embryos at 24,48,72,96, 120 hpf

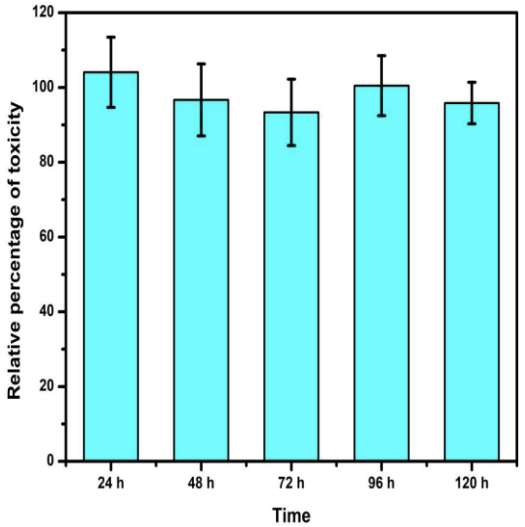


Figure 10. Represent the relative percentage of toxicity

DISCUSSION

The development of bioactive materials for bone regeneration has gained significant attention in periodontal and orthopedic research. Among these materials, bioglass has demonstrated promising potential due to its ability to stimulate osteogenesis, promote vascularization, and facilitate bone repair. Recent advancements in ion-doped bioglasses have further improved their regenerative potential, making them an ideal candidate for tissue engineering applications. In this study, erbium-doped bioglass (Er-BG) was investigated for its structural, biological, and osteogenic properties and the findings highlight the morphological, compositional, and biological advantages of Er-BG, positioning it as a superior biomaterial for periodontal applications.

The morphological and compositional characteristics of bioglass play a vital role in determining its bioactivity and regenerative properties. In this study, SEM analysis of Er-BG revealed a highly porous, rough, and interconnected surface, which is beneficial for cell adhesion, proliferation, and osteogenesis. When compared to previously studied bioglasses (BGp, BG85Sp, and BG45Sp), some key differences were observed. While BGp and BG45Sp displayed irregularly shaped particles with rough surfaces, similar to Er-BG, BG85Sp showed a higher tendency for particle agglomeration, which may limit cellular infiltration and nutrient diffusion.[17-19] In contrast, Er-BG maintained a more uniform porosity with well-defined structural integrity, allowing better vascularization and tissue integration. These morphological advantages position Er-BG as a more osteoconductive and bioactive scaffold for bone regeneration applications. Further elemental analysis (EDX) confirmed the successful incorporation of erbium in Er-BG, alongside essential components such as silicon (Si), calcium (Ca), and phosphorus (P), which are critical for bioactivity. Unlike commercially available BG45Sp, which contained high sodium levels (Na_2O: 25%), Er-BG lacked sodium, likely leading to a more controlled degradation rate and reduced cytotoxicity.

Studies on Ion modified-BG have demonstrated that low crystallization tendencies and controlled dissolution profiles contribute to better bioactivity and osteogenesis. Incorporating ions such as erbium into Ion modified bioglass could further enhance its therapeutic effects, making Er-BG a promising alternative to conventional bioglasses. Additionally, Boron (B)-doped bioglasses have been investigated for their osteogenic and angiogenic potential, but their bioactivity appears to be highly dose-dependent, with high concentrations leading to cytotoxicity. Comparatively, Er-BG maintained a stable biocompatibility profile, with high cell viability and minimal toxicity, suggesting a wider therapeutic window for clinical applications.[17-19]

The biocompatibility of Er-BG was validated through MTT assays, which demonstrated >90% cell viability across all tested concentrations. These findings align with previous bioglass studies, such as BG1 and BG2, which were well tolerated by periodontal cells without reducing viability below toxicity thresholds. While bioglass toxicity is often observed at higher concentrations (>2 µg/mL), Er-BG maintained high cell viability even at elevated concentrations, reinforcing its suitability for biomedical applications. [19] In comparison, B-doped BGs displayed variable cytotoxic effects, with high boron ion concentrations leading to a significant drop in cell viability over time. A previous study indicated that borate ion concentrations above 2.5 mM (27.03 mg/L ionized B) resulted in a 50% reduction in cell proliferation, suggesting a narrow therapeutic window for B-doped bioglasses. In contrast, erbium doping did not show any dose-dependent cytotoxic effects, making Er-BG a safer alternative.

The osteogenic potential of Er-BG was further supported by enhanced ALP activity (~130%) and increased calcium deposition (~145%), surpassing the osteogenic response observed in undoped BGs. While B-doped BGs demonstrated a positive impact on osteogenic differentiation, their effectiveness was limited at higher concentrations, potentially due to cytotoxic effects. In previous studies, B-doped BGs enhanced osteogenic marker gene expression (OPN, OCN, and BMP-2), but these effects were only slightly greater than those observed in undoped BGs. In contrast, Er-BG consistently outperformed undoped BGs in terms of ALP activity and mineralization, suggesting that erbium plays a more stable role in promoting osteoblast differentiation and bone formation.[19-20] Additionally, B-doped BGs exhibited limited pro-angiogenic effects in vitro, whereas Er-BG's unique microstructure could further support vascularization in vivo.

Another crucial factor for bone regeneration is angiogenesis, which plays a fundamental role in bone repair and tissue remodeling. Studies on B-doped bioglasses have shown that low to moderate B-ion release can positively influence angiogenesis, whereas high boron concentrations may negatively impact endothelial cell viability. In contrast, Er-BG demonstrated excellent biocompatibility and sustained osteogenic properties without cytotoxic effects, making it a more reliable candidate for long-term applications. Furthermore, studies using vascular endothelial growth factor (VEGF) assays have suggested that boron-doped BGs only exhibit a limited impact on angiogenesis, requiring additional modifications to enhance vascularization.[21]

Meanwhile, Er-BG may naturally promote better vascularization due to its biocompatible and osteoinductive properties, which warrant further investigation in in vivo models.

Future research should focus on evaluating the long-term stability, degradation kinetics, and mechanical strength of Er-BG in vivo. Additionally, investigating the combined effects of erbium with other bioactive ions (e.g., strontium or magnesium) could further enhance its osteogenic and angiogenic potential. The integration of advanced biomaterial models, such as 3D cell cultures, vasculature-on-a-chip, and bioreactors, may provide deeper insights into the angiogenic-osteogenic coupling of Er-BG in complex biological environments. With continued research and clinical validation, Er-BG could revolutionize periodontal and orthopedic regenerative therapies, offering a safer and more effective alternative to currently available bioglasses.

CONCLUSION

Erbium Doped Bioglass (Er-BG) exhibits high porosity, reduced agglomeration, and enhanced osteogenic potential, making it a promising biomaterial for periodontal regeneration. Its stable biocompatibility, increased ALP activity, and higher calcium deposition support efficient bone formation and tissue integration. Additionally, Er-BG demonstrated minimal cytotoxicity and sustained bioactivity, ensuring safe and effective application in regenerative dentistry. With its ability to promote osteogenesis without dose-dependent toxicity, Er-BG holds significant potential for bone tissue engineering and clinical applications, offering a biocompatible and osteoinductive scaffold for enhanced periodontal and orthopedic regeneration.

DECLARATIONS

Conflicts of Interest

The authors declare no conflicts of interest.

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Ethical Approval

Not Applicable

Informed Consent

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Acknowledgments

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RESEARCH ARTICLE

WOUND HEALING EVALUATION OF NOVEL CISSUS QUADRANGULARIS, BIOCERAMICS AND TENDON EXTRACELLULAR MATRIX INCORPORATED SCAFFOLDS FOR PERIODONTAL BONE REGENERATION WITH ZEBRAFISH MODELS

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ABSTRACT

Background: Wound healing is a complex biological process involving, proliferation, and tissue remodelling, requiring advanced biomaterials to facilitate effective tissue regeneration. This study evaluated the wound healing potential of composite scaffolds incorporating *Cissus quadrangularis* extract, carrageenan, tendon extracellular matrix (ECM) with bioceramics such as silver hydroxyapatite and silver tricalcium phosphate using a zebrafish model.

Materials and Methods: The scaffolds were extensively characterized using Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), and X- ray diffraction (XRD) to determine their physiochemical properties and their correlation with biological efficacy. The wild- type zebrafish were wounded and treated as following negative control (Group 1), *Cissus quadrangularis* extract, carrageenan, tendon extracellular matrix (TEM) (Group 2), Group 3 (contained silver hydroxyapatite + Group 2 components), Group 4 (Silver tricalcium phosphate + Group 2 components) respectively for 21 days. The wound closure ratio was assessed on 0, 7 and 14 days with histological observations.

Results: As the results, SEM analysis revealed a highly porous architecture with interconnected pores, enhancing cellular adhesion, proliferation, and migration at the wound site. The surface roughness provided an optimal microenvironment for fibroblast infiltration and re-epithelization, accelerating the healing process. FTIR analysis confirmed the presence of hydroxyl, carboxyl, amide, and phosphate groups indicating strong biomaterial interactions, hydration, and bioactivity. The observed phosphate peaks validated the incorporation of carrageenan, essential for calcium ion release and osteogenic differentiation. XRD patterns exhibited well- defined crystalline peaks corresponding to hydroxyapatite and bioactive glass, ensuring stability, controlled degradation, and prolonged bioactivity at the wound site. Zebrafish wound healing assays demonstrated significant improvements in wound closure, reduced inflammation, and enhanced tissue remodelling in scaffold treated groups. The histology observations revealed that the Group 4 treatment induced rapid epidermal formation, immune cell activity, and vascularization. Faster wound contraction and new scale formation were observed 4 to 21 days post injury, highlighting the scaffold's effectiveness compared to the slower recovery in untreated controls. The combined effects provided a synergistic approach for effective wound healing and tissue regeneration.

Conclusion: The study demonstrated that the application of silver tricalcium phosphate, *C. quadrangularis*, and TEM effectively promoted wound healing. Future research should focus on optimizing scaffold composition and validating efficacy in mammalian models for translational applications.

Keywords: zebrafish, wound healing, tissue regeneration, extra cellular matrix

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INTRODUCTION

Periodontal bone regeneration remains a significant challenge in the field of regenerative medicine and dentistry. The periodontal disease, which affects the supporting structures of teeth, can lead to severe bone loss and compromise oral function. Current treatment strategies include mechanical debridement, guided tissue regeneration (GTR), bone grafting, and the use of biologically active molecules[1]. However, these approaches have limitations, including inadequate integrations, infection risk, and limited regenerative capacity. Therefore, the development of novel biomaterials and scaffold- based therapies with enhanced regenerative potentials are critical for advancing the periodontal bone regeneration[2]. Tissue engineering has emerged as a promising solution for periodontal regeneration, integrating biomaterials, cells, and bioactive molecules to restore the lost tissue. Among various scaffold materials, natural plant extracts, carrageenan, and extracellular matrix (ECM) based components have gained attention due to their biocompatibility, bioactivity and ability to mimic the native periodontal environment. This study explores a novel scaffold incorporating plant extracts, carrageenan, bioceramics and tendon extracellular matrix (TEM) for periodontal bone regeneration[3,4]. Furthermore, the zebrafish model was employed to evaluate the wound healing and regenerative efficacy of these scaffolds, providing valuable insights into their biological performance.

Plant derived bioactive compounds have been widely studied for their anti- inflammatory, antimicrobial, antioxidant and osteogenic properties. Several polyphenols, flavonoids, alkaloids, and terpenoids have demonstrated potential in promoting osteogenesis and reducing oxidative stress, thereby facilitating periodontal tissue healing[5]. In other hand, carrageenan, including, hydroxyapatite (HA), beta-tricalcium phosphate (β- TCP), and bioactive glass, are widely used in bone regeneration due to their osteoconductive and osteoinductive properties. These materials closely resemble the mineral composition of native bones, facilitating cellular attachment, proliferation, and differentiation[6,7]. Hydroxyapatite, a calcium phosphate based carrageenan, has excellent bioactivity and integration with surrounding bone tissue. It provides a suitable framework for osteoblast adhesion and extracellular responses and stimulate bone tissue formation. The combination of these carrageenan with plant derived bioactive compounds in scaffold design can create a synergistic effect, promoting periodontal regeneration while maintaining structural integrity[8]. Extracellular matrix components play a crucial role in tissue development, regeneration and repair. TEM derived scaffolds are particularly advantageous due to their rich composition of collagen, glycosaminoglycans (GAGs), and growth factors, which support cell adhesion and differentiation[9,10]. Decellularized TEM provides an ideal microenvironment for periodontal ligament regeneration by retaining essential biological cues while eliminating immunogenic responses. The incorporation of TEM in scaffold based therapies aims to mimic the native ECM environment, allowing for better integration and functional recovery periodontal tissues. TEM based biomaterials have been reported to enhance cellular migration, differentiation, and matrix remodelling, making them a valuable component in periodontal bone regeneration[11].

The zebrafish (*Danio rerio*) model has emerged as a powerful tool studying wound healing and bone regeneration due to its remarkable regenerative capacity, genetic similarity to humans, and rapid tissue repair mechanisms[12]. Unlike mammals, zebrafish can efficiently regenerate lost tissues, including bone, skin, and muscle, making them an ideal model for evaluating scaffold efficacy. The objective of the study is to evaluate the wound healing and bone regeneration potential of novel scaffolds incorporating plant extracts, carrageenan, and tendon extracellular matrix using a zebrafish model. Key aims include synthesizing and characterising these scaffolds, assessing their biocompatibility and cytotoxicity *in vitro*, and analysing their regenerative effects in zebrafish through wound healing activity and histopathology studies[13,14]. This research aimed to develop bioactive, cost- effective, and clinically viable scaffolds for periodontal bone regeneration and tissue engineering applications.

MATERIALS AND METHODS

Fresh *Cissus quadrangularis* stems were collected, washed and shade dried. The dried stems were powdered and subjected to Soxhlet extraction, using ethanol (95%) for 48 hours. The extraction was filtered and concentrated using a rotary evaporator at 40°C under reduced pressure. The obtained crude extract was stored at -20°C until further use.

2.1 Synthesis of bioceramics

Bioceramic materials such as silver hydroxyapatite (HA) and silver Tricalcium phosphate (TCP) were synthesized using the sol- gel method. For HA, calcium, nitrate and ammonium phosphate were used as precursors and mixed in a 1.67:1 molar ratio. The pH was maintained at 10 using ammonia, followed by aging for 24h and calcination at 800°C.

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TCP was synthesized using a silica gel method, incorporating calcium and phosphate precursors[15].

2.2 Extraction and processing of Tendon ECM (TEM)

Porcine/ Achile tendon was decellularized using a detergent- based method (1% SDS and 01% Triton X-100) followed by washing with PBS. The extracellular matrix (TEM) was lyophized, ground into the powder, and reconstituted into a hydrogel[16].

2.3 Scaffold fabrication

Scaffold were fabricated by incorporating *Cissus quadrangularis* extract, carrageenan, and tendon ECM hydrogel using the electrospinning technique. Polycaprolactone (PCL) was used as a base polymer to provide- mechanical stability[17]. A 10% w/w solution of PCL was mixed with varying ratios of carrageenan, HA and TCP. TEM and plant extract followed by electrospinning under optimized conditions (voltage: 15 kv, flow rate 1.2 mL/ h). The scaffold were crosslinked using glutaraldehyde vapors and stored in a desiccator until further use. Scaffolds were divided into four groups which were as follows Group 1 was negative control – PERIO COL, Group 2 was *Cissus quadrangularis* extract, carrageenan, tendon extracellular matrix, Group 3 contained silver hydroxyapatite, *Cissus quadrangularis* extract, carrageenan and tendon extracellular matrix , Group 4 contained Silver tricalcium phosphate, *Cissus quadrangularis* extract, carrageenan and tendon extracellular matrix[16].

2.4 Characterization Techniques

The morphological characteristics and size distribution were analysed using Scanning electron Microscopy (SEM), (JEOL JSM-IT800) method. A small quantity of the dried samples was mounted onto an aluminium stub using carbon tape. To enhance conductivity and prevent charging effects, the samples was coated with thin layer of gold using a sputter coater. Imaging was performed at an accelerating voltage with multiple magnifications to assess samples shape, size uniformity and dispersion. Fourier Transform Infrared Spectroscopy (FTIR) (PerkinElmer, USA) analysis was performed to identify functional groups and confirm interactions between the scaffold components. Spectra were recorded in the range of 4000- 400 cm^{-1}. X- ray Diffraction (XRD) (Bruker D8 Advance) analysis was performed to determine the crystallinity of the carrageenan[18].

2.5 Zebrafish husbandry and wound healing evaluation

All the experimental protocols involving zebrafish were approved by the Institutional Animal Ethical Committee, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS) (Approval No.: BRULAC/SDCH/SIMATS/IAEC/06-2023/15). The experiments were conducted in compliance with laboratory animal care guidelines.Wild- type zebrafish (*Danio rerio*) were maintained under standard conditions (28°C, 14:10 light- dark cycle) in a recirculating aquaculture system. They were anaesthetized priorly to the experiment with 0.016% tricaine methansulfonate. A standardized full thickness (2mm) wound was created on the lateral flank of each fish using a sterile scalpel. The wound area was treated with scaffold suspension in PBS (5 mg/ mL) for 7 days. Scaffolds were divided into four groups which were as follows Group 1 was negative control – PERIO COL, Group 2 was *Cissus quadrangularis* extract, carrageenan, tendon extracellular matrix, Group 3 contained silver hydroxyapatite, *Cissus quadrangularis* extract, carrageenan and tendon extracellular matrix, Group 4 contained Silver tricalcium phosphate, *Cissus quadrangularis* extract, carrageenan and tendon extracellular matrix, these scaffolds were respectively treated for 21 days. Wound closure and tissue regeneration were monitored at 0, 7,14 and 21 days, post wounding using bright field microscope. Wound healing was quantified by measuring the wound area using ImageJ software[12,19].

2.6 Histological analysis

Wounded skin tissues were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. Hematoxylin and eosin (H&E) staining was performed to evaluate tissue regeneration.

2.7 Statistical analysis

All experiments were performed in triplicates. Data were analysed using Graphpad Prism software, and statistical significance was determined using one-way ANOVA with post hoc Tukey's test ($p < 0.05$ was considered significant).

3. RESULTS

3.1 scanning electron microscopy (SEM) Analysis

The scanning electron microscopy (SEM) images of scaffold incorporating *C. quadrangularis* carrageenan, and TEM (*Figure 1*) revealed predominantly spherical shapes with notable irregularities, suggesting successful incorporation in samples. The size distribution appeared relatively uniform across the sample. And the particles were well dispersed, with minimal signs of aggregation, indicating a stable colloidal formulation. Further characterization was conducted using various spectroscopic techniques.

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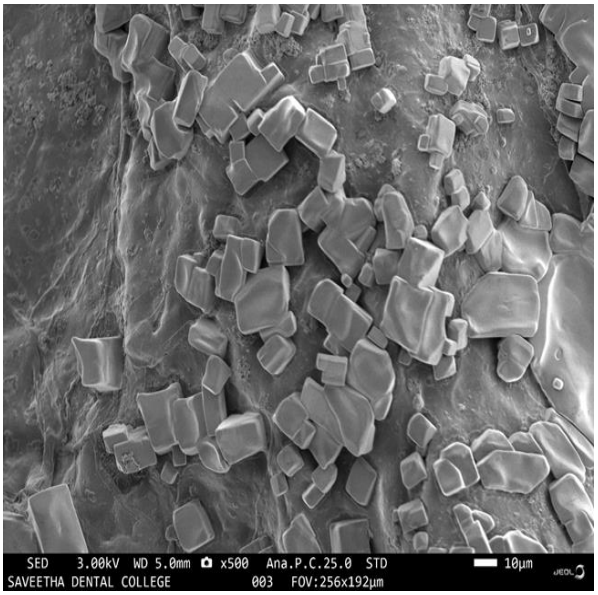


Figure 1. scanning electron microscopy (SEM) images of scaffold incorporating *C. quadrangularis* carrageenan, and TEM

3.2 Fourier Transform Infrared (FTIR) spectroscopy analysis

Fourier Transform Infrared (FTIR) spectroscopy was performed to identify the functional groups present in the samples, with characteristic absorption peaks observed on Group 1, 2, 3 and 4 respectively (*Figure 2*). These peaks correspond to various modes, confirming the presence of different functional groups in the sample. The broad peak at 3362.8 cm^{-1}, is indicative of O-H stretching vibrations, which suggests the presence of hydroxyl groups (-OH), possibly from water molecules or hydroxyl containing compounds such as alcohols or phenols (presented on CQ+CAR+TEM+AG HAP and CQ+CAR+TEM+AG TCP respectively). The peaks at 2883.6 cm^{-1} and 2740.6 cm^{-1} corresponded to C-H stretching vibrations of aliphatic and aldehydic groups, respectively, suggesting the presence of alkane or aldehyde functionalities (on negative control and CQ+CAR+TEM+ AG HAP). The peak at 1575.3 cm^{-1} was assigned to C=C stretching vibrations, which were characteristic of aromatic rings or conjugated systems (on Negative control and CQ+CAR+TEM+ AG HAP). The band at 1466.2 cm^{-1} was attributed to C-N stretching, indicated the presence of amines (CQ+CAR+ECM+ AG HAP, CQ+CAR+ TEM+ AG HAP and negative control). Peaks at 1280.6 cm^{-1} and 1240.4 cm^{-1} corresponded to C-O stretching vibrations, characteristic of esters, carboxyl groups, or ether linkages, which could be associated with polysaccharides or biomolecules. The peaks observed at 1145 $cm^{-1,}$ [1105] cm^{-1}, and 1059.8 cm^{-1} were typically associated with C-O-C stretching vibrations found in carbohydrates or ether bonds, confirming the presence of polysaccharide like- structures. The peaks at 959.44 cm^{-1} and 924.32 cm^{-1} suggested C-H bending vibrations in aromatic compounds or unsaturated hydrocarbons. Further, the peak at 841.53 cm^{-1} might be linked to out-of-plane bending vibrations of aromatic C-H bonds, whereas the absorption at 698.54 cm^{-1} was characteristic of C-Cl stretching vibrations, indicating the possible presence of organochlorine compounds. The peaks a 560.56 cm^{-1} and 442.65 cm^{-1} might correspond to metal-oxygen stretching vibrations (except on negative control), suggested the presence of metal oxide bonds, likely from nanoparticles or inorganic structures.

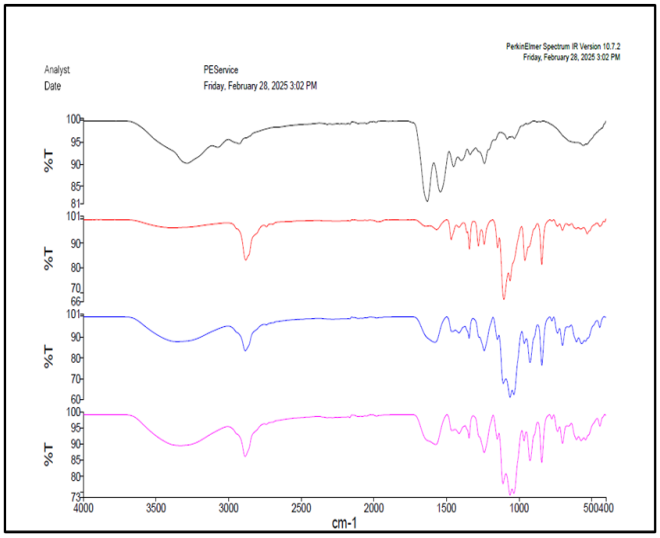


Figure 2. Fourier transform infrared spectroscopy (FTIR) spectra of Perio COL- Control (No 1 , Black color), *Cissus quadrangularis* extract, carrageenan, tendon extracellular matrix (TEM) (No 2 , Red color), No. 3 Blue color, (contained silver hydroxyapatite, *Cissus quadrangularis* extract (CQ), carrageenan (CAR), tendon extracellular matrix (TEM), (No 4 , Purple color) (Silver tricalcium (Ag TCP), *Cissus quadrangularis* extract, carrageenan, tendon extracellular matrix (TEM) respectively.

3.3 X- Ray diffraction (XRD) analysis

XRD analysis performed to determine the crystallographic structure, phase composition, and degree of crystallinity of the synthesised scaffold. The XRD (*Figure 3*) pattern of the scaffold exhibited characteristic peaks corresponding to bioceramic phases, particularly, hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP), which were essential for osteogenic properties. Prominent diffraction peaks were observed at 2θ values of 25.8°, 31.7°, 32.9° and 39.5°, confirming the presence of crystalline HA.

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The peaks at 29.5° and 34.1° were attributed to β- TCP, indicating its integration within the scaffold.
The presence of these carrageenan phases suggested that the scaffold maintains structural integrity. The peaks at 20°- 30° might exhibit the presence of polyphenols from the plant extract. 15° and 25° corresponded to the amorphous components such as collagenous and glycosaminoglycan-rich structures, *viz.*, tendon extracellular matrix. The XRD analysis showed the successful synthesis of the membrane.

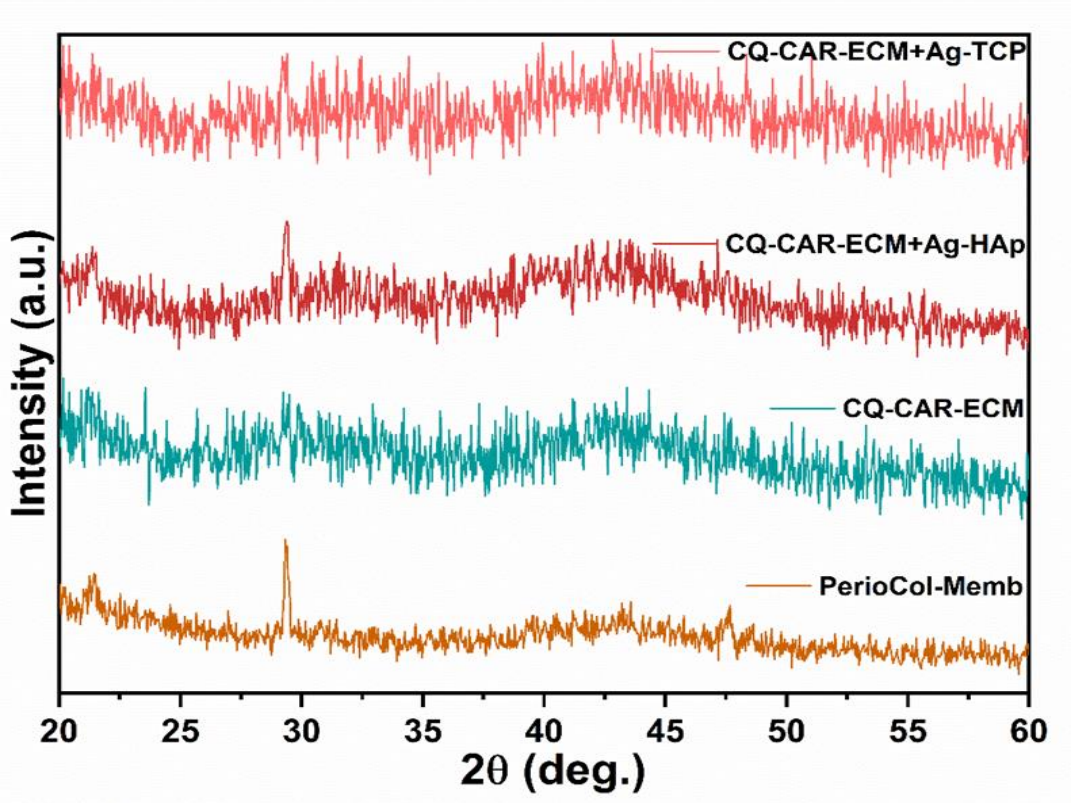


Figure 3. X- Ray diffraction (XRD) analysis of Perio COL- Control (NC), *Cissus quadrangularis* extract, carrageenan, tendon extracellular matrix (TEM) (No. 2), No. 3 (contained silver hydroxyapatite, *Cissus quadrangularis* extract (CQ), carrageenan (CAR), tendon extracellular matrix (TEM), No. 4 (Silver tricalcium (Ag TCP), *Cissus quadrangularis* extract, carrageenan, tendon extracellular matrix (TEM) respectively

3.4 Wound healing activity and survival rate analysis

The wound healing potential of scaffolds incorporating plant extracts, carrageenan, and tendon extracellular matrix (TEM) was evaluated using a zebrafish incisional wound model. The study included three treatment groups and one negative control, with wound progression monitored at 0-, 7-, 14- and 21-days post- treatment. The healing response was assessed based on wound closure at 0, 7, 14, and 21 days. The healing process was assessed based on wound closure rate, tissue regeneration and molecular markers indicative of inflammation and repair (*Figure 4*).
At day 0, all groups exhibited a uniformly inflicted incisional wound with no significant differences in initial wound size, confirming consistent baseline conditions. The negative control group, which received no treatment, showed minimal signs of tissue remodelling by day 7, with delayed wound contraction and persistent inflammatory markers. On contrast, all three treatment groups, demonstrated early signs of wound healing with noticeable reductions in wound size and increased epithelization. The scaffold incorporating plant extracts exhibited accelerated wound

closure, likely due to the anti-inflammatory ad antioxidant properties of the bioactive compounds.

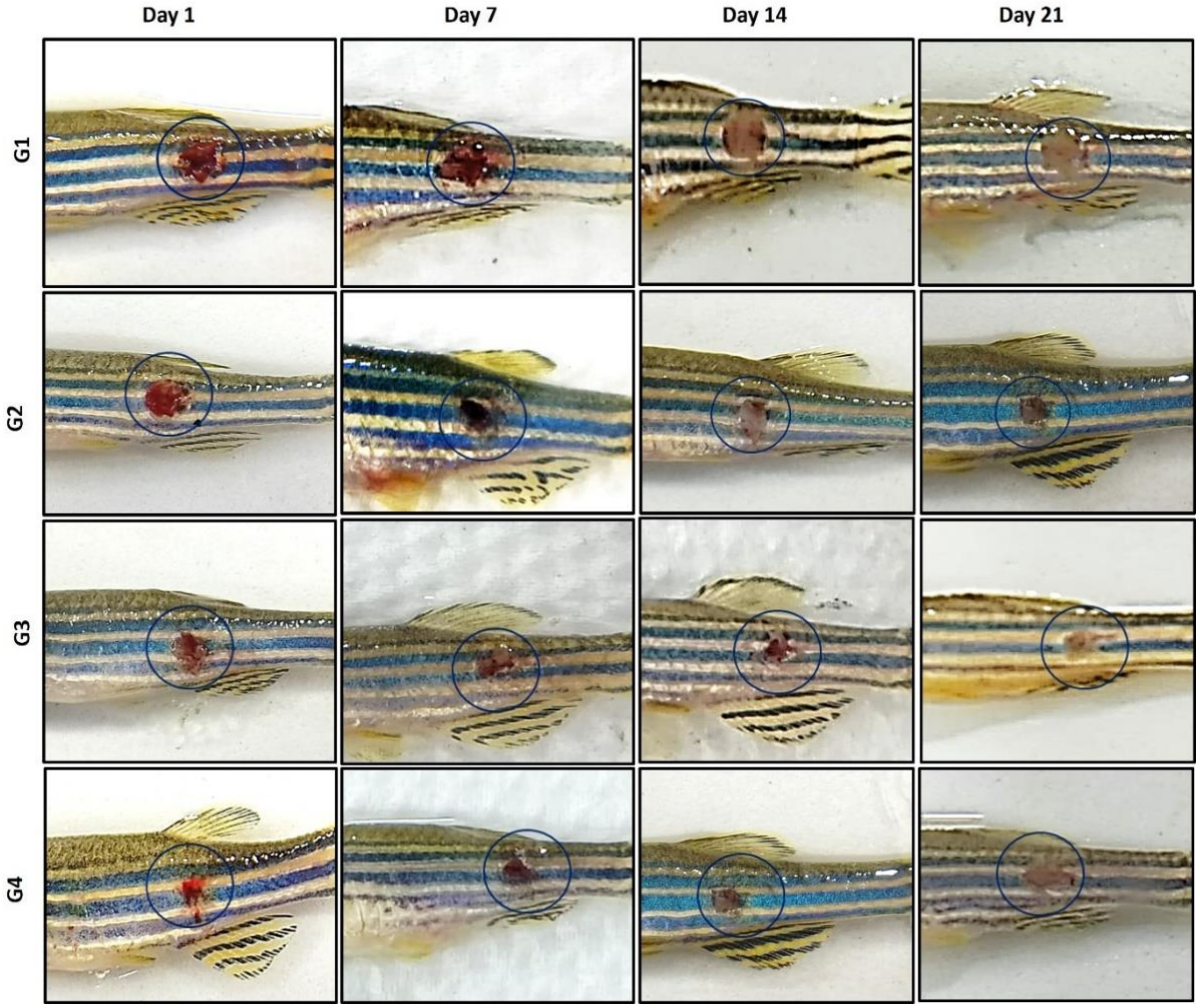


Figure 4. Representative photograph of wounded zebrafish treated with negative control (Group 1), *Cissus quadrangularis* extract, carrageenan, tendon extracellular matrix (TEM) (Group 2), Group 3 (contained silver hydroxyapatite + Group 2 components), Group 4 (Silver tricalcium phosphate + Group 2 components).

The bioceramics based scaffold also showed early regenerative responses, possibly due to its osteoconductive and mineralization enhancing properties, facilitating cellular attachment and proliferation.
The TEM incorporated scaffold displayed enhanced collagen deposition and matrix remodelling, indicating improved extracellular matrix formation and structural support for tissue regeneration.
By day 21, significant differences were observed between the treatment groups and the negative control. The plant extract- based scaffold exhibited an approximately 60%- 70% wound closure rate, with visible neovascularization and reduced inflammatory response, suggesting an immunomodulatory effect. The bioceramics scaffold group displayed moderate wound closure (up to 55%), with signs of osteoid formation and early mineral deposition, indicating potential application for bone- related tissue regeneration. The TEM incorporated scaffold group showed the most extensive tissue remodelling regeneration (up to 75%), with well- organized extracellular matrix deposition and enhanced fibroblast proliferation, supporting the role of ECM components in guiding cellular responses during tissue repair. Meanwhile, the negative control group showed only 30-40% would closure, with persistent inflammatory cell infiltration and incomplete epithelization, highlighting the necessary of bioactive scaffolds for effective wound healing.

At day 21, the treatment groups exhibited nearly complete wound closure, with well- integrated tissue architecture. The plant extract incorporated scaffold demonstrated over 90% wound closure, with minimal scar formation and enhanced epithelial regeneration. The carrageenan scaffold also exhibited substantial healing (85% closure), with increased mineralized tissue (*Figure 5*).

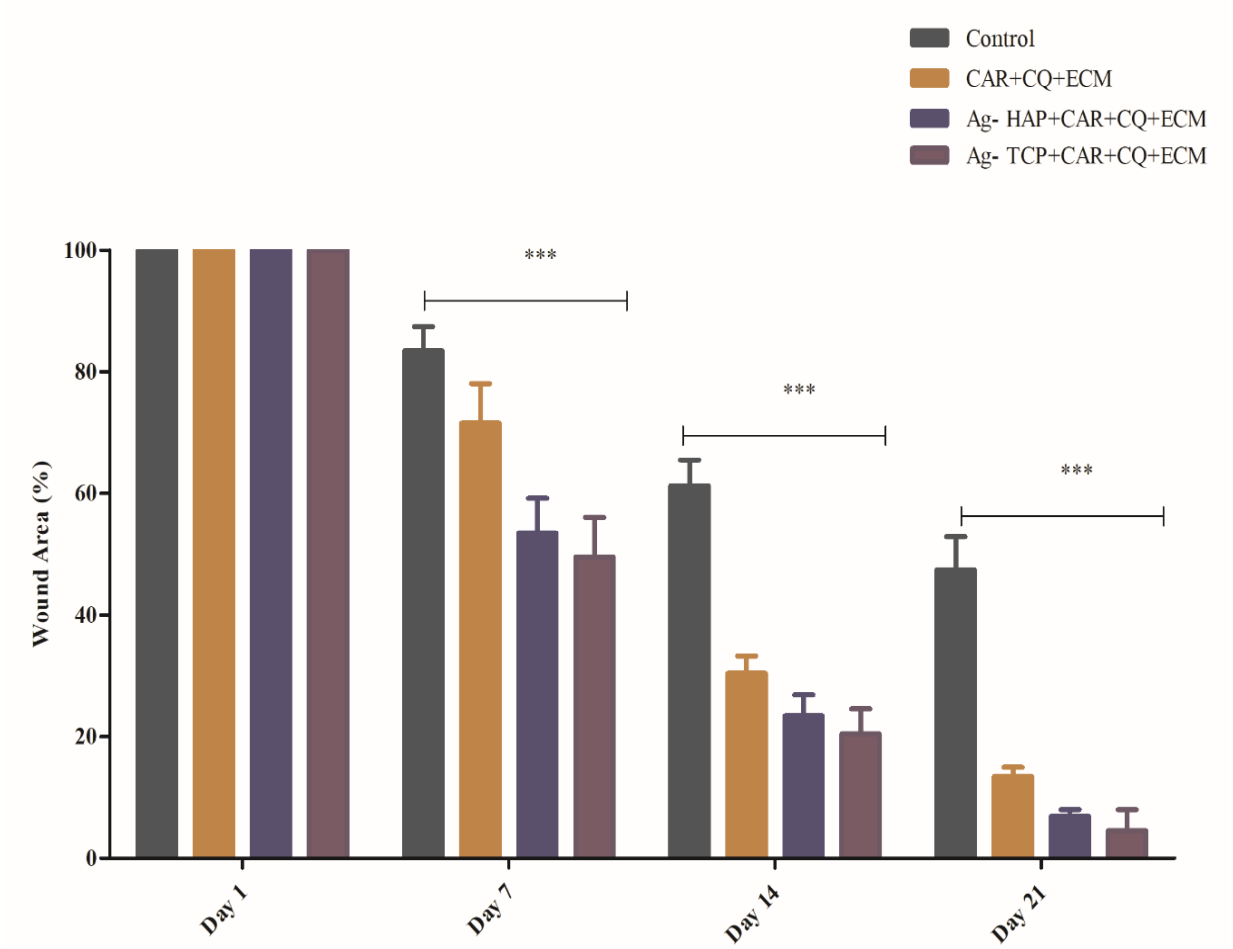


Figure 5. The graphical presentation of the wound closure of zebrafish treated with negative control (Group 1), *Cissus quadrangularis* extract, carrageenan, tendon extracellular matrix (ECM) (Group 2), Group 3 (contained silver hydroxyapatite + Group 2 components), Group 4 (Silver tricalcium phosphate + Group 2 components). It was measured using Image J software. The independent three experimental data were expressed as mean ± SD (n=6/group). The significant difference at $p < 0.05$ was expressed as ***

3.5 Histology analysis

Histological analysis of zebrafish tissue following wounding and treatment with the synthesized scaffold combined with *C. quadrangularis* and ECM showed significant structural differences compared to the control group. The unwounded (Group A) zebrafish displayed normal tissue organization including skeletal muscle, dermis, epithelial layer and a thin epidermis. The wounded zebrafish exhibited inflammatory and delayed tissue regeneration (Group B). The Group contained *C. quadrangularis*, carrageenan and TEM scaffold promoted a more pronounced healing response, characterized by a well-developed multi layered epidermis, increased granulation tissue, and early stage neoangiogenesis (Group E). Group with silver hydroxyapatite and *C. quadrangularis*, carrageenan and TEM showed dense new blood vessel formation and significant fibroblast activity within granulation tissue (Group C). Group treated with (silver tricalcium phosphate, *C. quadrangularis* and TEM, showed a new epidermal layer formation, characterized by a rapid healing process (Group D). The presence of numerous immune cells highlighted the inflammatory phase, while the emergence of new vasculature further facilitated tissue repair (Figure E). These findings suggested that the treatment with silver tricalcium phosphate, *C. quadrangularis* and TEM accelerated wound healing, as demonstrated by faster wound contraction and new scale formation within 4 to 21 days (*Figure 6*).

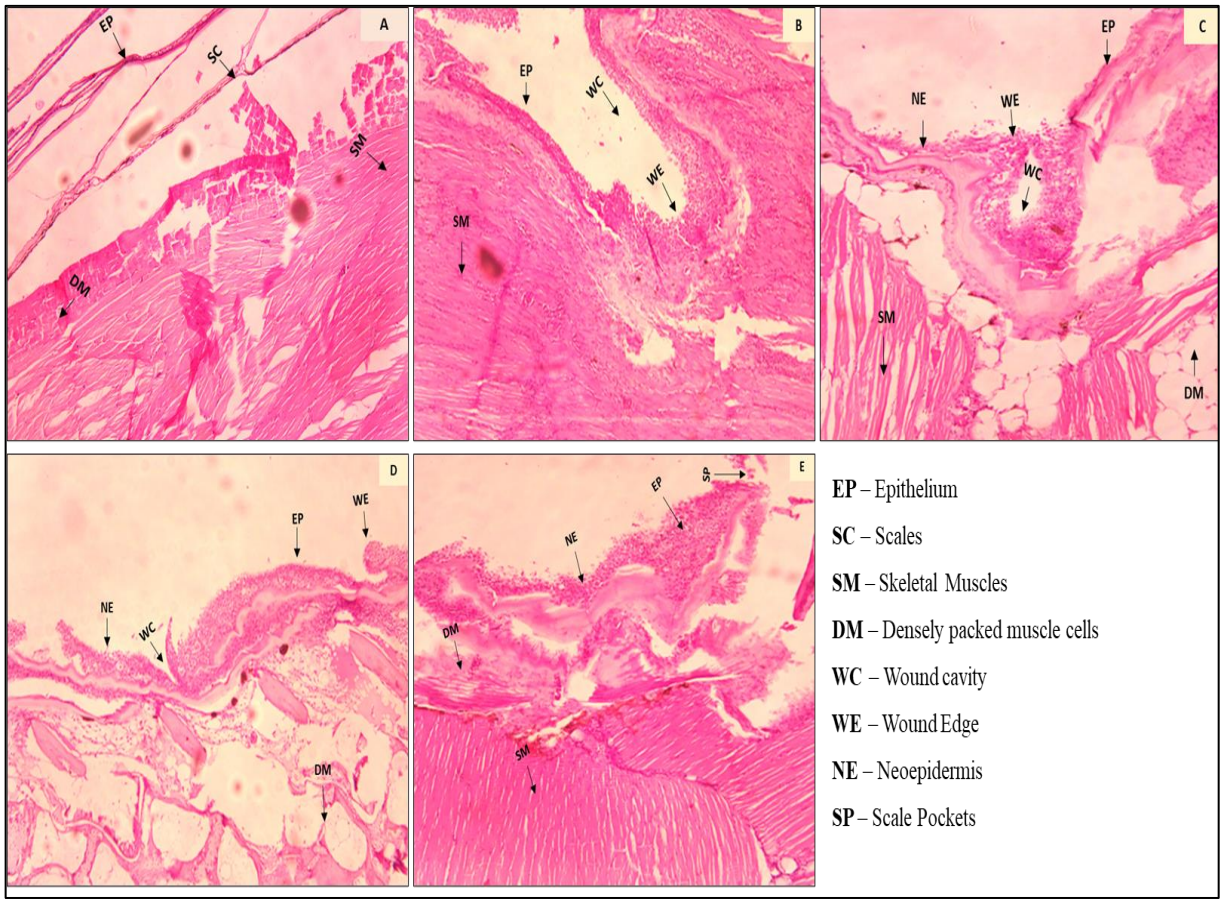


Figure 6. Histological analysis of wounded zebrafish tissue treated with Positive control (Group A), Negative control (Group B), *Cissus quadrangularis* extract, carrageenan, tendon extracellular matrix (TEM) (Group C), contained silver hydroxyapatite + Group 2 components (Group D), Silver tricalcium phosphate + Group 2 components (Group E).

DISCUSSION

The present study investigated the wound healing efficacy of scaffold incorporating *Cissus quadrangularis* extract, carrageenan, and tendon extracellular matrix (TEM) in an external wound zebrafish model. The findings demonstrated the significant regenerative potential of these scaffolds as evident from enhanced wound closure, histological improvements in zebrafish skin tissue with material characterization through SEM, FTIR and XRD analysis[20].

SEM analysis revealed the important of surface morphology and porosity that help for efficient wound healing. The SEM analysis also revealed that the scaffolds had porous architecture with the interconnected pores that enhance the adhesion, migration and diffusion of the nutrient materials. This porous architecture might help for wound healing in the zebrafish due to the increased surface area. The FTIR analysis revealed the confirmation of the bioactive functional groups in the scaffolds[21]. The presence of the characteristic peaks may correspond to the biological interactive groups such as -OH, -COOH, -CONH and -PO_4. These functional groups might also change the biological signalling to the benefit of wound healing. The observed the well -organized crystallinity should help in controlled degradation of the scaffolds that essential for tissue remodelling[22].

The wound healing is a complex biological process involving inflammation, proliferation, and tissue remodelling. The combination of *C. quadrangularis* extract, carrageenan, and TEM within scaffolds provided a multifaceted approach to accelerate wound healing. *C. quadrangularis* is known for its anti-inflammatory, antioxidant, and osteogenic properties, which likely contributed to reduced oxidative stress and enhanced fibroblast proliferation at the wound site. Previous studies have reported that flavonoid and triterpenes in *C. quadrangularis* can modulate inflammatory pathways and promote tissue repair. Carrageenan, silver hydroxyapatite and silver tricalcium phosphate, were incorporated to provide a favourable microenvironment for cellular attachment and proliferations. These materials are widely recognized for their bioactivity and ability to promote osteoconductivity[23]. Their presence in the scaffold likely contributed to calcium deposition and enhanced mineralization, facilitating tissue regeneration.

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Additionally, the inclusion of TEM provided a natural extracellular environment rich in collagen and bioactive molecules that support the cell adhesion, migration and differentiation.

The present study encouraged the usage of the *C. quadrangularis* incorporated scaffold could be a promising candidate for potential translational applications. The future studies should focus on optimizing the scaffold composition, for enhanced mechanical properties and degradation rates suited for clinical applications. Additional *in vivo* studies using mammalian models could provide further validation of their efficacy and safety. Furthermore, exploring the incorporation of additional bioactive molecules such as growth factors or antimicrobial agents could enhance the therapeutic potential of these scaffolds[7].

The study demonstrated that the application of silver tricalcium phosphate, *C. quadrangularis*, and TEM effectively promoted wound healing. The treatment led to a noticeable reduction in wound size and facilitated the regeneration of scales, indicating improved recovery. The accelerated wound contraction observed between days 4 and 21 suggests that these agents play a crucial role in tissue repair[24]. Additionally, the formation of new scales within this period further supports their regenerative potential.

The results highlight the therapeutic efficacy of these components in enhancing the natural healing process. The rapid healing response may be attributed to their bioactive properties, which contribute to cell proliferation and tissue regeneration. The consistent progress in wound closure observed throughout the study period reinforces the effectiveness of this combination. These findings suggest that the applied treatment can serve as a promising approach for improving wound healing outcomes by stimulating faster tissue recovery and regeneration[25].

CONCLUSION

The findings from this study demonstrated that composite scaffold incorporating *C. quadrangularis* carrageenan, silver tricalcium phosphate and TEM significantly enhance wound healing in zebrafish by modulating inflammation, promoting cell proliferation, and supporting tissue remodelling. The integration of SEM, FTIR, and XRD analyses provided comprehensive insights into the physiochemical properties of the scaffold and their correlation with wound healing outcomes. These results lay the ground work for future translational research aimed at developing effective wound healing therapies for clinical periodontal applications.

Acknowledgement

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Conflicts of Interest

Authors have no conflict of Interest

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RESARCH ARTICALE

DECODING AUTISM:THE SCIENTIFIC LINK BETWEEN SALIVARY VITAMIN D3 LEVELS AND NEURODEVELOPMENT-A CASE-CONTROL STUDY

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ABSTRACT

Objective: Autism Spectrum Disorder (ASD) involves challenges in social communication and repetitive behaviors. Emerging evidence suggests a link between vitamin D3, vital for brain function, and ASD, though research on salivary vitamin D3 as a biomarker in ASD is limited.
Aim: To compare salivary vitamin D3 levels between ASD patients and neurotypical individuals.
Materials and Methods: This case-control study collected unstimulated saliva samples from ASD patients and neurotypical controls. Vitamin D3 levels were measured using enzyme-linked immunosorbent assay (ELISA), with paired t-tests for group comparisons
Results: ASD patients had significantly lower salivary vitamin D3 levels than controls ($p = 0.001$), with ranges of 28.9–48.4 ng/mL in ASD versus 45.2–60.9 ng/mL in neurotypical individuals.
Conclusion:ASD patients showed lower salivary vitamin D3, suggesting a potential association with vitamin D3 deficiency. Further research could explore the role of vitamin D3 in ASD and assess the benefits of monitoring and supplementation.

Keywords: Keywords: Autism Spectrum Disorder, Vitamin D3, Saliva, ELISA, Vitamin D Deficiency, Neurodevelopmental Disorders, Case-Control Study.

INTRODUCTION

Autism is a neurodevelopmental disorder characterized by repetitive patterns of interest and behavior, along with deficits in social and communication development.[1] It typically manifests within the first three years of life and is considered a serious, lifelong developmental

condition. Approximately five out of every 10,000 children are affected, with boys being four times more likely to have the disorder than girls. No psychosocial factors in a child's environment have been linked to the development of autism; instead, physical abnormalities in the brain are believed to be responsible for the symptoms.[2] Autism spectrum disorders (ASDs) are neurological conditions with a significant genetic basis, though their precise origins remain unknown.[3] The genetic complexity of ASDs, along with the broad range of observable traits, has made it challenging to pinpoint a definitive cause. These disorders are influenced by multiple genes, resulting in considerable variability in their manifestation.[4] The prevalence of autism spectrum disorder (ASD) appears to be substantial among children and adolescents worldwide, making it important to understand the most recent prevalence rates and trends.[5] Individuals with disabilities, including those with ASD, may encounter health disparities and difficulties in securing employment as adults, potentially leading to a cycle of poverty.[6,7] Vitamin D is thought to be one of the oldest hormones, with its origins dating back over 750 million years. It is crucial for bone development from birth until death and can be synthesized in phytoplankton, zooplankton, and most plants and animals exposed to sunlight. Vitamin D plays a key role in maintaining calcium balance by enhancing the gastrointestinal tract's ability to absorb dietary calcium. Its metabolism is accelerated during pregnancy and lactation. The two main forms of vitamin D are ergocalciferol (vitamin D2), which is derived from irradiating plants or food materials, and cholecalciferol (vitamin D3), which is produced in the skin after exposure to sunlight or UV light.[8] In the summer, the skin synthesizes vitamin D3, which can also be obtained through diet, particularly from fatty fish like mackerel and herring. Cholecalciferol undergoes hydroxylation in the liver to form 25-hydroxyvitamin D3, and then in the kidneys to produce 1,25-dihydroxyvitamin D3, the active metabolite that boosts calcium absorption in the intestines. Risk factors for low vitamin D levels include premature birth, pigmented skin, limited sun exposure, obesity, malabsorption of dietary fat, impaired enzymatic activation of cholesterol, and older age. Severe vitamin D deficiency can lead to rickets or osteomalacia. The active form of vitamin D, 1,25-dihydroxyvitamin D, is vital for regulating calcium homeostasis and bone mineralization. Additionally, it induces cell death, halts the cell cycle, and promotes cancer cell differentiation, which helps inhibit growth.[9] In recent years, vitamin D insufficiency has been widely observed across all age groups. In developed countries, rickets has not been entirely eradicated, particularly affecting young immigrants.[10]

Previous studies have shown that children with autism tend to have lower vitamin D levels compared to their parents, siblings, and the general population. Low vitamin D levels at birth have been observed in children later diagnosed with ASD, while their healthy siblings did not exhibit the same deficiency. Low maternal vitamin D levels during pregnancy have been associated with adverse effects on cognitive development, early childhood development, and an increased risk of ASD diagnosis.[11] Vitamin D deficiency in mothers during pregnancy may be a risk factor for ASD in their children.[12,13] Additionally, children diagnosed with ASD have been found to have significantly lower vitamin D levels than neurotypical children, and it has been suggested that vitamin D supplementation could have beneficial effects for these children.[12,14,15,16]

There are several gaps in the existing literature on the relationship between vitamin D levels and autism spectrum disorder (ASD). Many previous studies are limited by small sample sizes, making it difficult to generalize their findings. Additionally, confounding factors such as diet, sun exposure, geographic location, and genetic differences are often not adequately controlled, which can affect the accuracy of the reported vitamin D levels. Moreover, the majority of research focuses on blood serum levels of vitamin D, with little exploration of alternative, less invasive biomarkers like salivary vitamin D. Few studies specifically examine how vitamin D3 levels correlate with neurodevelopmental milestones in children with autism. This study aims to address these gaps by focusing on salivary vitamin D3 levels, providing a non-invasive and practical measure. It also offers a direct comparison between individuals diagnosed with autism and a neurotypical population, thus contributing to a more comprehensive understanding of the potential link between vitamin D levels and ASD.

MATERIALS AND METHODS

A case-control study was conducted with a sample size of 32 students from a private educational trust for autism in Chennai, Tamil Nadu, India. The study adhered to the provisions of the declaration of

Helsinki and received approval from the scientific review board (IHEC/SDC/UG-2253/24/PHARM/097). Participants in the case group were children diagnosed with autism, aged 10 to 15 years. Exclusion criteria included those with systemic diseases, poor oral health, chronic kidney or liver conditions, individuals under vitamin D supplementation, and those who were malnourished. Unstimulated saliva (2 ml) was collected from participants between June and July 2024. The samples were centrifuged at 5000 rpm for 10 minutes and stored at 4°C for analysis. Vitamin D levels were measured using the Vitamin D Quanti Microlisa, based on delayed competitive ELISA. A diet history was also recorded, and participants consuming vitamin D supplements or fortified foods were excluded. On the day prior to and the morning of sample collection (between 8 to 9 A.M.), participants were instructed to follow their usual South Indian diet. Data analysis was performed using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp, Armonk, NY).

RESULTS

Salivary vitamin D3 levels were measured using a delayed competitive ELISA method. The mean salivary vitamin D3 levels ranged from 45.2 to 60.9 ng/mL in healthy subjects, while in patients with autism, the levels were significantly lower, ranging from 28.9 to 48.4 ng/mL. A paired sample t-test was done, yielding a highly significant p-value ($p = 0.000$, $p < 0.05$), indicating that the vitamin D3 levels in patients with autism were significantly lower compared to the healthy control group. This suggests a strong association between lower vitamin D3 levels and autism.(Table.1) (Fig.1, FIg.2)

Table.1 Table showing the statistically significant result (p<0.05) Paired-t test done using SPSS software version 23.0

Paired t test		
Study group	**Mean**	**Significance(2-tailed)**
Case group (Patients with autism)	53.05ng/ml	0.001
Control group (Healthy patients)	38.65ng/ml	

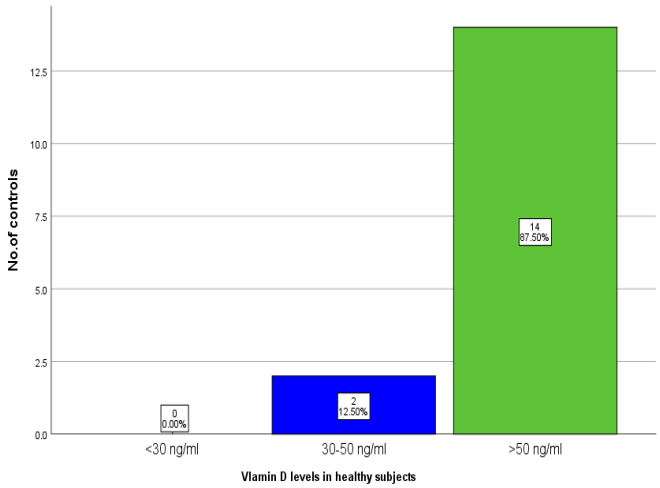


Figure1. Bar graph showing the vitamin D levels in healthy subjects

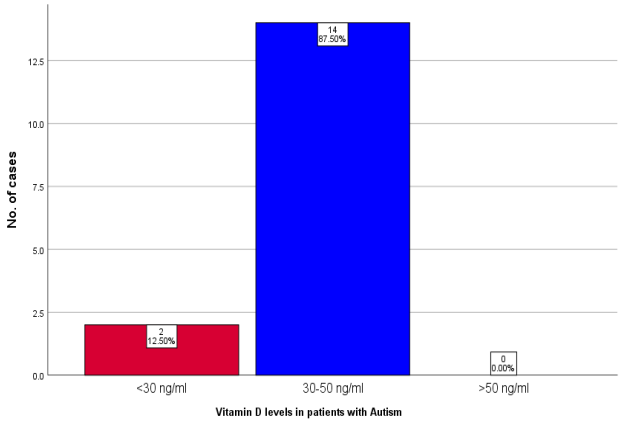


Figure 2. Bar graph showing vitamin d levels in autism patients

DISCUSSION

Vitamin D plays various biological roles, including reducing inflammation, and may influence the development of autism spectrum disorders (ASD).[17] Deficiency in vitamin D is often linked to limited sun exposure, inadequate dietary intake, and impaired liver function, which reduces the synthesis of this essential vitamin. In cases of severe obesity, lower vitamin D levels are commonly observed alongside elevated parathyroid hormone levels, which disrupt calcium metabolism and adversely affect bone health. This can also contribute to the onset of conditions like diabetes, cardiovascular disease, and hypertension.[18] Vitamin D3 is particularly crucial for brain development, and its deficiency during key periods such as pregnancy and early childhood could contribute to neurodevelopmental disorders,

including ASD.[19] Studies have indicated that children with ASD often present with vitamin D deficiency, and supplementing with vitamin D3 may improve outcomes, particularly in younger children. Vitamin D3 supplementation is a safe and cost-effective intervention that could benefit many children with ASD.[20] However, there remains limited data exploring the relationship between vitamin D deficiency and the severity of ASD symptoms.[21]

Our study found that children with autism had lower salivary vitamin D3 levels compared to their neurotypical peers. These findings align with prior research, which consistently reports lower vitamin D3 levels in children diagnosed with ASD. For example, a study by Zhang et al. (2022) found that children with ASD had significantly lower serum vitamin D levels and a higher prevalence of vitamin D deficiency (below 20 ng/ml) compared to healthy controls (67.7% vs. 34.1%).[22] Similarly, a 2022 study by Ganta Avani emphasized the need to screen children at risk of vitamin D deficiency, particularly those with ASD who may have restricted diets. The study also noted that reduced sun exposure during the COVID-19 pandemic heightened the risk of deficiency in this population.[23]

Additionally, low vitamin D levels have been reported in individuals with Down syndrome, indicating that vitamin D deficiency might be a common issue across neurodevelopmental disorders. [24] Maternal vitamin D deficiency during pregnancy, as well as early childhood deficiency, may increase the risk of developing neurodevelopmental disorders such as ASD.[25] Furthermore, research has suggested that vitamin D3 supplementation may improve autism symptoms, underscoring the importance of assessing and correcting vitamin D3 deficiencies in children with ASD.[26]

In this study, salivary samples were used to measure vitamin D3 levels. Saliva is increasingly recognized as a valuable tool in translational research and diagnostics, providing molecular biomarkers for both oral and systemic conditions.[27] The collection process was simple, involving participants spitting saliva into a container. This non-invasive method is particularly advantageous for children with ASD, who may have sensory sensitivities and behavioral challenges that make traditional blood sampling difficult.[28,29] Saliva sampling offers a cost-effective, painless, and less anxiety-inducing alternative to blood tests, while also providing abundant biological material for analysis, including DNA, proteins, hormones, and microRNA.[29] Despite these advantages, saliva sampling does have limitations, such as potential contamination from food or oral conditions, and some biomarkers may be present at lower concentrations compared to blood or urine. However, these limitations can be addressed with highly sensitive detection methods.[30-34]

Previous studies have also highlighted the risks associated with vitamin D deficiency during pregnancy, which can lead to various adverse outcomes. Maternal vitamin D3 levels during pregnancy significantly influence newborns' vitamin D3 levels, potentially affecting early brain development and increasing the risk of ASD.[35,36] This emphasizes the importance of monitoring vitamin D levels in expectant mothers to reduce the risk of neurodevelopmental disorders in their offspring.[32]

This study stands out due to its focus on salivary vitamin D3 levels in autism patients, particularly in the Indian population, where such research is limited. While the study contributes valuable insights, it has some limitations. Since only salivary vitamin D3 levels were assessed, the study lacks a comprehensive analysis that includes other potential biomarkers. Despite these limitations, this research is significant as it highlights the importance of monitoring vitamin D3 levels in children with autism. By identifying and addressing vitamin D3 deficiencies in this population, we could improve their overall health and potentially alleviate some autism symptoms, offering a better quality of life for individuals with ASD and their families. Future studies should expand the sample size to improve the generalizability of the findings and consider longitudinal research to explore how vitamin D3 levels fluctuate over time in children with autism. Incorporating other biomarkers and evaluating neurodevelopmental milestones in parallel with vitamin D3 levels could offer a more comprehensive understanding of the relationship between vitamin D3 deficiency and autism severity. Additionally, exploring the effects of vitamin D3 supplementation in different age groups and varying levels of autism severity would help determine its therapeutic potential. Finally, broader population-based studies, especially in diverse geographic regions, can further elucidate the role of environmental factors like sun exposure and dietary habits in contributing to vitamin D3 deficiency in individuals with ASD.

CONCLUSION

This study highlights the significant difference in salivary vitamin D3 levels between children with autism and their neurotypical peers, with autism patients exhibiting notably lower levels. The findings support previous research suggesting that vitamin D3 deficiency may play a role in the development and progression of autism spectrum disorders (ASD). By utilizing a non-invasive and practical method such as saliva sampling, this study offers a novel approach for monitoring vitamin D3 levels in children with ASD, especially those with sensory sensitivities. Despite the small sample size and limitations in assessing other biomarkers, the study underscores the potential value of vitamin D3 supplementation as an adjunct treatment for improving symptoms in children with autism.

DECLARATIONS

Author contributions

DD and SS wrote the manuscript. PR and GJ reviewed the manuscript.

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Data availability

All data and materials of the study are available and can be provided on request. The corresponding author can be contacted anytime to get the data of the study.

Conflict of interest

No conflict of interest is declared by all the authors.

Ethics approval and consent to participate

Ethical Committee Clearance Number: (IHEC/SDC/UG-2253/24/PHARM/097). “The study was approved by the institutional human ethical committee board of Saveetha Dental College and Hospitals. (IHEC/SDC/UG-2253/24/PHARM/097). The protocol of the study was approved by the Scientific Review Board (IHEC/SDC/UG-2253/24/PHARM/097) and it conforms to the provisions of the declaration of Helsinki. Informed consent was obtained from all the patients and their legal guardians by informing and clearly explaining the details of the study. All the methods in the study were performed in accordance with the relevant regulations and guidelines.

Consent to participate

Informed consent was obtained from all the patients and their legal guardians by informing and clearly explaining the details of the study.

Consent for publication

Informed consent was obtained from all the patients and their legal guardians by informing and clearly explaining the details of the study.

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COMPARATIVE ASSESSMENT OF SALIVARY ENDOTHELIN-1 LEVELS PERIODONTITIS SUBJECTS FOLLOWING NON-SURGICAL PERIODONTAL THERAPY (NSPT) WITH ADJUNCTIVE PHOTOBIOMODULATION AND NSPT ALONE: A PROSPECTIVE INTERVENTIONAL STUDY

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ABSTRACT

Background: Non-surgical periodontal therapy (NSPT) effectively reduces microbial load and inflammation in Periodontitis; however, adjunctive therapies such as photobiomodulation (PBM) have been proposed to enhance outcomes. Endothelin-1 (ET-1), an inflammatory biomarker, has been associated with periodontal disease progression, yet its levels in saliva remain under-investigated, thus we aimed to comparatively assess salivary ET-1 levels in periodontitis patients undergoing NSPT alone versus NSPT combined with photobiomodulation.

Material and methods: This prospective, randomized, single-blinded study involved 96 subjects divided equally into three groups: healthy controls, generalized periodontitis treated with NSPT alone, and periodontitis treated with NSPT and photobiomodulation. Clinical periodontal parameters and levels of salivary ET-1 were recorded at baseline and on the 21st day.

Results: Results demonstrated significantly elevated levels of ET-1 in periodontitis patients in comparison to healthy individuals (p=0.002). Both treatment modalities showed improvements in clinical parameters; however, NSPT combined with photobiomodulation significantly reduced ET-1 levels (p=0.048) and improved periodontal outcomes.

Conclusion: Adjunctive photobiomodulation enhances the efficacy of NSPT, with salivary ET-1 proving to be promising as a biomarker to monitor periodontal disease and therapeutic response.

Keywords: non-surgical periodontal therapy, endothelin-1, photobiomodulation, periodontitis

INTRODUCTION

Chronic Periodontitis (CP) is an inflammatory disorder affecting tissues supporting teeth. The standard treatment for CP is nonsurgical periodontal therapy (NSPT) as it can reduce bacterial load and microbe induced inflammatory responses, targeting subgingival biofilm elimination[1]. Therapeutic efficacy of this method is well-documented, and scaling and root planing(SRP) are the standard in CP treatment[2]. To improve the results of NSPT, additional therapies including photobiomodulation have been suggested. Photobiomodulation (PBM) is a therapeutic approach that employs appropriate dosages of laser photonic radiation. It is advocated for its anti-inflammatory properties, analgesic benefits, and promotion of wound healing[3].

Endothelin-1 (ET-1) is a commonly studied element of the Endothelin system in the human body, expressed in tissues during inflammatory states[4]. Evidence-based literature indicates a robust correlation between ET-1 and the aetiology of periodontitis[5,6]. Furthermore, ET-1 levels are considered a pivotal component in the outcomes of periodontitis.

Existing literature has assessed ET-1 concentrations

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in gingival crevicular fluid(GCF) and serum in CP and NSPT effects on these levels[7–9]. To our knowledge, just a single study has evaluated Salivary ET-1 levels in CP. This study aimed to assess Salivary ET-1 levels in individuals with periodontitis and to compare these levels before and after NSPT combined with PBM in CP patients. This will facilitate the investigation of Endothelin-1 as a salivary biomarker in periodontitis.

2. MATERIAL AND METHODS

2.1 Study design and Patient Selection

This prospective, randomised, single blinded interventional trial was executed in Department of Periodontology.

The sample size was determined using the subsequent formula:

$$n = \frac{Z1\text{-}\alpha/22\ SD2}{(M\ X\ \varepsilon)2}$$

A minimum sample of 29 was necessary in each category according to the given calculation. Nevertheless, given the likelihood of dropouts, a total of 32 patients were included in each of the groups. A selective sample method was employed for assigning a total of 96 patients for 3 groups. Group 1 comprised 32 periodontally and systemically healthy individuals, Group 2 included 32 individuals with generalised periodontitis undergoing NSPT exclusively, Group 3 consisted of 32 individuals with generalised periodontitis getting both NSPT and PBM. Subjects with periodontitis were selected based on 2017 AAP World Workshop on Classification of Periodontal and Peri-implant Diseases and Conditions. Participants in the study included systemically healthy individuals, those exhibiting bleeding on probing at≥10 % of sites, a Simplified Oral Hygiene Index of ≥ 1.2, Gingival Index (GI)≥1, Periodontal Probing Depth (PPD)≤5mm at over 30% of sites, Clinical Attachment Loss (CAL) of 3-4 mm at over 30% of sites, radiographic evidence of bone loss, and individuals aged between 30 and 65 years. Patients with systemic diseases, recent infections, pregnant or lactating females, individuals having previous history of periodontal therapy within the past six months, tobacco users, those using any drugs that affect periodontal tissues, and patients who did not provide consent for study enrolment were excluded.

A flowchart illustrating the recruitment and allocation of the research population (Figure 1).

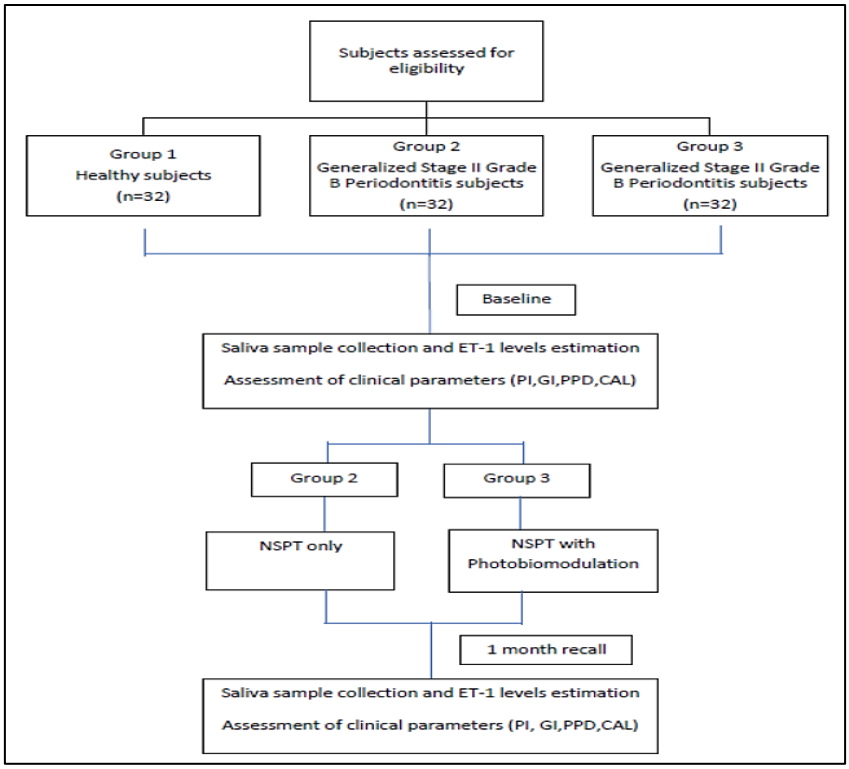


Figure 1. Recruitment and allocation of the study population

Sharayu Kulkarni, Sameer Zope, Girish Suragimath, Siddhartha Varma, Apurva Kale.Comparative Assessment Of Salivary Endothelin-1 Levels In Periodontitis Subjects Following Non-Surgical Periodontal Therapy (NSPT) With Adjunctive Photobiomodulation And NSPT Alone:A Prospective Interventional Study.Bulletin of Stomatology and Maxillofacial Surgery.2025;21(3).222-232.doi:10.58240/1829006X-2025.3-223

2.2 Collection of Saliva samples

Under aseptic conditions, 2ml of unstimulated whole saliva was collected via the spitting method two hours postprandially, between 10 am and 12 pm for standardising our collection. 2 millilitres of saliva sample were transferred into an Eppendorf tube. Cell debris was eliminated by centrifuging the samples at 1000 rpm for 20 minutes at a temperature of 2-8°C. For subsequent examination, 0.5ml supernatant was preserved in a 1.5ml plastic Eppendorf tube at -80°C in the Department of Genetics. The tubes were labelled and a tracking number was allotted.

2.3 Assessment of clinical parameters

Our study involved NSPT, which included a single-session scaling with a piezo-electric ultrasonic scaling unit (EMS PM100, Guangxi Ehall Medical Technology Co., Ltd.) and root planing using Standard Gracey Curettes (HuFriedy, Chicago, IL, USA), conducted after the collection of saliva samples in groups 2 and 3 by a blinded, trained operator. The operator provided self-administered plaque control techniques and oral hygiene instructions. The patients were called for follow up after 3 weeks for the reassessment of periodontal markers.

2.4 Photobiomodulation

Laser specifications and Laser irradiation: Indium Gallium Arsenic based diode laser (Photonplus diode laser®, ZolarTechnology and Mfg.Co.Inc.Canada) was utilised, featuring a 980nm wavelength, 100mW output power, .5cm²spot size area, and an irradiance of 0.2 W/cm². Cumulative energy administered was 8J. The provided dosage (energy density) was 2 J/cm² per application location. The laser functioned in continuous wave mode for 10 seconds/point. Laser tip was positioned perpendicular to tooth's long axis at four locations around the teeth, specifically at interdental papilla on buccal and lingual/palatal aspects, both mesially and distally. Standard postoperative directives were provided. All LASER safety protocols and measures were meticulously adhered to. The patients were summoned after 21 days for the re-evaluation of periodontal markers.

2.5 Estimation of salivary Endothelin-1 level

ET-1 levels were assessed utilising the Fine Test® Human Endothelin-1 enzyme linked immunosorbent assay (ELISA) kit. Assay was conducted with an ELISA reader (Lisa Quant, Department of Microbiology) in accordance with manufacturer's guidelines.

2.7 Statistical analysis

After obtaining all necessary information, the data was analysed to identify the impact of NSPT and PBM on ET-1 levels in saliva samples of Stage II Grade B Periodontitis. Data was imported into an MS Excel spreadsheet and subsequently evaluated using the statistical software application SPSS, IBM, INDIA 20.0. Variations in salivary ET-1 levels was assessed via Quadratic Regression Equation. A thorough comparative analysis of clinical parameters and Salivary ET-1 levels was conducted using a One-way ANOVA test, with Tukey's post hoc test applied for intergroup comparisons. The threshold for statistical significance was established at $p < 0.05$.

2.8 Study Ethics And Safety

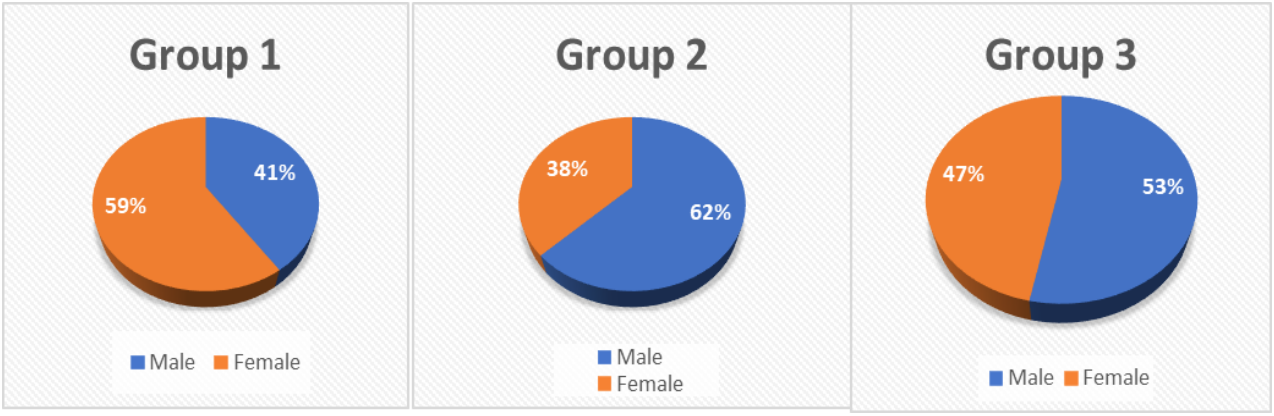
This clinical investigation received approval from the Ethical Committee and Institutional Review Board (No. 233/2020-2021). Participants were explained about study procedures and included upon receiving their agreement.

3. RESULTS

The current study was conducted among 96 subjects which were equally distributed among, the Healthy group (n=32), NSPT group (n=32), and NSPT along with the Photobiomodulation group (n=32).

3.1 Age and Gender wise distribution

Graph 1 shows gender-wise distribution of study population. The mean age was 41.65 years in group 1, in group 2 it was 48.43 years whereas for group 3 the average age was 53.37 years.



Graph 1. Age and Gender-Wise Distribution of the study population

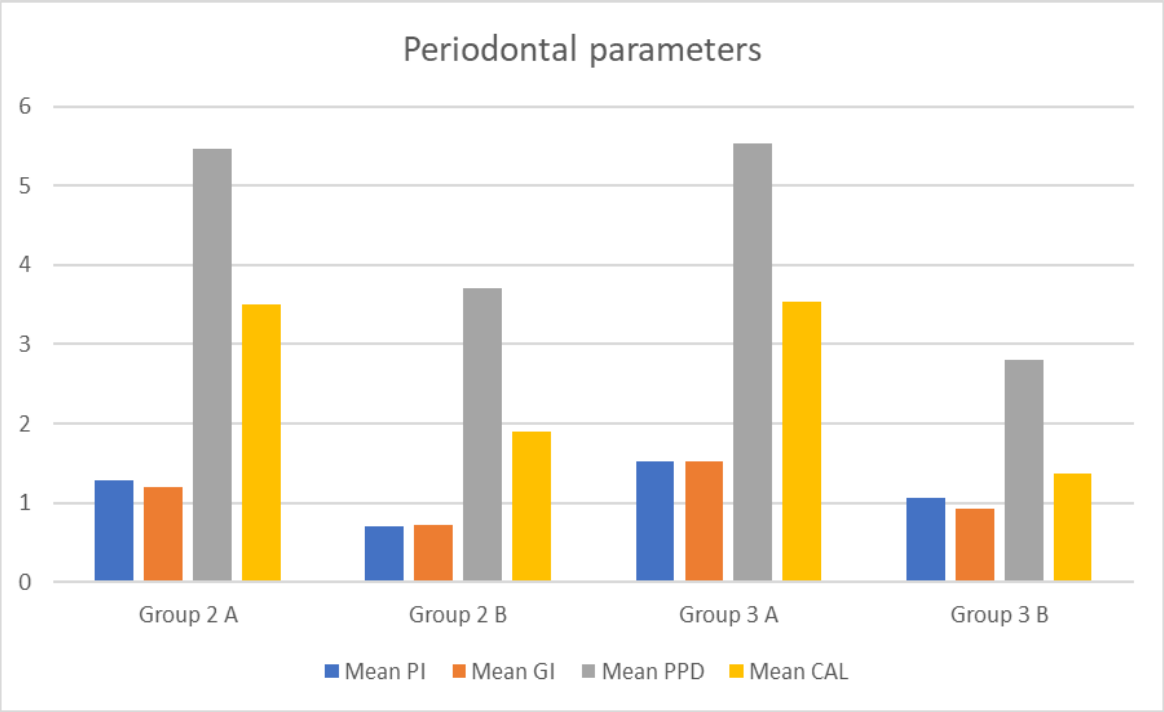
3.2 Comparison of Plaque index (PI) and Gingival Index (GI) at baseline and post 21 days in NSPT and NSPT with Photobiomodulation group

Graph 2 describes mean value of PI and GI while **Table 1** describes mean difference between PI and GI at baseline and after 3 weeks. A significant reduction in PI and GI was noted among both groups when compared to baseline scores. Intergroup comparative analysis revealed a dec decrease in PI score was significantly better in group 2 than in group 3. Results showed that in both groups there was a statistically significant reduction in mean GI values from the baseline to the 21st day ($p<0.001$). On comparison of both the study groups, the mean difference NSPT along with PBM showed more reduction in GI as compared to NSPT alone.

Table 1. Overall comparative statistics of mean PI and GI score in 3 groups

Group	Comparison Group	Plaque index		Gingival index	
		Mean Difference	P value, Significance	Mean Difference	P value, Significance
Group 2A (NSPT- Pre) vs	Group 2B (NSPT -Post)	0.571	p<0.001**	0.481	p<0.001**
Group 2B (NSPT -Post) vs	Group 3B (NSPT+PB Post)	0.346	p =0.009*	0.212	p <0.001**
Group 3A (NSPT+PB Pre) vs	Group 3B (NSPT+PB Post)	0.468	p<0.001**	0.6	p<0.001**

p>0.05-no statistically significant difference *p<0.05-significant **p<0.001-highly significant



Graph 2. Mean PI, GI, PPD, and CAL score at baseline and post 21 days in group 2 and group 3

3.3 Comparison of PPD and CAL at baseline and post 21 days in NSPT and NSPT with Photobiomodulation group

Graph 2 explains mean value of PPD and CAL while **Table 2** describes mean difference between PPD and CAL at baseline and on the 21st day. It was found that PPD reduced significantly from the baseline to the 21st day in both groups ($p<0.001$). Also, it was observed that the PPD reduced significantly for NSPT and PBM group than NSPT alone group. On intragroup comparison, it was found in both groups that there was a statistically significant difference from the baseline to the 21st day ($p<0.001$). Also, CAL gain was more significant in Group 3 than in Group 2 suggesting beneficial effects of PBM adjunctive to NSPT.

Table 2. Overall comparative statistics of mean PPD and CAL in 3 groups

Group	Comparison Group	PPD		CAL	
		Mean Difference	p value, Significance	Mean Difference	p value, Significance
Group 2A (NSPT- Pre) vs	Group 2B (NSPT -Post)	1.75	p<0.001**	1.59	p<0.001**
Group 2B (NSPT -Post) vs	Group 3B (NSPT+PB Post)	0.90	p =0.001*	0.53	p =0.062
Group 3A (NSPT+PB Pre) vs	Group 3B (NSPT+PB Post)	2.625	p<0.001**	2.15	p<0.001**

p>0.05 – no statistically significant difference *p<0.05 -significant **p<0.001 – highly significant

3.4 Comparison of Endothelin-1(pg/ml) Levels in group 1 and at baseline and post 21 days in group 2 and group 3

Table 3 and 4 summarizes the mean values of ET-1. For healthy group mean ET-1 levels were 201.74 pg/ml. At baseline and on 21st day for Group 2 it was 224.27 pg/ml and 208.94 pg/ml respectively. For Group 3, the mean

value at baseline was 226.31 pg/ml and 210.39 pg/ml at 21 days. Overall comparative analysis of all three groups reported higher levels of salivary ET-1 in CP patients than in healthy subjects (p =0.002). Intragroup comparison for Group 2 revealed that salivary ET-1 levels did not reduce significantly after NSPT alone (p=0.276). Whereas salivary ET -1 levels were significantly reduced post-NSPT with PBM (p=0.048). This suggested beneficial results of photobiomodulation as an adjunctive therapy to NSPT. Further intergroup comparative analysis suggested a statistically insignificant differences between Group 2 (p=0.828), Group 3(p=0.709) postoperatively.

Table 3. Overall comparative statistics of salivary endothelin-1 levels in 3 groups

	Mean (pg/ml)	SD	One-way Anova F test	p value, Significance
Group 1 (Healthy)	201.74	35.16	F = 4.448	p=0.002*
Group 2A (NSPT- Pre)	224.27	32.16		
Group 2B (NSPT -Post)	208.94	17.71		
Group 3A (NSPT+PB Pre)	226.31	30.78		
Group 3B (NSPT +PB Post)	210.39	13.24		

p>0.05 – no statistically significant difference *p<0.05 -significant **p<0.001 – highly significant

Table 4. Intergroup comparison of salivary endothelin -1 levels in 3 groups

Group	Comparison Group	Mean Difference	p value, Significance
Group1 (Healthy) vs	**Group 2 A (Pre)**	20.74	p=0.023*
	Group 2 B (Post)	7.19	p=0.828 (NS)
	Group 3 A (Pre)	24.56	p=0.004*
	Group 3 B (Post)	8.65	p=0.709 (NS)
Group 2A (NSPT- Pre) vs	**Group 2B (NSPT -Post)**	13.54	p=0.276 (NS)
	Group 3 A (Pre)	3.82	p=0.980 (NS)
Group 2B (NSPT -Post) vs	**Group 3B (NSPT+PB Post)**	1.45	p =1.000
Group 3A (NSPT+PB Pre) vs	**Group 3B (NSPT+PB Post)**	15.91	p=0.048*

4.DISCUSSION

ET-1 has been demonstrated to mediate the immune response at the endothelium level during periodontal disease by facilitating the generation of cross-reactive T cells through specific heat shock proteins[10,11]. ET-1 is a critical modulator that influences the host's defence system in this context. It subsequently induces the formation of endothelial cells, which increases the likelihood of future tissue injury caused by periodontal pathogenic bacteria in a variety of oral disorders[12]. The correlation between CP and levels of ET-1 in serum, GCF, & gingival tissues were evaluated in a few studies. However, based on our understanding, only one study has analysed ET-1 levels in saliva[13].

Concentrations of mean salivary ET-1 in NSPT and NSPT with PBM groups was significantly higher than the healthy group. Similarly, prior research has suggested that ET-1 levels have increased in periodontal and gingival tissues that are inflammatory. In comparison to the periodontally healthy group, Yamamoto E et al., Selvan T et al. and Ansai T et al., observed elevated levels of ET-1 in gingival tissue samples from CP cohort[14–16]. The Fujioka study observed that the ET-1 levels were higher in CP cases compared to those with periodontal health[15]. Nevertheless, Pradeep AR et al. were unable to identify ET-1 in GCF in their investigation[17].

The adoption of plaque management techniques in periodontitis treatment, in addition to the outstanding results associated with SRP, have been extensively documented[18].

Our study findings demonstrated substantial decline in CP metrics, such as PI, GI, PPD, and CAL. These results are in agreement with previous research that demonstrated NSPT to significantly increase CAL levels and decrease PPD in CP patients.

Kaur PK et al. and Gay IC et al. observed that PPD was increased at subsequent follow-up intervals following SRP[19,20]. In a similar vein, Smiley et al. conducted a meta-analysis of 11 RCTs and concluded that SRP was correlated with a statistically significant improvement in CAL of 0.49 mm in comparison to no therapy, as assessed at 6 months follow up[21]. A systematic review conducted by Needleman et al. observed correlation between SRP and a reduction in plaque levels[22].

Khare N et al. and Tawfig A et al. observed the changes in GI that occurred after SRP treatment from the baseline and showed a significant improvement in the SRP in 90days[23,24]. These findings are consistent our results, which similarly demonstrated a considerable reduction in PI, PPD, and GI, as well as a substantial increase in CAL, as a result of NSPT alone.

In CP patients, our study noted substantial decrease in salivary ET-1 post NSPT is similar to Khalid study, that reported a significant decrease in blood ET-1 levels of CP patients following NSPT[7]. A direct comparison of our findings is not feasible due to the lack of research that compares salivary ET-1 levels before and after therapy in CP.

PBM has been acknowledged for its anti-inflammatory, wound healing, alongwith analgesic properties[25,26]. Constituents of cellular metastatic chain are considered to be the primary mechanism of PBM at cellular level, as they absorb monochromatic visible light and near-infrared radiation[27]. Proton electrochemical potential, ATP synthesis, protein and RNA synthesis, mitochondrial membrane potential, synthesis of NADH, oxygen consumption, and ATP are all enhanced by PBM in mitochondria[28]. The dissociation of intracellular reserves, such as nitrosothiols, may result in the generation of a small quantity of nitric oxide from mitochondria during the transmission of laser light[29]. The modest concentration of NO produced by illumination is believed to be advantageous via cellular signalling pathways[30]. Consequently, PBM alters cellular behaviour by modulating mitochondrial respiratory chains or membrane calcium channels, thus promoting the angiogenesis, synthesis of collagen, release of growth factors, etc thereby expediting wound healing[31,32]. In periodontal practice, diode lasers can be securely employed for PBM.

Our study findings indicated both groups to have experienced statistically significant enhancements in clinical parameters following the implementation of NSPT in conjunction with PBM. The PI score and GI score showed a statistically significant decrease in both groups from baseline to 21 days post-intervention in the current study. In comparison to the NSPT along with photobiomodulation group, the PI score experienced a significant decline in NSPT group. These findings are consistent with the Michelli GD study[33]. In comparison to SRP alone, the photobiomodulation group demonstrated statistically significant improvements in GI levels for our study. This suggests that the supplementary use of photobiomodulation is more effective than SRP alone in reducing GI and PI scores. These results are consistent with the research by M Sopi et al., Gandhi et al., and Qadri et al[34–36].

Sharayu Kulkarni, Sameer Zope, Girish Suragimath, Siddhartha Varma, Apurva Kale.Comparative Assessment Of Salivary Endothelin-1 Levels In Periodontitis Subjects Following Non-Surgical Periodontal Therapy (NSPT) With Adjunctive Photobiomodulation And NSPT Alone:A Prospective Interventional Study.Bulletin of Stomatology and Maxillofacial Surgery.2025;21(3).222-232.doi:10.58240/1829006X-2025.3-223

The present study's intergroup comparison of mean PPD in the NSPT and NSPT with photobiomodulation groups, both pre- and post-intervention, demonstrated a substantial reduction in PPD for both groups. However, the comparative analysis determined that the photobiomodulation group experienced a significantly greater PPD reduction and CAL gain. The current study reported both groups to show a statistically significant increase in CAL from baseline to 21 days. After 21 days, photobiomodulation resulted in a statistically significant increase in CAL. A potential explanation is that laser irradiation increases cellular ATP production and decreases PGE2 levels[37,38]. These results are consistent with the research conducted by several authors. In periodontitis patients, NSPT + PBM showed significantly greater reduction in both clinical and microbiological parameters at 60 days follow up[35,39]. Our results are also consistent with the research conducted by Jiang et al. and Samulak R et al[40,41].

According to a study by Özdemir et al., PBMT + SRP groups exhibited a statistically significant decrease in PPD and CAL, but not in GI and PI, when contrasted with SRP alone[42].

On the other hand, Makhlouf et al., Lai et al., and Euzebio Alves et al. did not observe any substantial reduction in clinical measures following NSPT with photobiomodulation in comparison to NSPT alone[43–45]. According to Angiero et al., both groups demonstrated considerable clinical improvement when compared to controls; however, no statistically significant difference was observed[46]. Chandra et al. conducted a study that showed a statistically significant increase in PI. Although, there was no significant decrease in GI, PPD, and CAL gain in either laser or NSPT groups[47].

Dakhil et al. reported a statistically significant improvement in CAL, despite the absence of significant differences between cases and controls, which is in contrast to our results[48]. While Nguyen et al. & Hatipoğlum et al. observed a statistically significant decrease in PPD and an increase in CAL in a clinical trial, no significant differences were observed between the groups[49,50].

Our primary goal was to examine the function of ET-1 as a salivary biomarker in periodontitis. The results of this investigation will be beneficial to dentists in their evaluation of patients' periodontitis by evaluating salivary ET-1 levels. ET-1 is essential for regulation of blood pressure and vascular tone in healthy individuals, as previously mentioned. As a result, the sublingual evaluation of ET-1 in the dental context may aid in the prediction of cardiovascular issues and the facilitation of appropriate referrals to primary care specialists.

The current investigation has been limited by a relatively small sample size. In order to ascertain the precise role of ET-1 in CP aetiology and its influence on other systemic disorders, it is imperative to conduct additional interventional studies with a larger sample size. Additionally, research may be conducted using modified study designs, extended follow-up periods, and comparisons of different diode laser wavelengths with or without NSPT versus NSPT alone. This will enable the extrapolation of standardised laser techniques across a wide range of wavelengths.

CONCLUSION

The data that was previously discussed suggests that periodontitis is associated with elevated salivary ET-1 levels. Periodontitis can be effectively managed through conventional NSPT. Conventional NSPT is supplemented by photobiomodulation, which is a therapeutic approach that is advantageous. Salivary Endothelin-1 levels were significantly reduced by the combined use of NSPT and photobiomodulation. As a result, ET-1 may be identified as a diagnostic and prognostic indicator in assessment of therapy outcomes in CP cases.

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RESEARCH ARTICLE

COMPARATIVE EVALUATION OF SALIVARY ALPHA ENOLASE LEVELS IN PATIENTS WITH SUBMUCOUS FIBROSIS AND HEALTHY CONTROLS

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ABSTRACT

Oral submucous fibrosis (OSMF) is presently recognized as a potentially malignant disorder (PMD), with the likelihood of developing to oral squamous cell carcinoma ranging between 7 to 30%. Alpha-enolase, a lysosomal enzyme essential to glucose metabolism, is associated in disrupted energy production during carcinogenesis, guiding uncontrolled cell proliferation in numerous cancers. Identifying the connection between OSMF and salivary alpha-enolase could offer a helpful means for early diagnosis of malignancy, establishing the path for its application as a biomarker in both diagnosis and treatment. This study seeks to investigate and assess the salivary alpha-enolase levels in OSMF patients to healthy controls, proposing to find possible variations that might contribute to an early diagnostic breakthrough.

Material and methods: Saliva samples were taken from 20 participants in each group (OSMF sufferers and healthy controls). Salivary alpha-enolase levels were identified using the sensitive and precise enzyme-linked immunosorbent assay (ELISA). Statistical analysis were carried out using the Independent Sample t-test to establish significance.

Results: The results indicated that salivary alpha-enolase levels in OSMF patients were significantly higher in comparison to those in healthy people.

Conclusion: Salivary alpha-enolase could become a reliable biomarker for early OSF identification, thereby offering an opportunity for timely intervention, more efficient treatment planning, and improved outcomes for patients.

Keywords: Oral submucous fibrosis, Salivary alpha enolase, Oral premalignant disorders, ENO1 gene

1. INTRODUCTION

OSMF is defined by the excessive buildup of collagen and is recognised as a precancerous state, with the tendency to develop to malignant tumours.[1] This disorder provides a high likelihood (7–30%) of developing into oral squamous cell cancer.[2] The main feature of OSMF is its extensive detrimental impact on the oral cavity, which frequently results in major implications such as tissue scarring, dysphagia, and trismus.[3]

The specific origin of OSMF is uncertain; nevertheless, it is thought to develop from a combination of elements, such as areca nut chewing, eating spicy foods, genetics, immunological responses, and malnutrition.[4] These features lead to prevalent symptoms comprising restricted mouth opening, sensation of burning, and stiffness of the oral mucosa.[5] The epithelium often displays significant modifications, such as epithelial atrophy and, in certain instances, hyperplasia with or without dysplasia.[6]

Many molecular pathways play a part in the development of OSMF. Principal factors involve myofibroblasts, cytokines, and noncoding RNAs, all of which promote the progression of the disease.[7]

A biomarker is a discernible and quantifiable feature that indicates normal biological process, pathological process or respond to exposure or treatment.[8] Because molecular markers play a vital role in diagnosing, monitoring as well as treating OSMF, the screening of the biomarkers has become increasingly popular in recent years.[9] Enolase, an important enzyme in glycolysis has a

vital part in glucose metabolism, which is closely linked to altered energy production during cancer development. These changes contribute to abnormal cell growth and metastasis.[10] The ENO (Enolase) gene family includes three isoforms ENO1, ENO2, and ENO3 (Enolase1, Enolase2, Enolase 3) each with distinct functions. Alpha-enolase (ENO1) is particularly important as it is found on the cell surface, where it functions as a plasminogen receptor, progressing cell migration and promoting cancer metastasis.[11]ENO1 is involved in numerous pathological and biological processes, mainly in cancer and autoimmune diseases.[12] Its expression is strongly associated with decreased survival rates and poor detection across many cancer types.[13] Overexpression of ENO1 and its post-translational changes have significant diagnostic and predictive implications in several cancers.[14] Notably, ENO1 can be translated into a 37 kDa (kilo Dalton) protein known as (MBP-1) c-myc promoter-binding protein through an alternate start codon.[15]

ENO1 interacts with MBP-1 in the transcriptional regulation of the oncogene c-myc.[16] Additionally, ENO1 has the ability to bind to DNA (Deoxyribonucleic Acid), mRNA (messenger Ribonucleic Acid), lncRNA (Long noncoding RNA), and tRNA Lys (Lysin Transferase Ribonucleic Acid), thereby influencing gene transcription and translation in cancer cells.[17] This emphasizes on the significant role of ENO1 in the molecular mechanisms underlying cancer progression and metastasis.[18]

The current focus is on developing minimally invasive and non-invasive diagnostic procedures for detecting lesions. Recent discoveries in scientific technology has led to the discovery of specific salivary biomarkers for various clinical conditions, including cancer.[19] Saliva is an ideal diagnostic medium as it is easily accessed and can be collected in a non-invasive manner.[20]

Using saliva for diagnosis offers numerous benefits, such as ease of collection, minimal staff training, rapid sampling, convenient storage, simple transportation, lower susceptibility to clotting, and reduced health risks.[21] Salivary biomarkers are rapidly emerging as promising diagnostic tools, offering speed, accuracy, non-invasiveness, and higher patient acceptance.[22,23] These advantages make salivary diagnostics a valuable and patient-friendly alternative in clinical practice. Prior to this, no other previous studies have evaluated salivary alpha-enolase as a biomarker in OSMF patients. Therefore, the objective of this study was to assess the salivary alpha-enolase levels in patients with OSMF and healthy controls and comparing the results between the two groups.

2. MATERIAL AND METHODS

The study was approved by Krishna Vishwa Vidyapeeth Ethics Committee of Karad (ref no.-KVV/IEC/10/2024) India. Written informed consent was taken from all the participants before saliva collection in accordance with ethical guidelines.

2.1 Recruitment of Study Subjects

The study comprised 20 cases of OSMF and 20 healthy controls from the School of Dental sciences Karad Outpatient Department. Medical records and demographic details were collected. Patients with any infectious diseases within a month prior to saliva sampling, active dental abscesses, systemic illness, collagen vascular diseases, or were undergoing any form of medical or dental treatment were excluded. Pregnant and lactating individuals were also excluded. None of the control participants presented with oral lesions.

2.2 Sample collection

Saliva samples were obtained between 9:00 and 12:00 A.M. under non-stimulatory conditions. Participants were instructed to abstain from eating, chewing, or drinking for at least one hour prior to collection. A total of 4 to 5 milliliters of saliva was collected from OSMF patients before any therapeutic interventions. The samples were then centrifuged immediately to remove cellular debris, and the supernatant was stored at −80°C for subsequent analysis.

2.3 Estimation of alpha enolase

Alpha enolase concentrations in saliva were quantified using a commercial ELISA Kit from Bioassay Technology, following the manufacturer's instructions. The assay was performed according to the provided protocol, with absorbance measured at 450 nm using a Robonik ELISA plate reader. The results were expressed in units of nanograms per milliliter (ng/ml) for saliva samples.

2.4 Statistical analysis

The data were analyzed using IBM SPSS statistics 21. Independent sample t-test is used to compare means of two groups to determine if they are statistically different. The descriptive statistics such as mean and standard deviation were calculated for each group.

3. RESULTS

The study included 20 control participants and 20 participants with Oral Submucous Fibrosis (OSMF), matched for age and sex. The age range for the control group was 19 to 30 years, while for the OSMF group, it was 24 to 50 years. Both groups were predominantly male. All participants in the OSMF group had a history of tobacco or paan chewing, smoking, or alcohol use.

Table 1. Demographic Parameters in Healthy and OSMF Patients Descriptive statistics

	N	Range	Minimum	Maximum	Mean	Std Error	Std deviation
Healthy salivary α-enolase concentration	20	.26	.52	.78	.65	.02	.08
OSMF salivary α-enolase concentration	20	.18	.83	1.01	.93	013	.06

Table 1. shows OSMF patients salivary α-enolase concentration values (0.83–1.01) are consistently higher compared to healthy patients (0.52–0.78), indicating a potential biomarker for differentiation. OSMF patients demonstrate significantly high salivary α-enolase concentration compared to healthy individuals, which could serve as diagnostic markers. The higher variability in age among OSMF patients may reflect a broader demographic susceptibility. The minimal variability in measurements across groups suggests reliable data collection methods.

Table 2. Descriptive Statistics and Distribution Analysis of salivary α-enolase Concentrations in Healthy and OSMF Patients

			Statistic	**Std. Error**
Healthy patients salivary α-enolase concentration	Mean		.66	.01852
	95% Confidence Interval for Mean	Lower Bound	.62	
		Upper Bound	.69	
	5% Trimmed Mean		.66	
	Median		.66	
	Variance		.007	
	Std. Deviation		.083	
	Minimum		.52	
	Maximum		.78	
	Range		.26	
	Interquartile Range		.13	
	Skewness		-.211	.512
	Kurtosis		-1.13	.992
OSMF patients salivary α-enolase concentration	Mean		.93	.01309
	95% Confidence Interval for Mean	Lower Bound	.90	
		Upper Bound	.96	
	5% Trimmed Mean		.93	
	Median		.93	
	Variance		.003	
	Std. Deviation		.06	
	Minimum		.83	
	Maximum		1.01	
	Range		.18	
	Interquartile Range		.10	
	Skewness		-.09	.512
	Kurtosis		-1.22	.992

Table 2 shows α-enolase Concentration in Healthy Patients. The mean concentration is **0.66**, with minimal variability (std. deviation **0.083**) and a narrow range (**0.26**). The skewness (**-0.211**) and kurtosis (**-1.13**). The mean salivary α-enolase concentration (**0.93**) is significantly higher than in healthy patients (**0.66**), with minimal variability (std. deviation **0.06**) and a narrow range (**0.18**). The distribution metrics (skewness: **-0.09**, kurtosis: **-1.22**). OSMF patients have consistently higher salivary α-enolase concentration values than healthy individuals. These parameters could serve as biomarkers for diagnosing OSMF. Both groups show slight left skewness and flatter distributions, but the differences in means and ranges highlight clear distinctions between healthy and OSMF groups. Narrow confidence intervals and low standard errors across all metrics reflect reliable data and precise measurements.
Table 2 highlights the potential diagnostic significance of salivary α-enolase concentrations in distinguishing between healthy and OSMF patients.

Table 3. Normality Test Results for salivary α-enolase Concentration Measurements in Healthy and OSMF patients

Tests of Normality						
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
α-enolase concentration in healthy patients	.155	20	.200*	.931	20	.162
α-enolase concentration in OSMF patients	.101	20	.200*	.947	20	.327
*. This is a lower bound of the true significance.						
a. Lilliefors Significance Correction						

Table 3 indicate the tests of normality, including the Kolmogorov-Smirnov test with Lilliefors correction and the Shapiro-Wilk test, were conducted to evaluate the distribution of α-enolase concentration measurements in healthy and OSMF patients. For healthy patients α-enolase Concentration showed non-significant p-values in both tests ($p > 0.05$), indicating no significant deviation from normality. Similarly, for OSMF patients, salivary α-enolase Concentration also demonstrated non-significant results in both tests, confirming normal distribution.

Healthy patients salivary α-enolase concentration

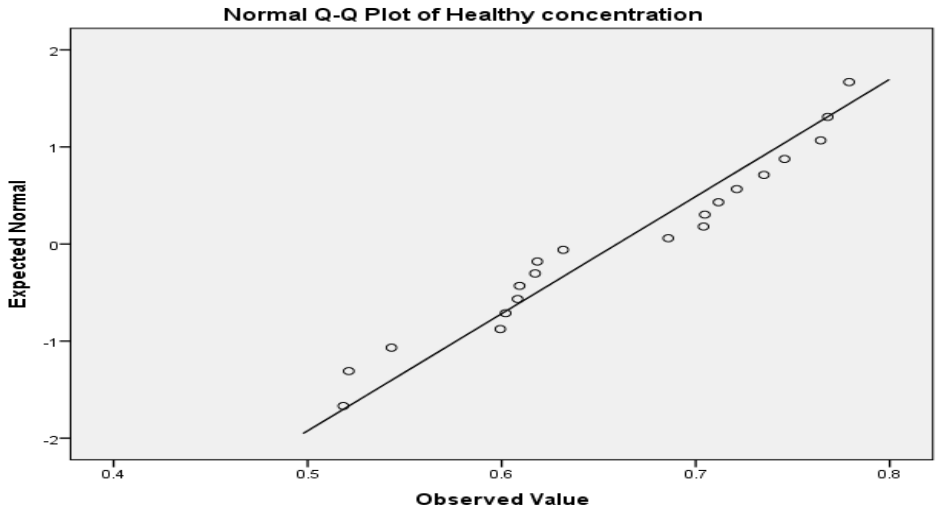


Figure 1. Normal Q-Q Plot for Healthy patients salivary α-enolase concentration

Figure 1 illustrates the Normal Q-Q Plot of salivary α-enolase concentration in healthy patients, comparing the measured values to a theoretical normal distribution. The points nearly align with the diagonal line, suggesting that the data seems to be regularly distributed. Minor deviations are noted at the extremes; nevertheless, they are insignificant, indicating that the assumption of normality applies for this dataset.

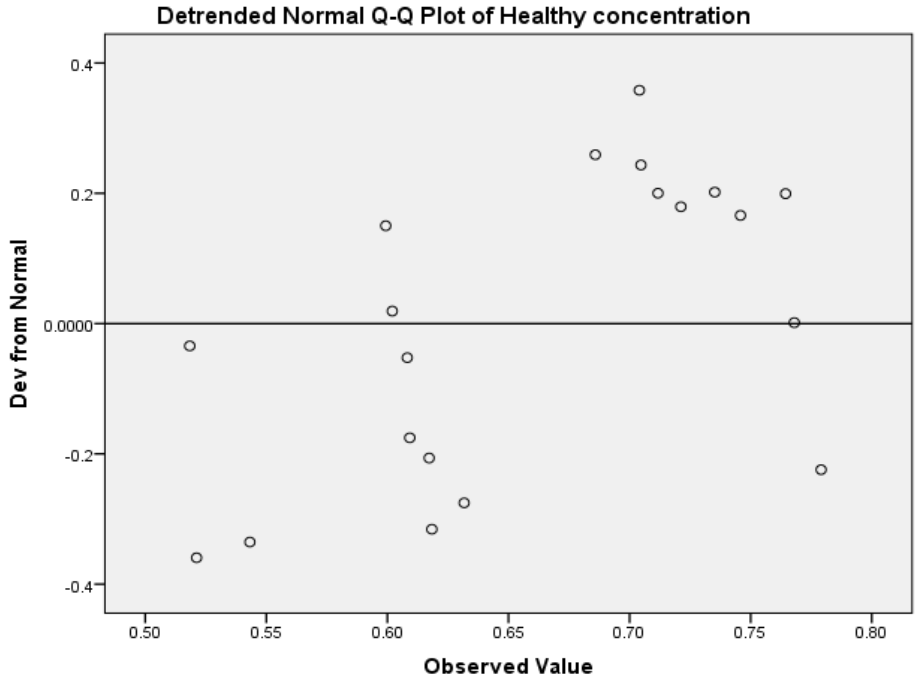


Figure 2. Detrended Q-Q Plot for Normality Assessment of Healthy patients salivary α-enolase concentration

Figure 2 illustrates the Detrended Normal Q-Q Plot of salivary α-enolase concentration deviations from normality in healthy patients by plotting the residuals (the differences between observed and predicted values under normality). The majority of dots are clustered near the zero line, signifying negligible deviations and corroborating the hypothesis of normalcy. Nevertheless, few points diverge marginally, especially near the extremes, indicating tiny deviations from an ideal normal distribution. The data seems to conform to the principles of normality.

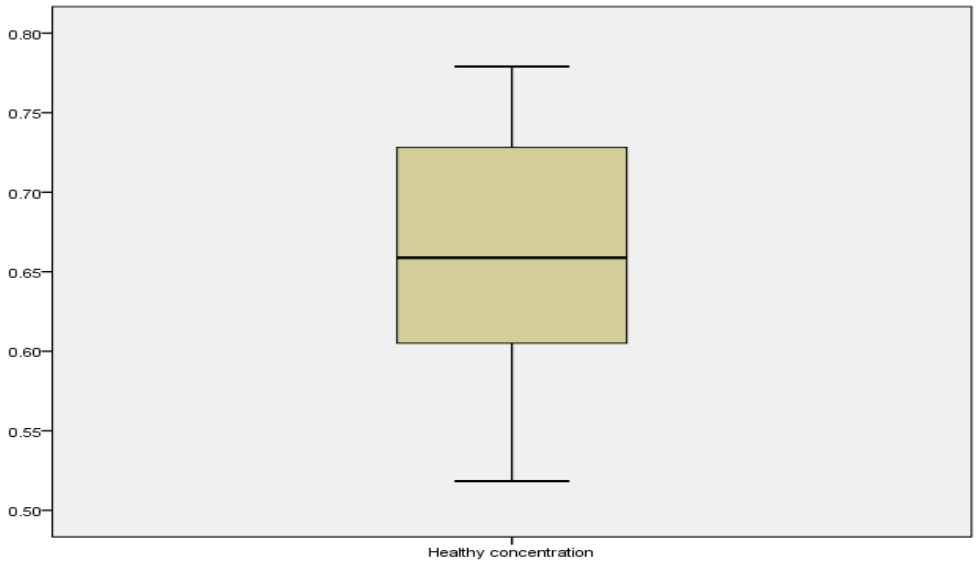


Figure 3. Boxplot of Healthy patients salivary α-enolase concentration

Figure 3 shows the boxplot for healthy patients salivary α-enolase concentration distribution of data through the interquartile range (IQR) and central tendency. The median, represented by the line inside the box, is around 0.66, indicating a central value close to 0.65. The box itself covers the middle 50% of the data, with the lower quartile around 0.05 and the upper quartile around 0.10. The whiskers extend from the box to the minimum (0.52) and maximum (0.77) values, capturing the range of the data. There appear to be no extreme outliers, as the data is relatively evenly distributed within the box and whiskers. This suggests a fairly normal distribution with moderate variability.

OSMF patients α-enolase concentration

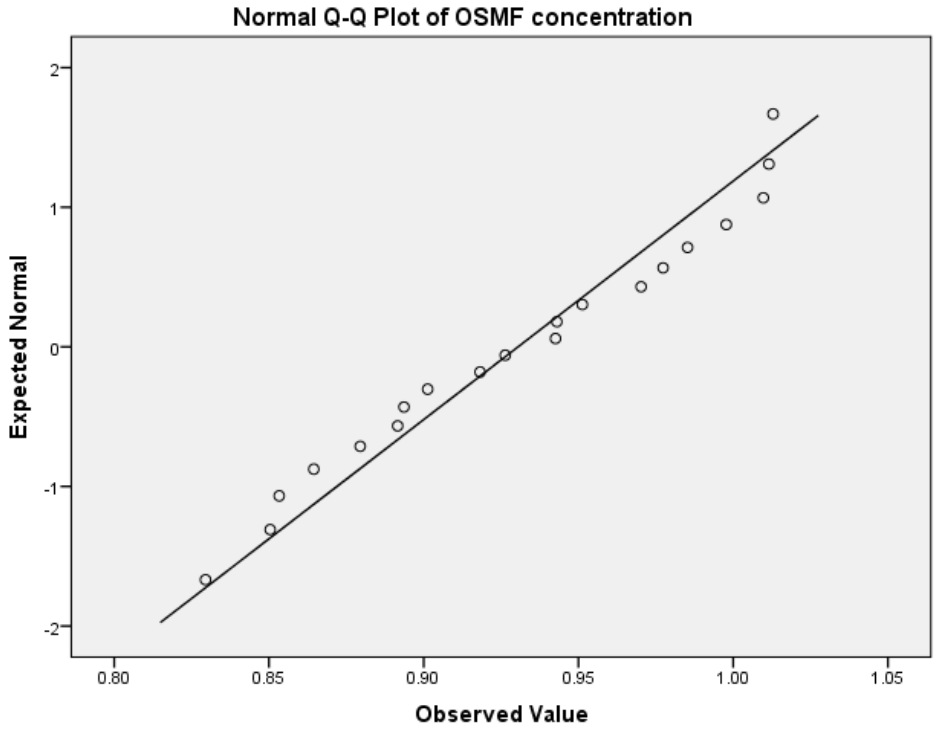


Figure 4. Normal Q-Q Plot for OSMF patients salivary α-enolase Concentration

Figure 4 illustrates the Normal Q-Q Plot of salivary α-enolase concentration in OSMF patients, comparing the observed values to a theoretical normal distribution. The points nearly align with the diagonal line, suggesting that the data is approximately regularly distributed. Minor deviations are noted at the extremes; nonetheless, they are insignificant, indicating that the assumption of normality holds for this dataset.

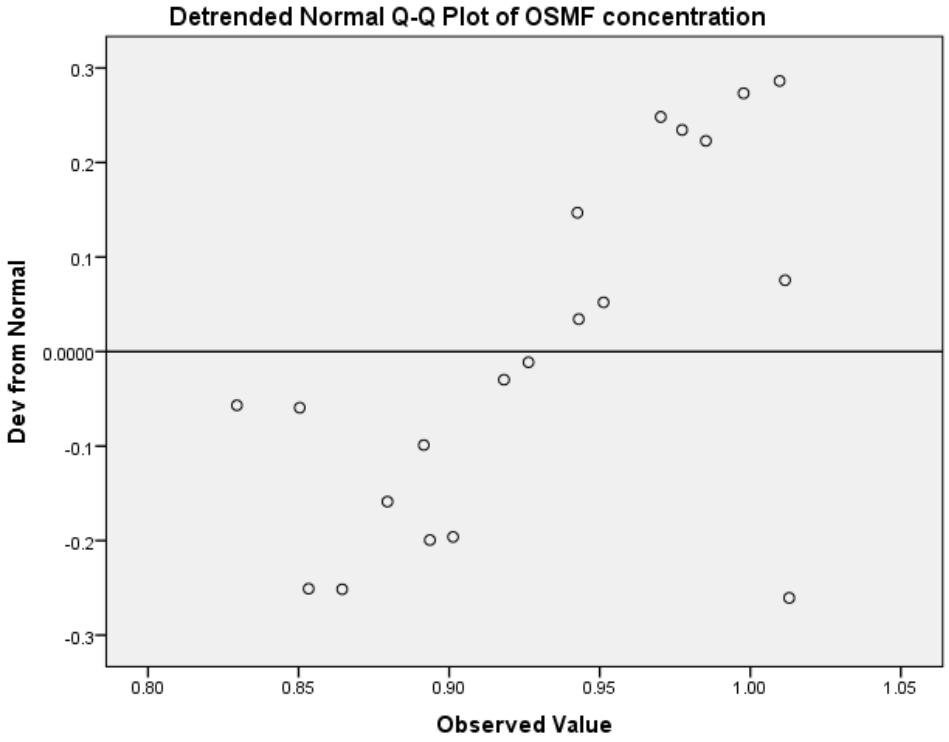


Figure 5. Detrended Q-Q Plot for Normality Assessment of OSMF patients salivary α-enolase Concentration

Figure 5 shows the Detrended Normal Q-Q Plot of OSMF patients salivary α-enolase concentration deviations from normality by plotting the residuals (differences between observed and expected values under normality). Most points are scattered closely around the zero line, indicating minimal deviations and supporting the assumption of normality. However, a few points deviate slightly, particularly at the extremes, suggesting minor departures from a perfectly normal distribution. Overall, the data appears to align well with normality.

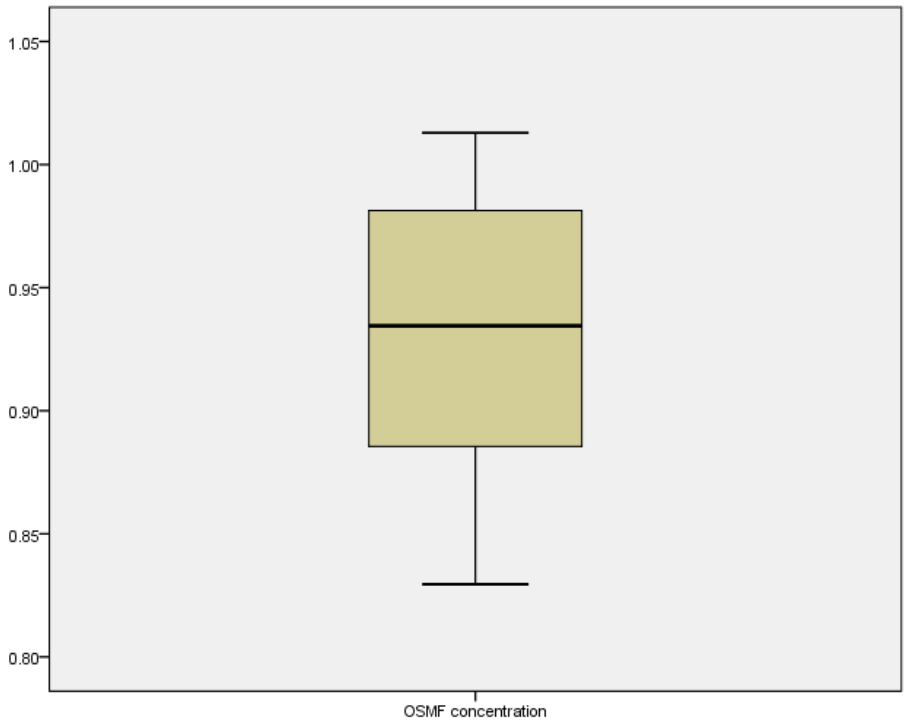


Figure 6 boxplot shows OSMF patients salivary α-enolase concentration average distribution of data through the interquartile range (IQR) and central tendency. The median, represented by the line inside the box, is around 0.93, indicating a central value close to 1. The box itself covers the middle 50% of the data, with the lower quartile around 0.05 and the upper quartile around 0.50. The whiskers extend from the box to the minimum (0.83) and maximum (1.20) values, capturing the range of the data.

Table 5. Comparison of salivary alpha enolase Concentration in ng/mL between Patients with Oral Submucous Fibrosis (OSMF) and Healthy Individuals

Comparison		N	Mean	Std. Deviation	Std. Error Mean	t	Sig. (2-tailed
Comparison of salivary alpha enolase Concentration in ng/mL in patient s with OSMF as compared to Healthy individuals	Healthy individuals	20	.66	.08	.018	-11.951	.000*

Table 5 compares the salivary α-enolase concentration (ng/mL) between patients with Oral Submucous Fibrosis (OSMF) and healthy individuals. Healthy Individuals (Mean - 0.66±0.08) had lower salivary α-enolase concentration with minimal variability. OSMF Patients (Mean - 0.93°±0.06) had higher salivary α-enolase concentration, suggesting an association with disease status. The difference (t = -11.951, Sig. (2-tailed) = 0.000) is statistically significant, indicating a clear distinction in concentration levels between groups.

The findings highlight significant differences between OSMF patients and healthy individuals in terms of concentration. Concentration levels (ng/mL) are elevated in OSMF patients. The statistical significance ($p < 0.001$) across all comparisons indicates a strong correlation between these measures and the presence of OSMF. These results suggest concentration may serve as reliable markers for identifying or studying OSMF.

Table 6. Independent Samples t-Test for Comparison of alpha enolase Concentration between Patients with OSMF and Healthy Individuals

Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Comparison of Concentration in ng/mL in patients with OSMF as compared to Healthy individuals	Equal variances assumed	4.868	.033	-11.951	38	.000*	-.27106	.02268	-.31698	-.22514
	Equal variances not assumed				34.185	.000*	-.27106	.02268	-.31714	-.22498

Table 6 shows the independent samples t-test results significant differences between patients with Oral Submucous Fibrosis (OSMF) and healthy individuals across all comparisons, for the salivary alpha enolase concentration in ng/mL, the t-test also revealed a significant difference ($t = -11.951$, $p < 0.000$), with a mean difference of -0.27106. The confidence intervals for all comparisons did not include zero, indicating robust statistical significance. Overall, these results demonstrate that patients with OSMF have notably higher concentrations than healthy individuals, highlighting potential biochemical or physiological changes associated with the condition.

4. DISCUSSION

Oral potentially malignant disorders (OPMDs), including erythroplakia, leukoplakia, and OSMF, are known to precede the development of oral squamous cell carcinoma (OSCC), with OSMF being the most commonly encountered, particularly in Southeast Asia.[24] OSMF is a chronic, insidious, and progressive condition, often leading to trismus due to submucosal fibrosis in various parts of the oral cavity. Individuals affected by OSMF face a significantly higher risk of developing oral cancer, with the malignant transformation rate to squamous cell carcinoma estimated to range between 7% and 30%.[25]

Enolase, or phosphopyruvate hydratase, was initially discovered in 1934 by Lohman and Mayerhof.[26] It is among the most widely expressed cytosolic proteins in many organisms and is essential for glycolysis, hence playing a critical role in glucose metabolism. Enolase has been associated with modified energy generation during carcinogenesis, as well as with dysregulated cellular proliferation and metastasis.[27] The ENO1 gene, encoding alpha enolase, is thought to be significantly linked to cancer formation in the oral epithelium.[28]

Assessing alpha-enolase (ENO1) levels provides an appealing strategy for monitoring potentially malignant oral illnesses and how they become malignancies. Oral submucous fibrosis (OSMF), a potentially malignant disorder, might greatly benefit from the discovery of salivary alpha-enolase as an essential biomarker for early identification and confirmation of malignant transformation.[29] Regarding the significant correlation between the ENO1 gene and oral epithelial carcinogenesis, it is important that researchers identify biomarkers that are both reliable as well as therapeutically efficient.

This study examined salivary alpha enolase levels in both groups, finding a mean value of 0.66 ng/ml for the

control group and 0.93 ng/ml for the OSMF group. The concentration of salivary alpha enolase increased in the OSMF group in comparison to the control group.

The results of this study align with previous research[30], which demonstrated an apparent spike in alpha enolase levels in biopsies of OSMF patients. The present research is the first to examine the use of saliva samples, as biopsies are intrusive and generate discomfort. This non-invasive method is a more comfortable option for patients while nevertheless providing important diagnostic data. In addition, salivary alpha-enolase levels were markedly elevated in patients with OSMF relative to the control group.

Future scope: These findings underscore the potential of salivary alpha-enolase as a reliable biomarker for identifying alterations in persons with oral submucous fibrosis and oral cancer. The found protein marker, α-enolase, may be pivotal in the early management of OSMF, potentially alleviating pain, decreasing treatment expenses, and markedly improving patients' overall quality of life.

This investigation revealed that salivary α-enolase levels in the control group exhibited relatively minor fluctuations, which lacked statistical significance. In the OSMF group, salivary α-enolase levels demonstrated a high degree of statistical significance, indicating that they may be a viable biomarker for the early detection of precancer and cancer. Nevertheless, additional research with larger sample sizes is necessary to assess salivary α-enolase levels in a variety of OSMF and OSCC grades.

Limitations: This research has specific limitations. The sample size is really limited and could not fairly reflect the general population; so, a larger sample would be required for additional validation. Furthermore, a refined comprehension of salivary alpha enolase levels in relation to clinical staging and histological grading of OSMF and OSCC may elucidate its role in the progression of oral cancer.

5. CONCLUSION

The level of alpha-enolase was markedly increased in the saliva of OSMF patients relative to the control group. A discernible, incremental rise in alpha-enolase levels was noted from the control group to OSMF patients, highlighting its potential as a dependable, non-invasive, and economical diagnostic biomarker. This finding highlights the promise of salivary alpha-enolase in detecting early changes in oral health, offering a simple yet powerful tool for clinical use**.**

List of abbreviations:

1. OSMF – Oral submucous fibrosis
2. ENO – Enolase
3. ENO1,ENO2,ENO3 – Enolase1, Enolase2, Enolase3
4. 37 kDa -37 kilo Dalton
5. MBP-1 - c-myc promoter-binding protein
6. DNA – Deoxy ribonuclic Acid
7. mRNA - messenger Ribonuclic Acid
8. lncRNA - Long noncoding RNA
9. tRNA Lys -Lysin Transferase Ribonucleic Acid
10. IQR - Interquartile range
11. PMD – Potentially malignant disorders
12. OSCC – Oral squamous cell carcinoma

DECLARATIONS

Ethical approval and consent to participate

The study was approved by the Krishna Vishwa Vidyapeeth Ethics Committee of Karad (ref no. KVV/IEC/10/2024), India. In line with ethical standards, written informed consent was obtained from all participants before saliva collection**.**

Consent for publication

This study does not include individuals any kind of information hence NOT Applicable

Availability of data and material

All data generated or analysed during this study are included in the published article [and its supplementary links are given in references].

Competing interest I declare that there are no competing interest.

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1.Study and collection - 400
2.Analysis - 25000
3.Interpretation of data
4.Manuscript writing

Authors contribution: Divyani Patil - Study of the topic data collection ‘analysis ‘interpretation of the data, manuscript writing**; Madhura Mahajan** - Study of the topic data collection ‘analysis, interpretation of the data, manuscript writing

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PREPARATION AND CHARACTERIZATION OF HYDROXYAPATITE AND CISSUS QUADRANGULARIS COMPOSITE ENTRAPPED HYDROXYPROPYL METHYLCELLULOSE SCAFFOLD FOR BONE REGENERATION-AN INVITRO STUDY

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ABSTRACT

Background: The integration of biomaterials, such as hydroxyapatite (HAp) and natural extracts like Cissus quadrangularis (CQ), into scaffolds can enhance osteogenic properties and support bone healing. This study evaluates the potential of an HAp-CQ composite embedded in a Hydroxypropyl methylcellulose (HPMC) scaffold for bone regeneration.

Material and methods: The HAp-CQ composite was synthesized using a sol-gel method. HPMC scaffolds were prepared through a solvent-casting and particulate-leaching technique, followed by the incorporation of the HAp-CQ composite. To prepare the hydroxypropyl methylcellulose scaffold with the impregnation of Cissus quadrangularis (CQ) and hydroxyapatite (HAp), 1 gram of CQ was dissolved in 10 mL of distilled water and heated at 80°C for 24 hours. Following scaffold formation, FTIR (Fourier Transform Infrared Spectroscopy), contact angle, tensile strength testing and anti-microbial activity was compared between the HAp-CQ and HPMC+HAp+CQ scaffold.

Results: The synthesized HAp-CQ composite displayed improved mechanical properties and bioactivity, with FTIR confirming functional group interactions. The tensile strength of the test group scaffold was 1.51MPa and the contact angle of the test group scaffold showed an average angle of 53.07 degrees.

C**onclusion:** The present study showed that the HPMC scaffold with the impregnation of CQ and HAp showed high tensile strength and increase in hydrophilicity. The mechanical property was increased and the antimicrobial activity showed an enhanced zone of inhibition.

Keywords: Hydroxyapatite, Cissus quadrangularis, Hydroxypropyl methylcellulose, Bone regeneration, Composite scaffold, Guided regeneration

INTRODUCTION

Periodontitis is a multifactorial inflammatory disease characterized by the progressive destruction of the supporting structures of the teeth, including the periodontal ligament and alveolar bone. It is primarily caused by bacterial infection,It causes the periodontal tissues to break down by inciting an inflammatory reaction. Periodontitis is a significant global health concern, affecting a substantial portion of the adult population. If left untreated, it can result

in tooth mobility and eventual loss, severely impacting an individual's oral health, functional capacity, and quality of life. Additionally, periodontitis has been linked to systemic health issues, including cardiovascular disease, diabetes, and respiratory disorders, highlighting the importance of effective management and treatment.[1] Periodontal bone loss is a significant pathological process associated with periodontitis, a chronic inflammatory disease that affects the supporting structures of the teeth. This condition results from the complex interaction between pathogenic bacteria in dental plaque and the host's immune response, leading to inflammation and subsequent destruction of the alveolar bone surrounding the teeth.[2] As the condition worsens, tooth mobility, diminished appearance, and eventually tooth loss may arise from the loss of periodontal attachment and the resorption of alveolar bone.

The prevalence of periodontal disease highlights the need for a deeper understanding of the mechanisms driving bone loss and the development of effective treatment strategies. Periodontal bone regeneration refers to the therapeutic strategies aimed at restoring lost alveolar bone and periodontal tissues resulting from periodontal disease, particularly periodontitis.[3] This condition leads to significant destruction of the supporting structures of teeth, including the alveolar bone, periodontal ligament, and cementum. Given the critical role of alveolar bone in maintaining tooth stability and function, effective regeneration of these tissues is essential for restoring oral health, preventing tooth mobility, and improving overall quality of life. The focus on periodontal bone regeneration has gained momentum due to the increasing prevalence of periodontal disease and its association with systemic health issues, underscoring the need for effective treatment modalities.[4]

Bone regeneration is a critical challenge in orthopedics and dentistry, necessitating the development of effective biomaterials that can promote healing and restore functionality.[5] Traditional approaches to bone repair, such as autografts and allografts, often face limitations including donor site morbidity, risk of infection, and inadequate supply.[6] Consequently, there is an increasing interest in synthetic and natural biomaterials that can serve as scaffolds for bone regeneration, providing not only structural support but also biological cues to enhance healing.[7] Hydroxyapatite (HAp) is one of the most extensively studied biomaterials for bone applications due to its biocompatibility and similarity to natural bone mineral. HAp promotes osteoconduction, allowing for the attachment and growth of bone cells.[8] However, while HAp provides a favorable environment for bone regeneration, it lacks intrinsic biological activity. To overcome this limitation, there is a growing trend to incorporate bioactive compounds into HAp-based materials, enhancing their osteogenic potential. One such compound is Cissus quadrangularis, a medicinal plant known for its traditional use in promoting bone health. Its phytochemicals have demonstrated osteogenic properties, stimulating osteoblast differentiation and enhancing collagen synthesis.[9]

To improve the stability and release profile of the bioactive components, hydroxypropyl methylcellulose (HPMC) is employed as a matrix material. HPMC is a hydrophilic polymer that offers excellent film-forming capabilities and controlled drug release properties.[10] By entrapping HAp and Cissus quadrangularis extract in an HPMC matrix, this study aims to create a composite biomaterial that synergistically combines the structural support of HAp with the bioactive properties of Cissus quadrangularis. This innovative approach has the potential to enhance bone regeneration, making it a promising candidate for clinical applications in treating periodontal bone defects and enhancing healing processes.

MATERIAL AND METHODS

To prepare the hydroxypropyl methycellulose scaffold with the impregnation of Cissus quadrangularis (CQ) and hydroxyapatite (HAp), 1 gram of CQ was dissolved in 10 mL of distilled water and heated at 80°C for 24 hours. This process facilitated the extraction of bioactive compounds from the plant material. After heating, the solution was filtered through Whatman filter paper to obtain a clear CQ extract, which was set aside for later incorporation into the hydroxypropyl methylcellulose matrix. Simultaneously, a 5% hydroxypropyl methylcellulose solution was prepared by dissolving 5 grams of hydroxypropyl methylcellulose in 100 mL of distilled water, and this mixture was kept overnight to ensure complete dissolution. Once fully dissolved, the hydroxypropyl methylcellulose solution was divided into two equal parts. The first 50 mL served as the base membrane scaffold, consisting of pure hydroxypropyl methylcellulose. To the second 50 mL, 50 mg of hydroxyapatite was added, and the mixture was stirred for 5 hours to achieve a uniform dispersion of HA. This HAp-impregnated hydroxypropyl methylcellulose solution

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was then poured into a petri dish and placed in a deep freezer at -20°C for 24 hours to promote solidification. After freezing, the scaffolds were lyophilized to remove any residual: moisture, yielding stable gelatin scaffolds ready for guided tissue regeneration applications. The materials were divided into two groups: the control group consists of hydroxypropyl methylcellulose and the test group consists the addition of cissus quadrangularis and hydroxyapatite(HAp) and hydroxypropyl methylcellulose(HPMC)

CHARACTERIZATION OF THE SCAFFOLDS

Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was used to analyze the scaffolds' functional groups (Perkin Elmer, USA). The scaffold samples were positioned on a crystal surface with attenuated total reflectance, and spectra were recorded between 400 and 4000 cm-1. To look at the existence of different components in the sample, scans were conducted over different sections of the sample.

Tensile strength

The universal testing machine (Instron Electroplus E3000, USA) was used to measure the tensile strength. 10 × 15 mm test samples were used for analysis, with a crosshead speed of 5 mm/min. Forces were applied to the specimens when the scaffolds were fitted into the analyzer. The breaking force was defined as the force at which the scaffold broke. Tensile stress was calculated by multiplying this breaking force by the specimens' area, and tensile strain was defined as the deformation at the breaking force.

Contact angle analysis

Sessile drop method analysis was performed to measure the wettability of the scaffolds. A droplet of distilled water (2 µL) was horizontally dropped on the surface of the scaffolds with the help of a micropipette. Ossila goniometer was used to determine the contact angle of the water drop formed at the interface with the scaffold sample.

Antimicrobial activity

The agar disk diffusion method was used to assess the produced scaffold's antibacterial efficacy against S. mutans. On BHI agar, the bacterial culture was uniformly swabbed. After that, the plates were incubated anaerobically for 24 hours at 37°C. Following incubation, the inhibitory zone's diameter was assessed.

RESULTS

The graph shows the Fourier-transform infrared (FTIR) spectra of two samples, labeled as HPMC (in green) and HAp-HPMC (in red). The x-axis represents the wavenumber (cm^{-1}), which is a measure of the frequency of infrared light absorbed by the sample, while the y-axis shows the transmittance (%) of infrared light through the sample. The differences between the HPMC and HAp-HPMC spectra suggest that HAp (likely hydroxyapatite) has modified the structure or composition of HPMC (hydroxypropyl methylcellulose). The green line represents the pure HPMC spectrum. The characteristic peaks here would be attributed to HPMC's molecular structure, such as C-H, C-O, and O-H stretches, depending on their wavenumbers. The red line represents the HAp-HPMC complex, showing peaks that differ from pure HPMC. The presence of HAp might introduce additional peaks or shift existing ones due to interactions between HPMC and HAp, indicating potential bonding or structural changes. To precisely interpret each peak, the wavenumber positions could be compared with a reference table of common functional groups. For instance, peaks around 1000-1200 cm^{-1} might correspond to C-O stretches, while those near 1400-1600 cm^{-1} could be related to C=C or C=O bonds (Figure 1).

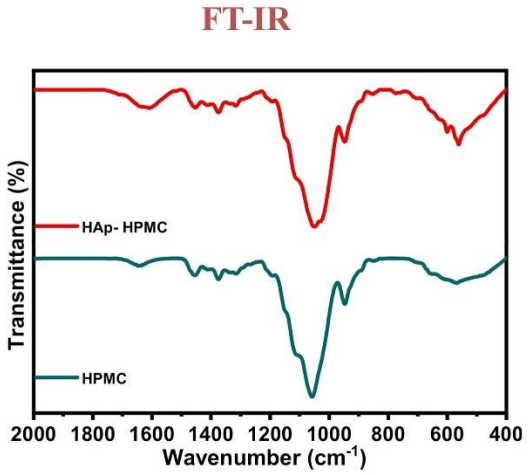
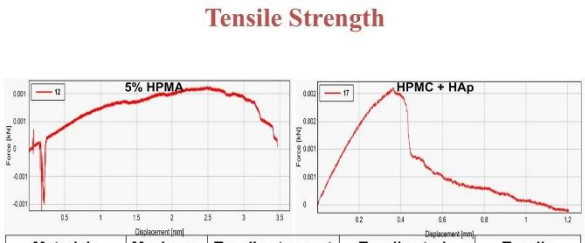


Figure 1. FTIR analysis

Snigdha Bharadwaz, Balaji Ganesh S, Vishnuvardhan Vengatachalam, Gurumoorthy Kaarthikeyan. Preparation and characterization of hydroxyapatite and cissus quadrangularis composite entrapped hydroxypropyl methylcellulose scaffold for bone regeneration - An invitro study.Bulletin of Stomatology and Maxillofacial Surgery.2025;21(3).244-249.doi:10.58240/1829006X-2025.3-244

Tensile strength was measured in newtons and it was tested using the INSTRON 3000 universal testing machine. The tensile stress for HPMC (control group) was 0.81MPa with tensile strength 8.67% at break and tensile stress of 0.12% at break, whereas with the addition of HPMC+HAp (test group) was 1.51MPa with tensile strength 3% at break and tensile stress of 0.06% at break this proves that an enhanced property of tensile strength in the test group was observed. The tensile strength of the scaffolds of HPMC and HPMC+HAp mechanical properties of material is increased (Figure 2).



Materials Name	Maximum Force [N]	Tensile stress at Tensile strength [MPa]	Tensile strain (Displacement) at Break (Standard) [%]	Tensile stress at Break (Standard) [MPa]
HPMC	1.13	0.81	8.67	0.12
HPMC+HAp	2.11	1.51	3.00	0.06

Figure 2. Tensile strength analysis

Contact angle was measured using an ossila goniometer. Test group had a contact angle of 37.75 degrees and the control group had an angle of 53.07 degrees. Hydrophilic property is more in the test group compared to the control group, which shows the test scaffold has good wettability. (Figure 3).

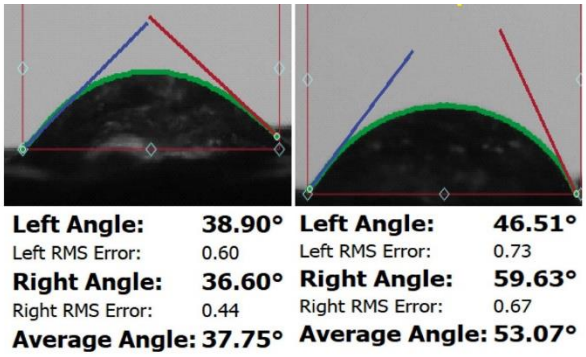


Figure 3. Contact angle analysis

The zone of diameter of S.mutans in the control group was 9 mm whereas in the test group it was 15 mm. The antibacterial activity in a cultured medium that shows the zone of inhibition of S.mutans, amoxicillin and erythromycin has been used as the antibody medium (Figure 4).

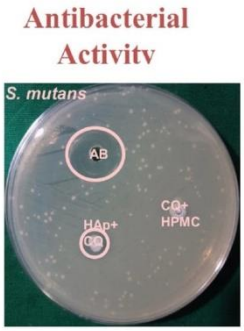


Figure 4. Antimicrobial activity

DISCUSSION

The development of hydroxyapatite (HA) Cissus quadrangularis (CQ) composite entrapped in hydroxypropyl methylcellulose (HPMC) scaffolds represents a significant advancement in the field of bone tissue engineering. Hydroxypropyl Methylcellulose (HPMC) is a versatile polymer widely used in biomedical applications due to its excellent biocompatibility, biodegradability, and ability to form hydrogels. In the context of bone tissue engineering, HPMC serves as a crucial scaffold material that provides mechanical support while allowing for the controlled release of bioactive agents. Its hydrophilic nature promotes the absorption of water, which is essential for maintaining a conducive environment for cell proliferation and differentiation. Additionally, the ability of HPMC to form a gel-like structure enhances the scaffold's porosity, facilitating nutrient diffusion and waste removal key factors in promoting osteogenesis. The incorporation of HPMC with Hydroxyapatite and Cissus quadrangularis not only enhances the scaffold's structural integrity but also enables the sustained release of bioactive compounds from the Cissus quadrangularis, potentially amplifying its osteogenic effects. Cissus quadrangularis is believed to possess anti-inflammatory and antioxidant properties that may enhance the healing process.[11] Its bioactive constituents are thought to stimulate osteoblast activity and promote the synthesis of bone matrix, crucial for effective bone regeneration.[12] Cissus quadrangularis was found to be useful for tissue engineering. Previous study showed that the cissus quadrangularis exhibits better microbial activity suitable for biomedical application.[13]

Contact angle measurements help determine the surface characteristics, with higher angles indicating more hydrophobic surfaces and lower angles indicating more hydrophilic surfaces. One crucial

factor that indicates this composite's wettability and, in turn, its biological performance is its contact angle. Greater hydrophilicity, which is necessary to encourage cell attachment and proliferation, is shown by a smaller contact angle.[14] The material's overall wettability can be increased by encasing HAp in HPMC, a hydrophilic polymer. Surface morphology and composition are also pivotal in determining the contact angle. The ratio of HAp to Cissus quadrangularis and HPMC affects the overall surface characteristics, influencing porosity and roughness.[15] These structural features can enhance surface area contact with cells and fluids, promoting better adhesion and integration with host tissue. Additionally, factors such as processing conditions and the method of composite formation can significantly alter the surface properties, necessitating careful optimization during material development. This study shows that the contact angle in the test group is better than the control group.

The addition of HAp to HPMC improves the material's tensile strength and maximum load capacity, making it stronger but also more brittle. The reduced strain at break indicates that the composite material is less stretchable and breaks at lower deformation, a common trade-off when reinforcing materials with rigid particles like HAp.[16] This analysis suggests that the HPMC+HAp composite may be more suitable for applications requiring higher strength with less concern for flexibility. Furthermore, intriguing insights are revealed when this composite is contrasted with other biomaterials, including chitosan-based composites and poly(lactic-co-glycolic acid) (PLGA). Although PLGA-based composites frequently exhibit greater tensile strength (between 50 and 70 MPa), they might not have the osteoconductivity and bioactivity that HAp provides.[17] Conversely, chitosan composites may offer superior bioactivity, but they frequently lack the mechanical strength needed for load-bearing applications.[18] In conclusion, while the tensile strength of HAp-Cissus quadrangularis-HPMC composites may not reach the levels of some synthetic polymers, the balance of mechanical properties, biocompatibility, and bioactivity positions it as a promising candidate for bone regeneration.

Overall, the HAp-CQ-HPMC composite scaffold demonstrates significant promise for clinical applications in bone regeneration. However, further research is necessary to optimize the formulation, including variations in the HAp and CQ ratios and the exploration of additional bioactive agents that could enhance its properties. Long-term studies assessing the scaffold's performance and integration with surrounding bone, as well as potential immunological responses, are essential for determining its clinical viability. This innovative approach could pave the way for more effective treatments of bone defects and ultimately improve patient outcomes in dental applications. despite current limitations in biocompatibility, mechanical strength, and regulatory pathways.

CONCLUSION

The present study showed that the HPMC scaffold with the impregnation of cissus quadrangularis and hydroxyapatite showed high tensile strength and increased hydrophilicity. In conclusion, the antimicrobial activity is more evident in HPMC+HAp as compared to HPMC control group, the tensile strength is more in HPMC+HAp. Cissus quadrangularis entrapped in a hydroxypropyl methylcellulose matrix holds promise as a good scaffold for bone regeneration.

DECLARATIONS

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Conflict of Interest

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Ethics approval and consent to participate
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Consent for publication
Not applicable.

Declaration of competing interest
The authors declare None of the authors have any relevant financial relationship(s) with a commercial interest.

Data Availability
Not applicable

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RESARCH ARTICALE

IMPACT OF BOSINTHETICALY DERIVED β-CHITOSAN TITANIUM NANOPARTICLES ON THE ENHANCMENT OF CAUDAL FIN REGENERATION ZEBRAFISH MODELS

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ABSTRACT

Background: Tissue regeneration is a crucial biological process with significant implications for developmental biology and regenerative medicine. Zebrafish (*Danio rerio*), known for their remarkable regenerative abilities, serve as an excellent model for studying tissue regeneration, particularly through the caudal fin regeneration model.
Materials and Methods: β-Chitosan-TiO_2 nanoparticles were biosynthesized using plant extracts and microbial cultures. Characterization was performed using SEM, UV-Vis and FTIR. Zebrafish caudal fin regeneration was assessed post-amputation, with nanoparticle treatment in 16 µg/L and 32 µg/L. Regeneration was analyzed via microscopic observation and gene expression. Data were statistically analyzed using ANOVA, considering $p < 0.05$ significant.
Results: β-Chitosan-TiO_2 nanoparticles exhibited uniform size, high stability, and strong biocompatibility. Zebrafish treated with β-Chitosan-TiO_2 nanoparticles showed significantly enhanced caudal fin regeneration compared to controls. Gene expression analysis supported upregulation of regenerative markers. Regenerated fin length measurements showed a dose-dependent improvement, with optimal effects at moderate nanoparticle concentrations. No significant toxicity or abnormalities were observed in treated zebrafish. Statistical analysis confirmed significant differences ($p < 0.05$) between control and treated groups, demonstrating β-Chitosan-TiO_2 NPs' potential in tissue regeneration.
Conclusion: β-Chitosan-TiO_2 nanoparticles significantly enhanced zebrafish caudal fin regeneration by promoting cell proliferation and angiogenesis without toxicity. These findings highlight their potential as a biocompatible and eco-friendly nanomaterial for tissue regeneration applications.

Keywords: tissue regeneration, zebrafish, caudal fin, chitosan, β-Chitosan Titanium nanoparticles, biosynthesis, regenerative medicine, cell proliferation, angiogenesis

INTRODUCTION

The process of tissue regeneration is a fascinating and complex biological phenomenon that has significant implications for developmental biology and regenerative medicine[1].Among various model organisms, zebrafish (*Danio rerio*) have emerged as a prominent model due to their remarkable ability to regenerate various tissues, including the caudal fin, heart, and spinal cord. The zebrafish caudal fin regeneration model is particularly valuable for studying the molecular and cellular mechanisms underlying

tissue regeneration because of its simplicity, accessibility, and the rapidity with which regeneration occurs [2,3]. This model system provides insights into the conserved mechanisms of regeneration that might be applicable to higher vertebrates, including humans. Chitosan, a naturally occurring biopolymer derived from chitin, has garnered significant attention in biomedical research due to its biocompatibility, biodegradability, and non-toxic nature.

Chitosan and its associated derivatives has been undergoing major research development in different fields on biomedical applications such as wound healing , specialized drug delivery as well as tissue engineering and enhancement[4]. In recent years, chitosan and its nanoparticle has been researched due to its superior and its enhanced attributes such as increased surface area, improved solubility and better cellular uptake, which makes them suitable for biomedical applications. β-Chitosan Titanium nanoparticles, a specific form of chitosan, is derived from the chitin of squid pen and is known for its superior solubility and lower molecular weight compared to α-chitosan derived from crustacean shells[5]. These unique properties make β-Chitosan Titanium nanoparticles an excellent candidate for nanoparticle synthesis aimed at enhancing tissue regeneration[6]. The biosynthesis of nanoparticles using biological materials, such as plants, bacteria, and fungi, offers an eco-friendly and sustainable alternative to conventional chemical synthesis methods. Biosynthetically derived nanoparticles often exhibit unique physicochemical properties and enhanced biological activities due to the presence of bioactive compounds from the biological sources used in their synthesis. In case of of tissue regeneration, biosynthetically derived nanoparticles have shown promise in promoting pluripotent cell growth, differentiation, as well as angiogenesis, which are critical in functioning because of their efficacy in tissue repair and regeneration [7]. To enhance caudal fin regeneration in zebrafish represents a novel and innovative approach in regenerative medicine. This strategy is justified by chitosan's capacity to regulate a number of biological processes that are critical for effective tissue regeneration, including inflammation, cell migration and extracellular matrix remodelling. Moreover, the nanoscale size of the particles ensures better penetration and interaction with the cellular and molecular components of the regenerating tissue. Previous studies have demonstrated the potential of chitosan and its derivatives in promoting wound healing and tissue repair. Numerous cell types, including fibroblasts and keratinocytes which are essential for tissue regeneration have been demonstrated to proliferate and migrate more readily when exposed to chitosan[8]. Furthermore, by encouraging the release of anti-inflammatory cytokines and lowering the concentration of pro-inflammatory mediators, chitosan can regulate the inflammatory response. This immunomodulatory effect is particularly important in the context of tissue regeneration, as excessive inflammation can impair the regenerative process. The biosynthesis of β-Chitosan-TiO NPs involves the use of biological agents that not only reduce the chitosan to nanoscale but also impart additional bioactive properties to the nanoparticles[9]. For instance, plant extracts used in the biosynthesis process can provide a rich source of antioxidants, phenolic compounds, and other bioactive molecules that can enhance the regenerative potential of the nanoparticles. These bioactive molecules can promote cell proliferation, protect against oxidative stress, and enhance the overall healing process. In the context of zebrafish caudal fin regeneration, the application of β-Chitosan-TiO_2 NPs can be particularly beneficial. The zebrafish caudal fin is composed of a complex structure of bone, blood vessels, nerves, and connective tissue. Successful regeneration of the fin requires the coordinated activity of various cell types, including osteoblasts, endothelial cells, and fibroblasts[10]. The ability of β-Chitosan-TiO_2 NPs to enhance the proliferation and migration of these cells can significantly improve the regenerative process. Additionally, the anti-inflammatory properties of chitosan can help in modulating the inflammatory response, thereby creating a favourable environment for tissue regeneration. Furthermore, the nanoscale size of the β-Chitosan-TiO_2 NPs allows for better interaction with the cellular components of the regenerating fin[11]. Nanoparticles can easily penetrate the cellular membranes and deliver bioactive molecules directly to the site of action. This targeted delivery can enhance the effectiveness of the nanoparticles in promoting tissue regeneration. Moreover, the sustained release of bioactive molecules from the nanoparticles can provide a prolonged stimulatory effect on the regenerating tissue. Utilizing zebrafish as a model organism to investigate the regeneration capacity of β-Chitosan-TiO_2 NPs. Zebrafish are highly amenable to genetic manipulation, which allows for the investigation of the molecular pathways involved in regeneration. Additionally, the transparent nature of zebrafish larvae enables real-time imaging of the regenerative process, providing valuable insights into the cellular and molecular dynamics of tissue regeneration[12]. The rapid regeneration of the zebrafish caudal fin, which occurs

within a few days, allows for the timely evaluation of the effects of β-Chitosan-TiO_2 NPs on the regenerative process. To evaluate the effectiveness of β-Chitosan-TiO_2 NPs in enhancing caudal fin regeneration in zebrafish, several experimental approaches can be employed. The regeneration process can be monitored using histological analysis, which provides detailed information about the structural changes occurring during regeneration. Immunohistochemical staining can be used to identify specific cell types and molecular markers involved in the regenerative process. Additionally, gene expression analysis can provide insights into the molecular pathways modulated by the β-Chitosan-TiO NPs. The gene expression level involved in cell proliferation, inflammation, and extracellular matrix remodelling can be quantified to understand the mechanisms by which the nanoparticles enhance regeneration. The synthesis, characterization, and evaluation of biosynthetically derived β-Chitosan-TiO NPs for enhancing caudal fin regeneration in zebrafish represent a promising and innovative approach in regenerative medicine. The unique properties of β-Chitosan-TiO_2 NPs, combined with the bioactive molecules from the biosynthesis process, offer significant potential for promoting tissue regeneration. The use of zebrafish as a model system provides a valuable platform for investigating the molecular and cellular mechanisms underlying the regenerative potential of these nanoparticles. This study aims to offer an in depth understanding and analysis of β-Chitosan-TiO NPs influence tissue regenerative and open avenue for their possible use in clinical applications of tissue injury and degenerative diseases[13].

2.MATERIALS AND METHODS

2.1 Synthesis of β-Chitosan Titanium Nanoparticles

The β-Chitosan-TiO_2 NPs were synthesized using a green biosynthesis approach. Initially, β-Chitosan-TiO_2 NPs was extracted from squid pen through a process involving deacetylation with sodium hydroxide. The resultant β-Chitosan-TiO_2 NPs was then dissolved in a 1% acetic acid solution to form a homogenous chitosan solution. For the biosynthesis of nanoparticles, an aqueous extract of a selected plant known for its reducing properties was prepared. The chitosan solution received the plant extract slowly while being stirred continuously[14].The blend was kept at room temperature and mixed for hours until a colour change indicated the formation of nanoparticles. The suspension obtained was subjected to centrifugation at 10,000 rpm's for a duration of 15 minutes to isolate the nanoparticle. Following this, the nanoparticles were rinsed in deionised water and subsequently dried at a temperature of 60^0C. FTIR and UV-Vis were used to analyse the produced chitosan particles in order to verify their size and crystalline properties.

2.2 Characterization of β-Chitosan Titanium Nanoparticles

FTIR analysis was conducted to determine the different functional groups present in the β-Chitosan-TiO_2 NPs. The spectra were captured between the range of 4000–400 cm^{-1}. UV-Vis spectroscopy was utilized to determine the optical characteristics of the nanoparticles, with absorbance was identified from 200 to 800 nm.

2.3 Zebrafish Maintenance and Caudal Fin Amputation

Adult zebrafish (*Danio rerio)* were maintained under standard laboratory conditions at 28°C with a maximum 14/10 hour light/dark cycle. Fish was fed twice every day using commercial fish food. For the caudal fin regeneration studies, adult zebrafish were anesthetized using 0.02% tricaine methanesulfonate (MS-222) solution. Under a dissecting microscope, approximately one-third of the total length of the caudal fin was amputated with the help of a sterile scalpel. The fish were then returned to fresh water for recovery. Post-amputation, the fish was then separated into two groups: the control and experimental groups. The experimental group used β-Chitosan-TiO_2 NPs by immersing the fish in water containing 16 µg/mL and 32 µg/mL of nanoparticles, while the control group was immersed in nanoparticle-free water[15].

2.4 Assessment of Caudal Fin Regeneration

The regeneration of the caudal fin was monitored over a period of 14 days. At 0, 3, 7, and 14 days post-amputation, the zebrafish were anesthetized and an image was taken using a digital camera. The length of the regenerated fin was calculated using ImageJ software. The percentage of fin regeneration was measured by comparing the length of the regenerated fin to the original fin length before amputation.

2.5 Gene Expression Analysis by RT-PCR

To understand the molecular mechanisms underlying the enhanced regeneration, gene expression analysis was conducted. Total RNA was extracted from the regenerating fin tissues using TRIzol reagent. The RNA was quantified using a Nanodrop spectrophotometer, and its structure was confirmed by gel electrophoresis, cDNA was obtained from 1 µg of total RNA using a reverse transcriptase kit. Quantitative real-time PCR (RT-PCR) was performed to assess the expression

levels of gene expression involved in regeneration, including Bax, Bcl-2, TNF-α, NF-κB, and TGF-β. Specific primers were designed for each gene, and their sequences are provided in Table 1. The RT-PCR reactions are carried out using a SYBR Green Master Mix in a real-time PCR system. The comparative level for target gene was normalized due to the expression of beta- actin, used as an internal control agent. The comparative Ct methods (ΔΔCt) calculates the fold change in gene expression [16]

RESULTS

3.1. Scanning Electron Microscopic (SEM)

The structure and size of the synthesized material was examined using the Scanning Electron Spectroscopy (SEM). The obtained particle β-Chitosan-TiO_2 NPs two main morphological structure, ie, a long rod and small cubes. Both appeared to be well distributed within, as well as little to no agglomeration. The size of β-Chitosan-TiO_2 NPs was measured using SEM micrographs. The particle had the diameter of 120-150nm and 70-90 nm respectively. The average size was calculated from multiple images and approximately,130±15 nm and 75±10 nm respectively. Due to van der Waals force produced in sample preparation; few aggregated samples of particles were noticed in the result. However, the overall distribution was achieved. These results confirmed the formation and stability of the nanoparticle (Figure 1).

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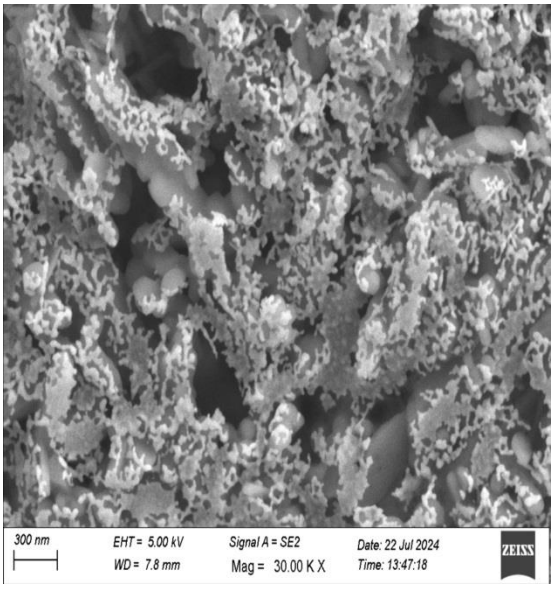


Figure 1. SEM Image of β-Chitosan Titanium Nanoparticles (β-Chitosan-TiO_2 NPs)

3.2 UV-Vis spectroscopy analysis

UV-Vis spectroscopy showed a distinct absorption peak around 300 nm, indicative of the nanoparticles' presence and stability in aqueous solution (Figure 2).

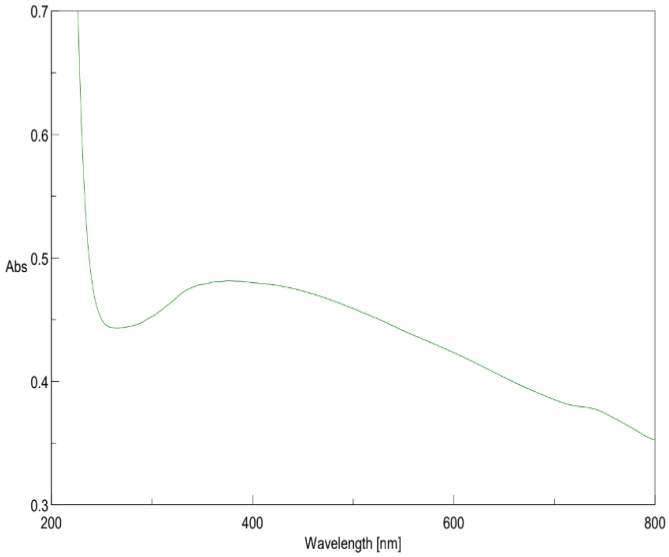


Figure 2. UV-Vis absorption spectra of β-Chitosan Titanium Nanoparticles (β-Chitosan-TiO NPs)

3.3 FTIR analysis

FTIR analysis of biosynthesized β-Chitosan-TiO_2 NPs was conducted to identify presumed functional groups present in the extracts as well as to determine the involvement of potential bioactive compounds in reducing Ti^{4+} to Ti^0, and in the capping and stabilization of bio-reduced β-Chitosan-TiO NPs. in Figure 3 of the IR spectrum, a broad peak at 3,371 cm^{-1} was attributed to the O–H stretching vibration of the alcohol functionality. In contrast, a broadened peak with lower intensity into the IR spectrum of TiO_2 NPs, in comparison to the FTIR of the extract, is recorded in and around 3,400 cm^{-1}, indicating the participation of bioactive compounds with OH groups in the formation of TiO NPs. Some other significant peaks were recorded at 2,890 cm^{-1} and a slightly split peak at 1,639 cm^{-1}, corresponding to C–H and C═C fused with C═O stretching vibrations of alkane groups and ketones, respectively. A prominent peak around 499 cm^{-1} in the FTIR spectrum of TiO_2 NPs, matching the metal–oxygen (M–O) vibration, supported the formation of nanoparticles. Spectral records of the extract suggest the presence of phytochemicals such as phenols, terpenes, as well as flavonoids likely played an integral role in the reduction of the metallic ions to metal (Figure 3).

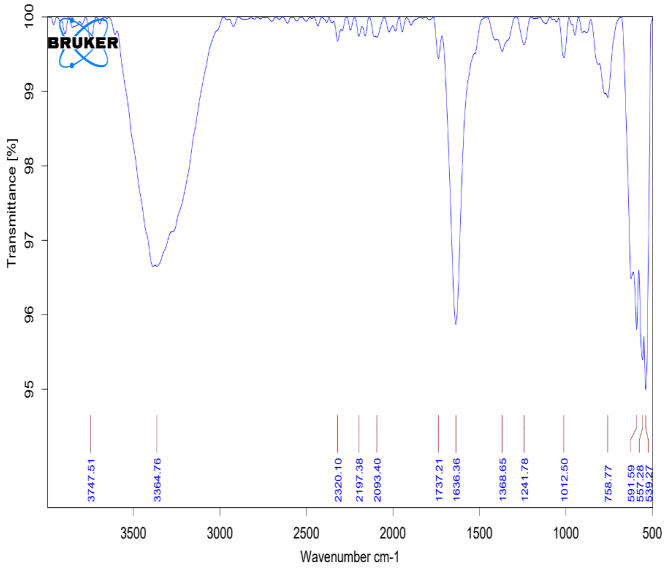


Figure 3. FTIR spectra of β-Chitosan Titanium Nanoparticles (β-Chitosan-TiO NPs)

3.4 Zebrafish Caudal Fin Regeneration

The application of β-Chitosan-TiO_2 NPs significantly enhanced the regeneration of the caudal fin in zebrafish. Quantitative measurements of the fin length at 0-, 3-, 7-, and 14-days post-amputation showed that the experimental groups of 1 and 2, treated with β-Chitosan-TiO_2 NPs in 16 µg/mL and 32 µg/mL (based on the less toxicity range of above synthesized nanoparticles) respectively exhibited a markedly higher rate of regeneration than the control group (Figure 4).

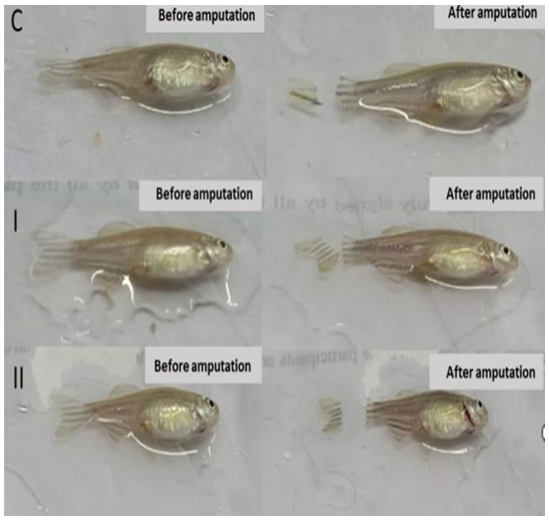


Figure 4. Caudal fin amputation in control and β-Chitosan Titanium Nanoparticles(β-Chitosan-TiO NPs) treated groups

By day 14, the experimental group 2 had shown an increased rate of average fin regeneration by 85%, whereas the control group demonstrated only 60% regeneration (Figure 5).

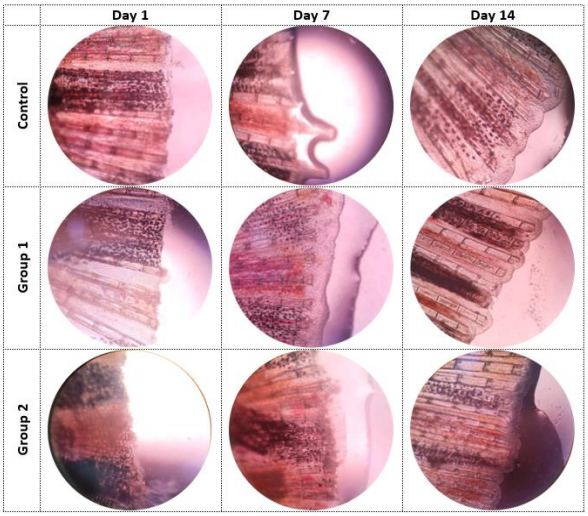


Figure 5. Microscopic observation of Caudal fin amputation in control and β-Chitosan Titanium nanoparticles (β-Chitosan-TiO_2 NPs) treated Groups

3.5 Gene Expression Analysis

The RT-PCR results provided insights into the molecular mechanisms by which β-Chitosan-TiO_2 NPs enhanced fin regeneration. The expression levels of Bax, a pro-apoptotic gene, were significantly upregulated in the nanoparticle-treated group, indicating increased apoptosis in damaged cells, which is a crucial step for tissue regeneration. In contrast, the expression of Bcl-2, an anti-apoptotic gene, was downregulated, further supporting the induction of apoptosis. These changes in apoptotic markers suggest that β-Chitosan-TiO NPs facilitate the removal of damaged cells thereby promoting the regeneration process. The anti-inflammatory properties of β-Citosan TiO NPs likely contribute to creating a favourable environment for tissue regeneration by reducing inflammation and promoting healing.

3.5.1 Expression of Bax in fin regeneration

β-Chitosan-TiO_2 NPs have been shown to enhance Bax expression during caudal fin regeneration, suggesting their role in modulating apoptosis. Bax, a pro-apoptotic protein, plays a crucial role in programmed cell death, which is essential for tissue remodelling and regeneration. The upregulation of Bax may indicate the involvement of β-Chitosan-TiO_2 NPs in facilitating controlled cell turnover,

ensuring proper regeneration of the caudal fin (Figure 6).

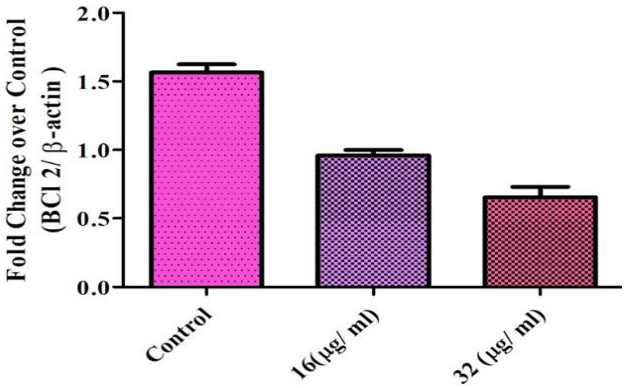
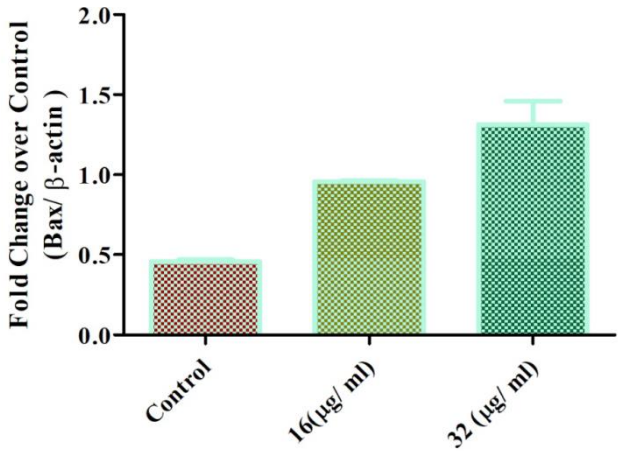


Figure 6. β-Chitosan Titanium Nanoparticles (β-Chitosan-TiO NPs) increased Bax expression on Caudal fin regeneration

3.5.2 Expression of Bcl2 in fin regeneration

β-Chitosan-TiO_2 NPs have been found to reduce Bcl-2 expression during caudal fin regeneration, indicating their influence on apoptotic regulation. Bcl-2, an anti-apoptotic protein, plays a key role in cell survival by inhibiting programmed cell death. The downregulation of Bcl-2 suggests that β-Chitosan-TiO_2 NPs may promote a balanced apoptotic response, which is crucial for effective tissue remodelling and regeneration of the caudal fin (Figure 7).

Figure 7. β-Chitosan Titanium Nanoparticles (β-

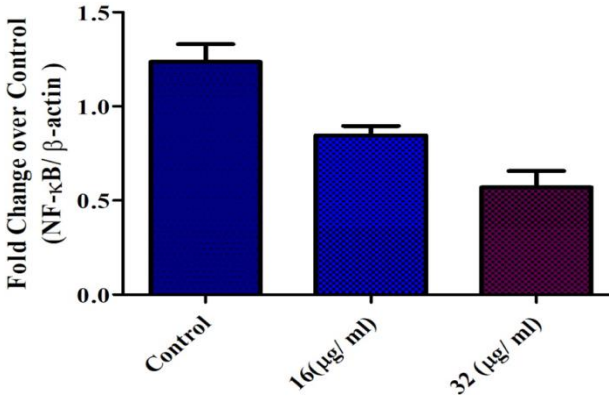


Chitosan-TiONPs) decreased BCl2 expression on Caudal fin regeneration

3.5.3 Expression of NF-kB in fin regeneration

β-Chitosan-TiO_2 NPs have been observed to suppress NF-κB expression during caudal fin regeneration, suggesting their potential role in modulating inflammatory pathways. NF-κB is a key transcription factor involved in regulating inflammation and cell survival. Its reduced expression may indicate that β-Chitosan-TiO_2 NPs contribute to a controlled inflammatory response, which is essential for efficient tissue repair and regeneration of the caudal fin (Figure 8).

Figure 8. β-Chitosan Titanium Nanoparticles (β-

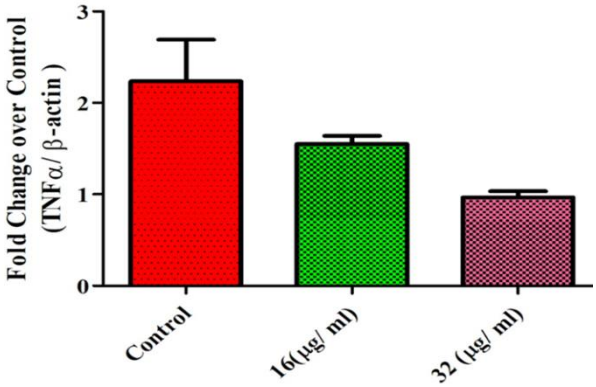


Chitosan-TiO_2 NPs) decreased NF-kB expression on Caudal fin regeneration

3.4.4 Expression of TGF-β in fin regeneration

The expression of TGF-β, a multifunctional cytokine involved in cell proliferation, differentiation, and extracellular matrix production, was moderately increased in the nanoparticle-treated group. TGF-β plays a critical role in tissue regeneration by promoting the growth of new blood vessels (angiogenesis) and the formation of extracellular matrix components necessary for tissue repair. The upregulation of TGF-β in the treated group indicates that β-Chitosan-TiO_2 NPs may enhance these regenerative processes, further supporting the observed improvements in fin regeneration (Figure 9).

Figure 9. β-Chitosan Titanium Nanoparticles (β-

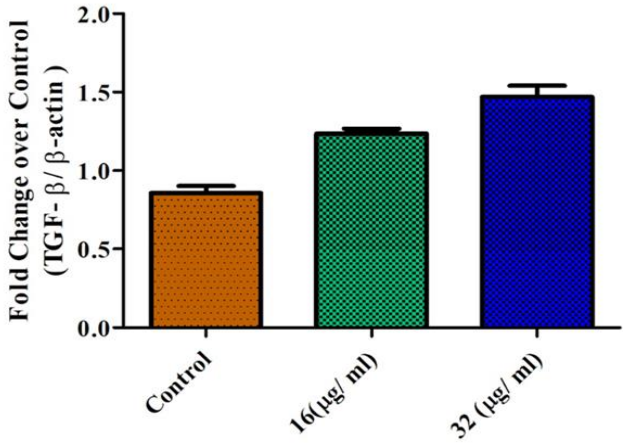


Chitosan-TiO_2 NPs) increased TGF-β expression on Caudal fin regeneration

3.5.5 Expression of in TNF-α fin regeneration

β-Chitosan-TiO_2 NPs have been shown to reduce TNF-α expression during caudal fin regeneration, indicating their potential anti-inflammatory effects. TNF-α is a pro-inflammatory cytokine that plays a crucial role in immune responses and tissue remodelling. Its downregulation suggests that β-Chitosan-TiO_2 NPs may help in minimizing excessive inflammation, thereby promoting a favourable environment for efficient fin regeneration (Figure 10).

Figure 10. β-Chitosan Titanium Nanoparticles (β-Chitosan-TiO NPs) decreased TNFα expression on



Caudal fin regeneration

DISCUSSION

The intersection of zebrafish caudal fin regeneration, chitosan-based nanoparticles, and biosynthetic nanoparticle technology presents a compelling narrative in the field of regenerative medicine. Zebrafish (*Danio rerio*) serve as a remarkable model organism due to their innate ability to regenerate complex tissues like the caudal fin, heart, and spinal cord. This regenerative capacity offers valuable insights into the underlying molecular and cellular mechanisms that could potentially translate to therapeutic applications in humans. Chitosan, derived from chitin, has garnered significant attention in biomedical research owing to its biocompatibility, biodegradability, and non-toxic nature[17]. The emergence of chitosan nanoparticles has further rise in its applicability in biomedical fields such as wound healing, specialized drug delivery, and tissue repair and engineering. β-Chitosan-TiO_2 NPs, derived from squid pen chitin, stand out due to their superior solubility and lower molecular weight compared to α-chitosan from crustacean shells. These nanoparticles are particularly promising for enhancing tissue regeneration, given their enhanced properties like increased surface area and improved cellular uptake[18]. The biosynthesis of nanoparticles using biological materials offers a sustainable and eco-friendly alternative to conventional chemical synthesis methods. These biosynthetically derived nanoparticles often exhibit unique physicochemical properties and biological activities due to the presence of bioactive compounds from their biological sources. In the context of tissue regeneration, such nanoparticles have shown potential in promoting critical processes like cell proliferation, differentiation, and angiogenesis, all crucial for effective tissue repair[19]. Enhancing caudal fin regeneration in zebrafish represents a novel approach in regenerative medicine, leveraging the multifaceted properties of chitosan nanoparticles. Chitosan's ability to modulate biological processes such as inflammation, cell migration, and extracellular matrix remodelling is pivotal for successful tissue regeneration. Moreover, the nanoscale dimensions of these particles facilitate deeper penetration and interaction with cellular and molecular components during tissue regeneration. Previous studies underscore chitosan's role in promoting wound healing and tissue repair through its effects on cell proliferation and migration. These attributes make chitosan and its derivatives promising candidates for therapeutic applications aimed at enhancing tissue regeneration in clinical settings. By harnessing the regenerative insights from zebrafish models and combining them with advanced nanoparticle technology, researchers are poised to develop innovative strategies that could revolutionize regenerative medicine[20].

Moving forward, extensive research is needed to enumerate precise functioning through which chitosan nanoparticles facilitate tissue regeneration. Understanding these mechanisms will enable the design of targeted therapies that optimize regeneration outcomes in diverse clinical scenarios. Additionally, the development of scalable methods for nanoparticle synthesis and rigorous preclinical studies will be crucial steps towards translating these findings into clinical applications. The convergence of zebrafish caudal fin regeneration, chitosan-based nanoparticles, and biosynthetic nanoparticle technology holds immense promise for advancing regenerative medicine. By capitalizing on nature's regenerative models and cutting-edge nanotechnology, researchers can pave the way for transformative therapies that enhance tissue repair and regeneration in humans[21,22].

CONCLUSION

The results of this study have had significant implications for the field of regenerative medicine. The capacity of β-Chitosan-TiO_2 NPs to enhance tissue regeneration in zebrafish suggests their potential application in treating injuries and degenerative conditions in humans. The biocompatibility, biodegradability, and non-toxic nature of chitosan make it an attractive material for clinical applications. Moreover, the green synthesis approach used in this study aligns with the principles of sustainable and environmentally friendly medical practices. Future studies should focus on elucidating the detailed molecular pathways involved and conducting in vivo studies in mammalian models to further validate the therapeutic potential of β-Chitosan-TiO NPs.

DECLARATIONS

Funding

No sources of funding.

Data availability

Conflict of interest

No conflict of interest is declared by all the authors.

Consent to participate

Informed consent was obtained from all the patients and their legal guardians by informing and clearly explaining the details of the study.

Consent for publication

Informed consent was obtained from all the patients and their legal guardians by informing and clearly explaining the details of the study.

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RESEARCH ARTICLE

PERIODONTAL ASSESSMENT IN PATIENTS WITH ORAL POTENTIALLY MALIGNANT DISORDERS – A RETROSPECTIVE STUDY

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ABSTRACT

Background: Periodontal disease is a chronic multifactorial disease while potential malignant disorders are precancerous lesions mostly associated with habits. The aim of the study is to assess the periodontal health in patients diagnosed with oral potentially malignant disorders(PMD) in a private hospital in Chennai, India.

Materials and Methods: The study was a retrospective study and was done under a university setting. Total number of sample size includes 209 patients who have been diagnosed with PMD. The data was obtained from the category of Mucosal lesions, leukoplakia, OSMF, lichen planus , actinic cheilitis and lupus erythematosus. General history including age, sex, adverse oral habits associated and different types of periodontal diseases were tabulated. The data was all tabulated and exported to SPSS for association and analysis using Chi-square test.

Results: There was a significant association found between tobacco usage and periodontal diseases(p=0.038). The association between periodontal diseases and potentially malignant disorders was not statistically significant since p=0.090.

Conclusion: The study concluded that a positive relation is established between smokeless tobacco with generalised chronic gingivitis and smoking tobacco with generalised chronic periodontitis. In patients with leukoplakia and lichen planus, generalized chronic periodontitis was commonly seen. In patients with oral submucous fibrosis, generalised chronic gingivitis was commonly seen. The study also concluded that a possible relationship can be established in a wide scale research between periodontal disease and oral potentially malignant disorders.

Keywords: Actinic Cheilitis; Leukoplakia; Oral Submucous Fibrosis; Periodontal assessment; Potentially Malignant Disorders

INTRODUCTION

Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity.[1] In accordance with this definition by the According to World Health Organization, periodontal health should be defined as a state free from inflammatory periodontal disease that allows an individual to function normally and not suffer any consequences (mental or physical) as a result of past disease.[2] However, while this definition is holistic and patient outcome based, it seems an impractical and limiting definition for the purposes of clinical management of periodontal diseases. Therefore, a more practical and absolute definition of periodontal health would be a state free from inflammatory periodontal disease. This, in turn, means that absence of inflammation associated with gingivitis or periodontitis, as assessed clinically, is a prerequisite for defining periodontal health. Periodontal disease is the second-most prevalent oral pathology, after dental caries, and has been described among populations of all ages throughout the world.[3]

A wide range of factors has been demonstrated as significantly associated with periodontal disease,including dental plaque, systemic diseases, medications, psychosocial factors and smoking.[4,5]

Many inflammatory biomarkers are also associated with periodontal diseases such as endothelin-1 and tumour necrosis factor which were reported in literature.[6-9]

Potentially Malignant Disorders (PMD) is defined by WHO 2005 as the risk of malignancy being present in a lesion or condition either at time of initial diagnosis or at a future date. No factor has been identified to be the causative factor for potentially malignant disorders. But a number of high risk factors have been put forward which have greater than normal risk of malignancy at a future date.[10,11]

Many etiological factors have been put forward by various authors, some etiological factors are Tobacco, Alcohol-synergistic action along with tobacco, Virus infection(HSV,HPV,EBV,HIV), Bacterial infection(*Treponema pallidum*), Fungal infection(Candida), Electro-galvanic restorative metals, Ultraviolet radiation from sunlight - associated with lip lesions(actinic chelitis), Chronic inflammation or irritation from sharp teeth or chronic cheek-bite (tissue modifiers rather than true carcinogens), Genetic (5% are hereditary), Immunosuppression – organ transplant, HIV, Malnutrition – iron (anemia), vitamin A, B, C deficiency.[12]

Average age of the population affected with PMDs is 50-69yrs, occurring about five years earlier than oral cancer. However recent studies conducted show that 1- 5% of PMDs affect the younger age group of 30 years. This may be due to the fact that various extrinsic and intrinsic etiological factors are now more prevalent in today's younger population.[13] Most common site in the oral cavity for PMDs in India are buccal mucosa followed by tongue, palate and floor of the mouth. Location of PMDs differs from the distribution of OSCC, for which the tongue, alveolar ridge and floor of mouth are the most common sites.[14]

The PMDs considered in this study are leukoplakia, oral submucous fibrosis (OSMF), actinic cheilitis, erosive lichen planus and lupus erythematosus. Leukoplakia - 'The term leukoplakia should be used to recognize white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer. Furthermore leukoplakia is a clinical term and the lesion has no specific histology.[15]

Oral Submucous Fibrosis - Oral submucous fibrosis (OSMF) is a chronic disorder characterized by fibrosis of the lining mucosa of the upper digestive tract involving the oral cavity, oropharynx and frequently the upper third of the oesophagus.[16]Lichen Planus - Lichen planus is a chronic inflammatory disorder demonstrating some immune pathology.[17] Actinic cheilitis - Actinic cheilitis is considered to represent a potentially malignant condition of the lip .

The squamous epithelium of the lip vermilion may be hyperplastic or atrophic and shows disordered maturation, varying degrees of keratinization, cytological atypia and increased mitotic activity on microscopic examination.[18] Lupus erythematosus - Lupus erythematosus is a chronic autoimmune disease which can be subdivided into three forms; the systemic, drug-induced and discoid. It is the latter benign variant that commonly affects the skin and may involve the mucosal surface of lips and the oral cavity. The disease is driven by an immune complex deposition in affected sites, leading to vasculitis.[19]

No previous articles were present in literature regarding the periodontal status in patients diagnosed with potentially malignant disorders. Studies on prevalence, case reports and periodontal management of leukoplakia, OSMF, Lichen planus, actinic cheilitis and lupus erythematosus were present in the database.[20-23] The idea for this research is stemmed from the current interest in our community and its cultural habits. The study will help in emphasis of periodontal assessment and importance of their assessment in patients diagnosed with PMD of oral cavity. The aim of the study is to assess the periodontal health in patients diagnosed with oral potentially malignant disorders in a private hospital in Chennai, India.

MATERIALS AND METHODS

The study was a retrospective study and was done under a university setting. The study was approved by the scientific review board of the institution. One principal investigator and 2 co-investigators were involved in the study. Total number of sample size includes 209 patients who have been diagnosed with PMD. The case sheets were verified with the help of photographs. The data was obtained from the category of Mucosal lesions, leukoplakia, OSMF, lichen planus , actinic cheilitis and lupus erythematosus. General history including age, sex, adverse oral habits associated and different types of periodontal diseases were tabulated. Data was verified by one external reviewer. If a variant of the particular PMD was not mentioned, the sample was excluded from the study. The data was imported to SPSS and the variables were verified. Chi-square test was done on the data obtained using SPSS software by IBM.

RESULTS

The data collected from the patient management software were tabulated in SPSS and the descriptive statistics were obtained. Out of 209 patients who were taken under study, 88 patients were diagnosed with leukoplakia, 91 patients were diagnosed with OSMF, 28 patients with Erosive lichen planus, 1 patient each under the category of actinic cheilitis and lupus erythematosus (Table1,Graph1).

Table 1.The table shows the frequency and percentage of various potentially malignant disorders included in the study.

POTENTIAL MALIGNANT DISORDERS		
	Frequency	Percentage
Leukoplakia	88	42.1
Lichen Planus	28	13.4
Oral Submucous Fibrosis	91	43.5
Actinic Cheilitis	1	.5
Lupus Erythematosus	1	.5
Total	209	100.0

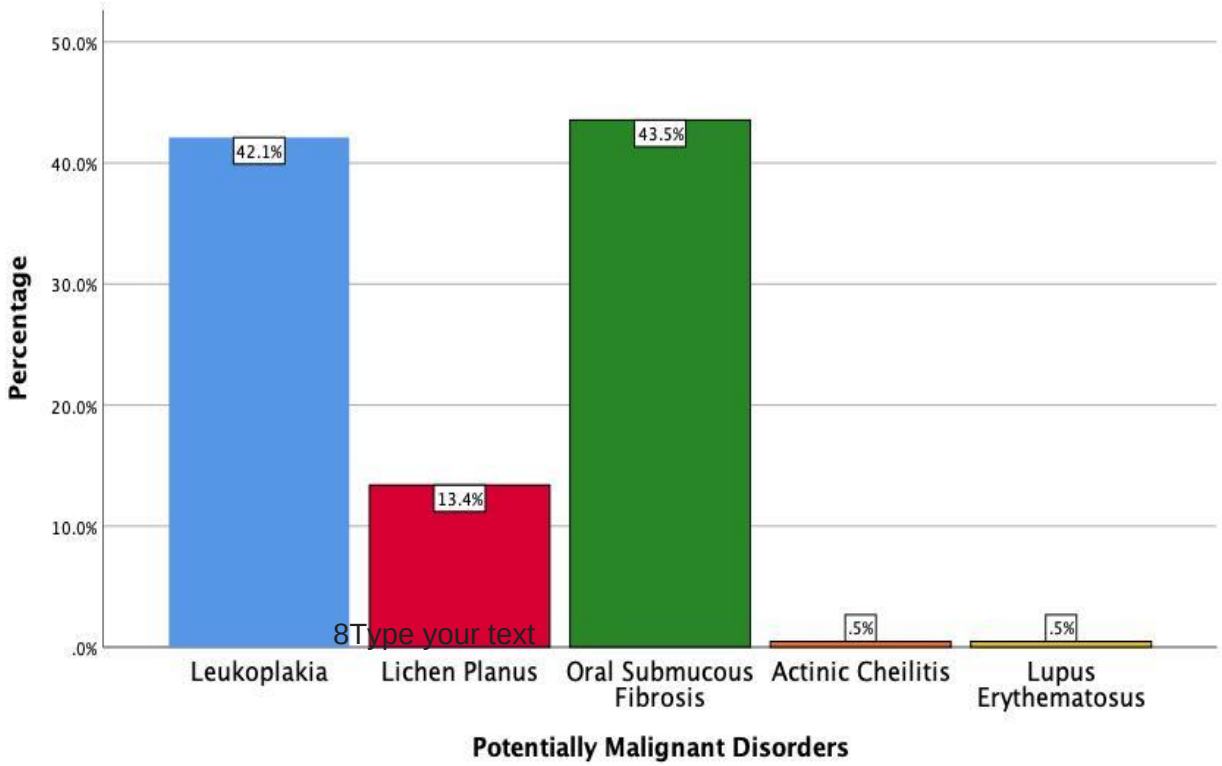


Figure 1. This graph represents the percentage of various potentially malignant disorders involved in the study population, X axis denotes all the potentially malignant disorders in the study and Y axis denotes its percentage, where blue denotes leukoplakia, red denotes lichen planus, green denotes oral submucous fibrosis, orange denotes actinic cheilitis and yellow depicts lupus erythematosus. This graph shows the most common potential malignant disorder involved in the study was oral submucous fibrosis(43.54%).

85 patients were diagnosed with generalised chronic gingivitis, 90 patients were diagnosed with generalised chronic periodontitis, 31 patients were diagnosed with generalised chronic gingivitis with localised periodontitis, 2 patients were diagnosed with localised chronic gingivitis and 1 patients was diagnosed with clinically healthy gingiva under periodontal assessment (Table 2 & Graph 2).

Table 2. This table shows us the frequency and percentage of the periodontal diseases in potentially malignant disorders from the study population.

PERIODONTAL DISEASES		
	Frequency	Percentage
Generalised Chronic Gingivitis	85	40.7
Generalised Chronic Periodontitis	90	43.1
Generalised Chronic Gingivitis with Localised Periodontitis	31	14.8
Localised Chronic Gingivitis	2	1.0
Clinically Healthy Gingiva	1	.5
Total	209	100.0

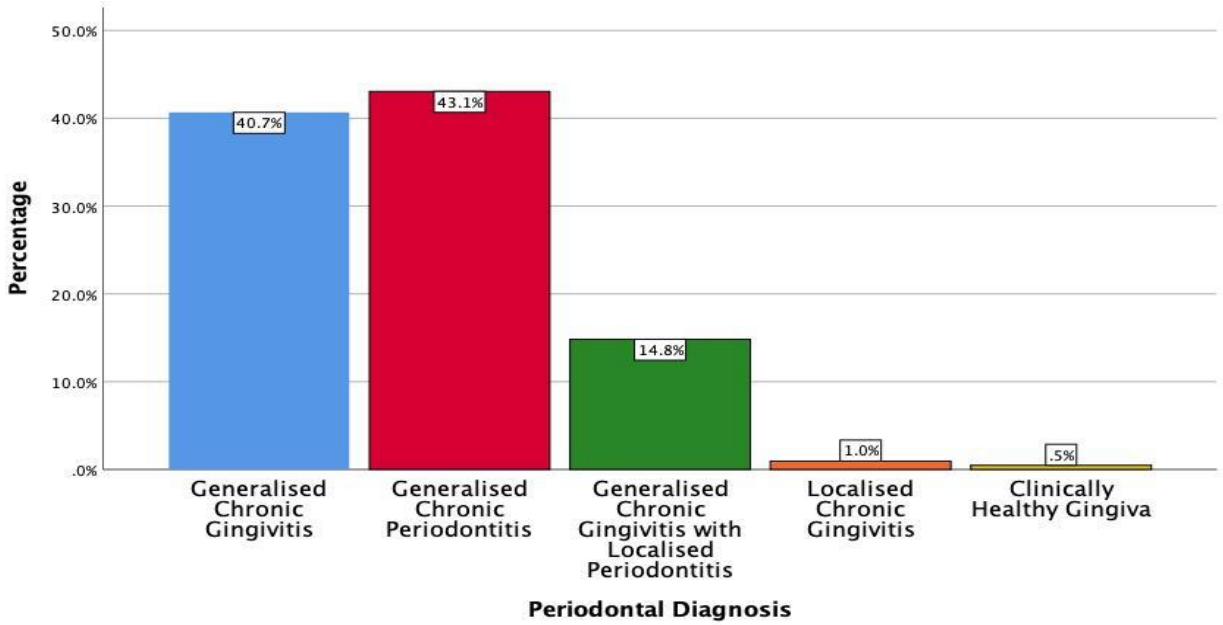


Figure 2. This graph represents the percentage of the periodontal diseases in potentially malignant disorders, X axis depicts the periodontal diseases and Y axis denotes its percentage, where blue denotes generalised chronic gingivitis, red denotes generalised chronic periodontitis, green denotes generalised chronic gingivitis with localised periodontitis, orange denotes localised chronic gingivitis and yellow denoted clinically healthy gingiva. This graph shows that the most commonly seen periodontal disease in potentially malignant disorders was generalised chronic periodontitis(43.06%).

Under the studied population 84 patients claimed to have been using smokeless tobacco, 40 patients were using smoking tobacco, 31 patients used both smoking and smokeless form of tobacco and 54 patients claimed to have no adverse oral habits (Table 3 & Graph 3).

Table 3. The table shows the frequency and percentage of adverse oral habits involved in potentially malignant disorders.

ADVERSE ORAL HABITS		
	Frequency	Percentage
Smoking Tobacco	40	19.1
Smokeless Tobacco	84	40.2
Both	31	14.8
NA	54	25.8
Total	209	100.0

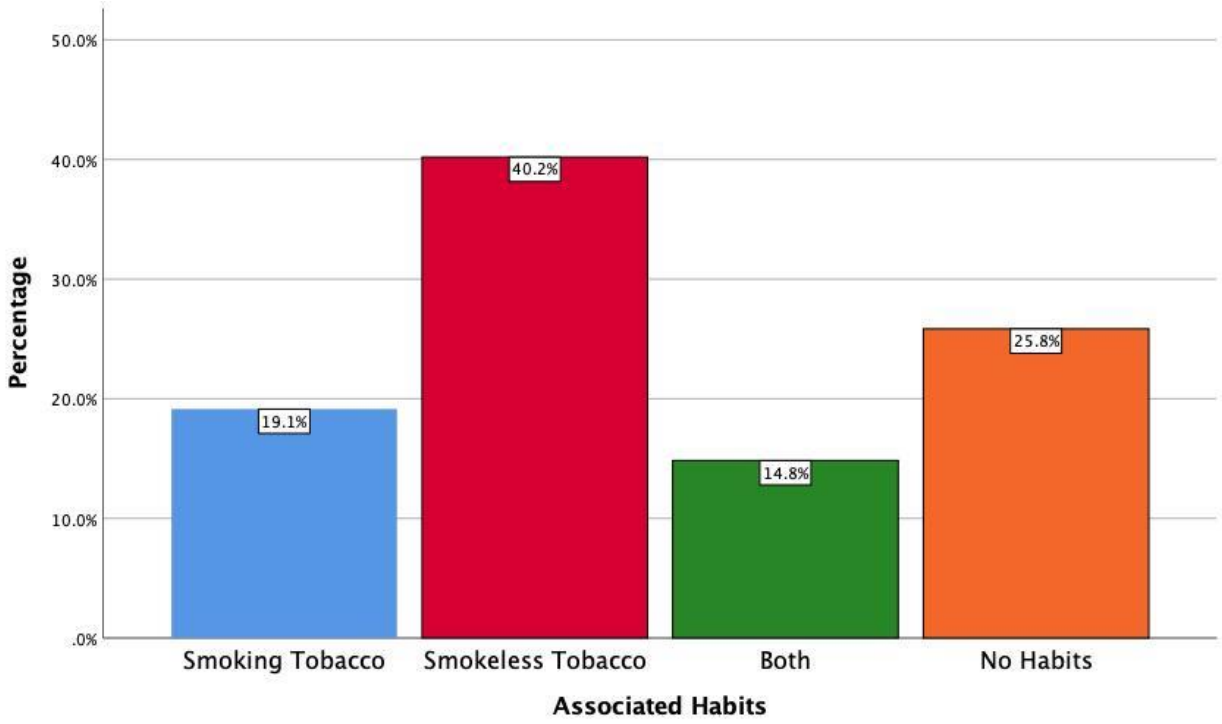


Figure 3. This graph represents the percentage of adverse oral habits in potentially malignant disorders. X axis depicts adverse oral habits among the population and Y axis depicts its percentage, where blue denotes smoking tobacco, red denotes smokeless forms of tobacco, green denotes both smoking and smokeless forms of tobacco and orange denotes no habits associated. This graph shows that the most common habit in the population was the usage of smokeless tobacco(40.19%).

On Chi-square analysis between adverse oral habits and periodontal diseases, it was established that patients using smoking tobacco, both smoking and smokeless tobacco and patients with apparently no adverse oral habits showed high prevalence of generalised chronic periodontitis and patients using smokeless tobacco showed high prevalence of generalised chronic gingivitis.This showed positive association and was significant since p=0.038(Table 4 & Graph 4).

Table 4. This table represents the chi-square analysis between adverse oral habits within the population and periodontal diseases. The association is significant since p=0.038.

Habits-Periodontal Diseases Chi-square Test			
Value		df	Asymptotic significance(2-sided)
Pearson Chi-square	21.968[a]	12	.038
Likelihood Ratio	24.533	12	.017
Linear-by-Linear Association	.129	1	.720
N of Valid Cases	209		

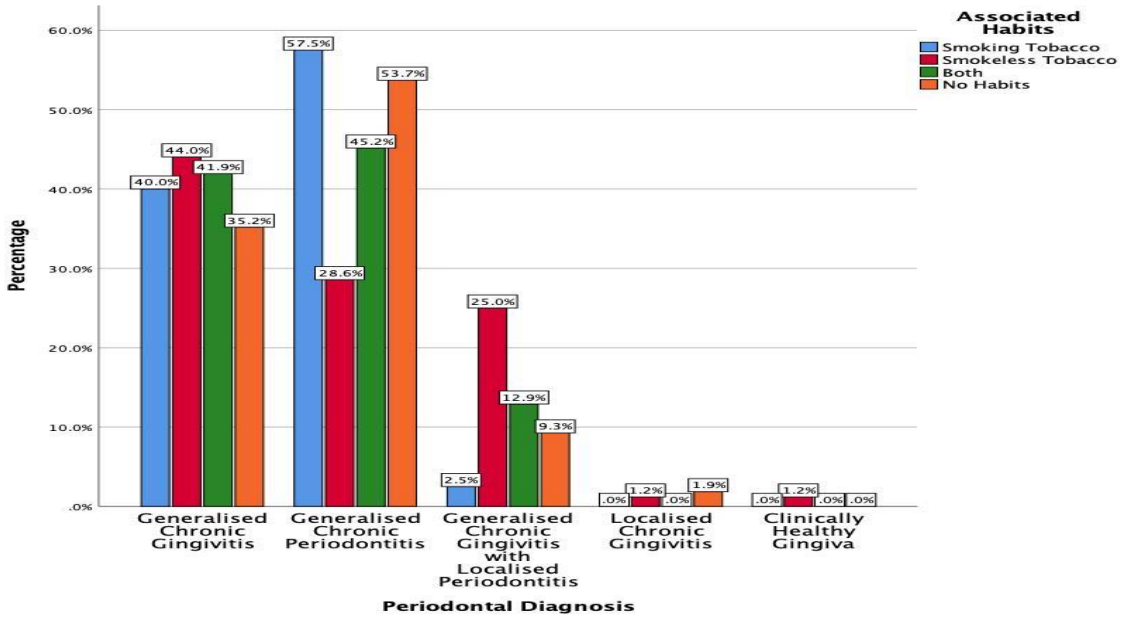


Figure 4. This bar graph represents the association between adverse oral habits and the periodontal diseases in the potentially malignant disorders. X axis denoting the various periodontal diseases and Y axis denoting the percentage of potentially malignant disorder patients.This graph shows generalised chronic periodontitis was mostly seen in patients using smoking tobacco(57.5%) and generalised chronic gingivitis was mostly seen in patients using smokeless tobacco(44%). Association between adverse oral habits and periodontal diseases was done using Chi-square test, Pearson Chi-square is 21.968-p value-0.038($p<0.05$) was found to be statistically significant.

On Chi-square analysis between associated PMDs and periodontal diseases , it was established that patients diagnosed with lupus erythematous and OSMF showed high incidence of generalised chronic gingivitis and patients diagnosed with lichen planus, leukoplakia and actinic cheilitis showed high prevalence of generalised chronic periodontitis. It was not statistically significant since p=0.090 (Table 5 & Graph 5).

Table 5. This table represents the chi-square analysis between potentially malignant disorders within the population and periodontal diseases. The association is non-significant since p=0.090.

Potentially Malignant Disorders*Periodontal Diseases Chi-square Test			
Value		df	Asymptotic significance(2-sided)
Pearson Chi-square	23.978[a]	16	.090
Likelihood Ratio	23.510	16	.061
Linear-by-Linear Association	.029	1	.866
N of Valid Cases	209		

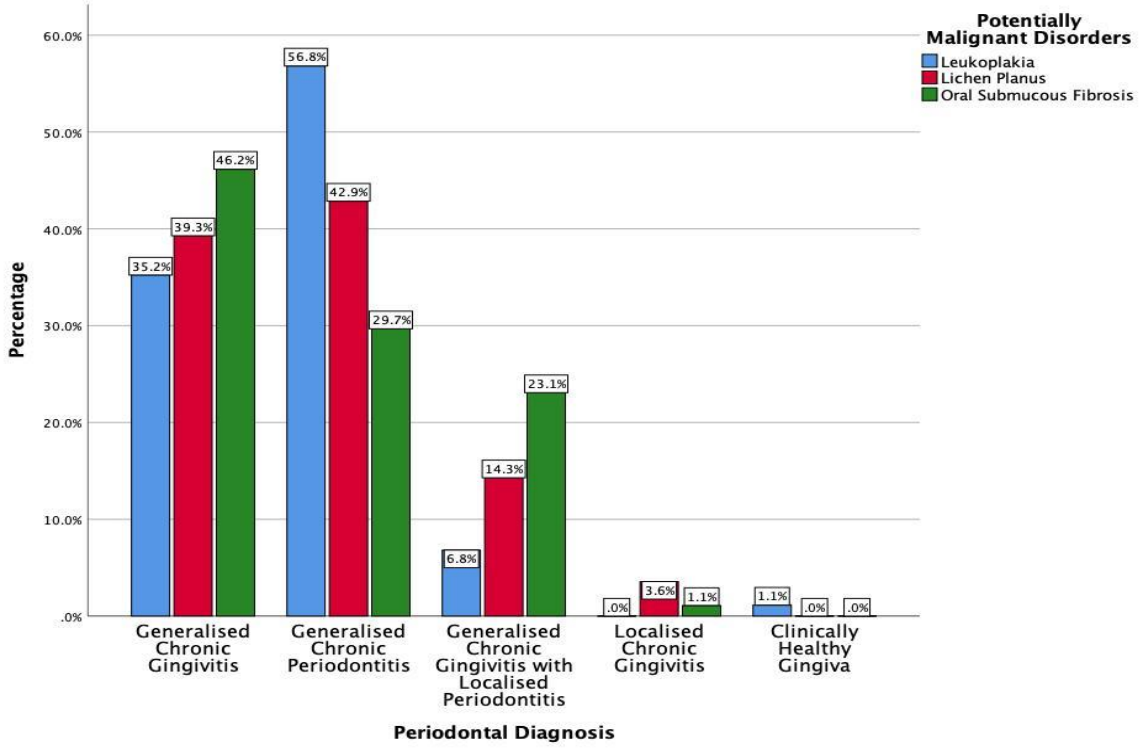


Figure 5. This graph represents the association between potentially malignant disorders and the periodontal diseases in the population, x axis denoting the various periodontal diseases and y axis denoting the percentage of potentially malignant disorders.This graph shows leukoplakia is more among population with generalized chronic periodontitis(56.8%) and oral submucous fibrosis is more among population with generalised chronic gingivitis(46.2%) and generalised chronic gingivitis with localised periodontitis(23.1%). Association between periodontal diseases and potentially malignant disorders was done using Chi-square test, pearson chi-square value is 23.978, p- value- 0.090(p>0.05) was found to be not statistically significant.

DISCUSSION

This study was the first of its kind, to find about the periodontal disease and its association with oral potentially malignant disorders among the chennai population and provides significant new information. A study done by Tezal et al, suggested that there may be a possible relationship between periodontitis and precancerous lesions, which in the present study has been represented with positive association between periodontal diseases and various PMDs involved in the study population.[24] The reason for this similarity is due to the similar sample size of the two studies.

According to the present study, significant association was established between adverse oral habits with the usage of smoking and smokeless tobacco with periodontal diseases, but in contradiction a study conducted by Robertson et al concluded that there was no consistent association between adverse oral habits and periodontal disease.[25] The reason for this difference may be due to the age group of the population involved in the study. Few previous studies suggested a link between dental factors and oral hygiene to oral cancer [26-28] and only three assessed the role of periodontal disease on oral cancer [29-31]. In all three of these studies, measurements of periodontal disease were not quantitative, subjective, or prone to bias. Periodontal disease was represented by self-report of presence or absence of bleeding gums by interview or frequency of bleeding gums by questionnaires.[32]

The association between periodontal disease and oral neoplasm is biologically plausible and may be explained by: 1) broken mucosal barrier in the presence of periodontal disease and consequent enhanced penetration of carcinogens such as tobacco and alcohol. Increased cellularity in blood vessels and connective tissue in chronic inflammation. Conditions such as Lichen Planus, Actinic Cheilitis and Lupus erythematosus can be caused due other etiological factors such as sunlight, genetic or autoimmunity. The most representative limitations of the study was that the patients involved in the study were confirmed cases clinically, to give a more significant relation all the lesions should be confirmed histopathology and their systemic diseases were not recorded. The study was single centered and was done in a population with the same ethnicity and geographic location. To improve the significance of the study, the study should be done extensively with a large amount of sample size, so that the results are reliable.

CLINICAL SIGNIFICANCE: The study helps us to understand the inter-relationship between periodontal diseases and oral potentially malignant disorders as well the adverse oral habits which can provide an indicator in clinical practice.

CONCLUSION

In conclusion, our findings give new hints into the complex inter-relationship between periodontal diseases and oral potentially malignant disorders. The study concluded that a positive relation is established between smokeless tobacco with generalised chronic gingivitis and smoking tobacco with generalised chronic periodontitis. In patients with leukoplakia and lichen planus, generalized chronic periodontitis was commonly seen. In patients with oral submucous fibrosis, generalised chronic gingivitis was commonly seen. Further longitudinal studies are needed to provide evidence of the association between periodontal diseases and oral potentially malignant disorders.

DECLARATIONS

Acknowledgement

The authors would like to acknowledge the help and support rendered by the department of periodontics and also the management of Saveetha Dental College and hospitals for their constant assistance with the research.

Conflict of interest

All the authors declare that there was no conflict of interest in the present study.

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ORIGINAL RESEARCH

EFFECT OF SKELETAL MALOCCLUSIONS ON TMJ HEALTH-A DESCRIPTIVE CROSS-SECTIONAL STUDY

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ABSTRACT

Background: Temporomandibular joint disorders (TMD) are musculoskeletal conditions that represent a group of conditions that involve the masticatory muscles, TMJ, and other related tissues. TMD is associated with various factors and skeletal malocclusion is implicated as one of the etiological factors cited inadequately in the literature. To formulate a hypothesis that skeletal malocclusion plays a crucial role in TMJ health.
Aim: To assess the effect of skeletal malocclusions on TMJ health.
Materials and Methods: A Descriptive Cross-Sectional Study was conducted with 180 samples divided into three groups of 60 each with skeletal class I, II, and III malocclusions reported to the Oral and Maxillofacial Surgery Department from June 2022 to December 2023. In each group, data was obtained through clinical examination, radiographs, and a DC/TMD chart. The severity of the functional TMJ is recorded using a modified Helkimo Anamnestic index and the observations were analysed to assess the relation between skeletal malocclusion and TMJ health.
Results: Skeletal malocclusion significantly affected TMJ function and morphology. Disc displacement was more prevalent in Class I ($p < 0.05$), while trauma from occlusion was more frequently observed in Class III ($p < 0.05$). Headache symptoms were high in Class II, showing a significant correlation with TMJ dysfunction ($p < 0.05$). These findings highlight the varying TMJ impacts across different skeletal malocclusion classes.
Conclusion: The present study supports the association of different skeletal malocclusions that contribute uniquely to TMD symptoms, with Class I being most associated with intra-articular TMD and Class III showing more muscle-related pain. Vertical growth patterns and occlusal trauma were also significant factors affecting TMJ health. This study provided a positive relationship between TMD and skeletal malocclusion.

Keywords: Temporomandibular joint disorders, Skeletal malocclusion, Temporomandibular joint, DC/TMD chart, TMJ

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INTRODUCTION

Temporomandibular Joint (TMJ) allows the jaw to move and perform various dynamic activities of the facial skeleton during mastication and speech. It is a ginglymoarthroidal joint that attaches the mandible to the squamous part of the temporal bone by allowing the jaw's orientation and position to be controlled by the neuromuscular frameworks surrounding the joint [1].

TMJ is a modified synovial joint with a biconcave fibrous articular disc separating the lower compartment from the upper compartment of the joint space. The articular disc is held in its functional position within the capsule by ligaments and muscles. The articular disc moves in synchronous with the condyle simultaneously on both sides [2].

Temporomandibular joint disorders (TMD) are clinical conditions involving the masticatory muscles, TMJ, and other related tissues. It has been categorized as a musculoskeletal condition that includes the head, face, and associated structures. Although the etiology of TMD is poorly understood and thought to be multi-factorial, it is frequently argued that malocclusion is one of the etiological factors of the condition [3]. However, the literature's citation of skeletal malocclusion's role in TMD is inadequate.

Skeletal malocclusions are one of the triggering factors in the complex etiology of TMD, resulting in an imbalance of the stomatognathic system. TMD risk is further increased by additional risk factors such as psychological problems, trauma, parafunctional habits, stress, genetics, and hyperlaxity of TMJ [4].

Studies have shown that the altered morphological relationship between the maxilla and mandible leads to changes in muscles of mastication, condyle-fossa, and disc-condyle relationship resulting in disc displacement and temporomandibular joint clicking [5].

Skeletal malocclusion particularly class II malocclusion was more likely to cause muscle strain problems in the joint that result in TMD [6]. Skeletal patterns and condylar asymmetry have a higher risk for TMD [7].

One of the main contributing factors to the etiology of TMD is mandibular retrusion because it causes the condyle to shift posteriorly resulting in excessive pressure on the joint and ultimately leading to joint pain, disc displacement, and degenerative changes [8].

Skeletal alterations cause prolonged strain on the TMJ and can cause degenerative changes including clicking or locking of the jaw, flattening of the condyle, arthritic changes, and asymmetrical growth patterns [9].

The main aim was to assess the effect of skeletal malocclusion on TMJ health and the objective was to evaluate the correlation between skeletal malocclusion and TMJ health.

MATERIALS AND METHODS

A Descriptive Cross-Sectional Study was conducted with 180 samples divided into three groups of 60 each with skeletal class I, II, and III malocclusions with age group 18-35 years reported to the Oral and Maxillofacial Surgery Department from June 2022 to December 2023. All the participants were informed about the study and written consent was acquired from all the participants. The Case history and the data obtained with the examination were recorded and examination results were analyzed by specially prepared forms using a DC/TMD chart which were taken as a reference. The severity of the functional TMJ is recorded using a modified Helkimo Anamnestic index [10] and the observations were used to assess the relation between skeletal malocclusion and TMJ health. The study was performed with the approval of the Institutional Review Board (IRB)/ Institutional Ethics Committee (IEC) 202204005 and the approval date was 22/04/2022.

1. Case history
2. Helkimo Anamnestic Index
3. Class I patient



Figure1.Orthopantomogram

4. Class I patient

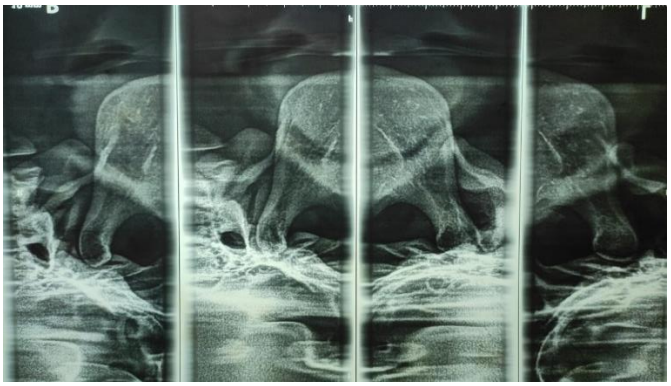


Figure 2.TMJ Open and closed views

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5. **Class I patient**



Figure 3. Lateral Cephalogram

6. DC/TMD chart

	Presence/Absence	**Class I**	**Class II**	**Class III**
Pain related TMD	Presence	4	1	2
	absence	56	59	58
Intra-articular temporo mandibular disorder	Presence	3	0	0
	Absence	57	60	60
Headache	Presence	40	44	21
	Absence	20	16	39
Age	Range	18-35 years	18-35 years	18-35 years

Inclusion criteria: 1. Physical status ASA I and II 2. The age group of 18-35 years with skeletal or dental malocclusion 3. Patients with a full set of dentition. 4. Patient who had given consent.
Exclusion criteria: 1. Patients undergoing orthodontic treatment 2. Patients who underwent orthodontic treatment 3. Patients who underwent orthognathic surgery. 4. Patients having missing teeth. 5. Patients who are using occlusal splints or underwent prosthetic rehabilitation 6. Patients with a history of trauma. 7. Patients having periodontal problems. 8. Patient who underwent any restorative dental treatment. 9. Patients with any systemic disorders /condition affecting TMJ health.
Primary variables were trauma from occlusion, TMJ disorders (pre-existing), Pain, intra-articular disease (IAD), and Headache.
Secondary variables were muscular pain, TMJ signs on palpation, mandibular plane angle, mandibular mobility, and TMJ plane movement.

RESULTS

Statistical methods: Statistical analyses were performed using version 25.0 of the Statistical Package for Social Sciences (IBM Corporation, Armonk, New York, USA). The data was categorical and Pearson's chi-square test was used.

p-value < 0.05 was considered statistically significant. The data was entered in Microsoft Excel 2016 for Windows.

Table 1. Comparison of Primary Variables with Skeletal Malocclusion Class I,II,III:

Primary Variables		Class I	Class II	Class III	chi-square	p-value
Trauma from occlusion	Presence	19(10.6%)	23(12.8%)	39 (21.7%)	15.018	**0.001***
	Absence	41(22.8%)	37(20.6%)	21 (11.7%)		
Pain related TMD	Presence	4 (2.2%)	1 (0.6%)	2 (1.1%)	2.081	0.353
	absence	56 (31.1%)	59 (32.8%)	58 (32.2%)		
Intra-articular temporo mandibular disorder	Presence	3(1.7%)	0 (0.0%)	0 (0.0%)	6.102	0.04*
	Absence	57 (31.7%)	60 (33.3%)	60 (33.3%)		
Headache	Presence	40 (22.2%)	44 (24.4%)	21 (11.7%)	20.70	0.000*
	Absence	20 (11.1%)	16 (8.9%)	39 (21.7%)		

Trauma from occlusion was observed in Class III skeletal malocclusion with a statistically significant p-value of 0.001. Trauma from occlusion was observed in Class III skeletal malocclusion with a statistically significant p-value of 0.001. (Table 1)

Distribution of pain-related TMD with skeletal class I, II, and III malocclusions showed no significant p-value (0.353). Intra-articular TMD was a statistically significant p-value (0.04) in skeletal class I malocclusion. The presence of headache was significant in class II skeletal malocclusion. (Table 1)

Table 2. Comparison of Secondary Variables with Skeletal Malocclusion Class I,II,III:

Secondary Variables		Class I	Class II	Class III	chi-square	p-value
High-angle or low-angle	High	1 (0.6 %)	16 (8.9%)	31 (17.2%)	43.192	0.000*
	low	35 (19.4%)	31 (17.2%)	24 (13.3%)		
	Normal	24 (13.3%)	13 (7.2%)	5 (2.8%)		
Mandibular mobility	Score- 0	31 (17.2%)	49 (27.2%)	45 (25.0%)	15.066	0.005*
	Score -1	28 (15.6%)	11 (6.1%)	15 (8.3%)		
	Score-2	1 (0.6%)	0 (0.0%)	0 (0.0%)		
Temporomandibular plane movement	Score- 0	43 (23.9%)	60 (33.3%)	47 (26.1%)	20.126	0.000*
	Score -1	16 (8.9%)	0 (0.0%)	13(7.2%)		
	Score-2	1 (0.6%)	0 (0.0%)	0 (0.0%)		
Muscular pain	Score- 0	54 (30.0%)	54 (30.0%)	45 (25.0%)	7.059	0.02*
	Score -1	6 (3.3%)	6 (3.3%)	15 (8.3 %)		
	Score-2	0 (0.0%)	0 (0.0%)	0 (0.0%)		
TMJ Tenderness on palpation	Score- 0	36 (20.0%)	52 (28.9%)	45 (25.0%)	18.332	0.001*
	Score -1	20 (11.1%)	2 (1.1%)	12 (6.7 %)		
	Score-2	4 (2.2 %)	6 (3.3%)	3 (1.7%)		

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The mandibular plane angle with skeletal malocclusion shows a significant p-value (0.000) in skeletal class III malocclusion patients. The distribution of mandibular mobility was statistically significant with a p-value (0.005) highest in class 1 skeletal malocclusion with moderate temporomandibular disorder n=28 (15.6%) and severe TMD was observed in one patient. The temporomandibular plane movement was highest in skeletal class 1 malocclusion with a significant value of p = 0.000. (Table 2)

Muscular pain was observed in skeletal class III with n = 15 (8.3%) with p-value (0.02). The association between the malocclusion and pain on TMJ palpation was significant with a p-value of 0.001 and pain was highest in class II skeletal malocclusion. (Table 2)

DISCUSSION

Treatment of TMD at an early stage requires in-depth knowledge and research about the cause-and-effect relationship which was inadequately reported in the current literature. The relationship between functional TMJ disorders and the occlusal factors was neither widely studied nor cited. The alterations in surrounding facial musculature and the occlusal factors were thought to be one of the causative agents and contributing factors for temporomandibular joint dysfunction. Occlusal alterations were described as a co-factor rather than an etiological factor [11].

The research on the association between occlusal features and disorders of TMJ considers nine variables- overjet, overbite, midline deviation, pain-free opening, maximum assisted opening, maximum unassisted opening, lateral excursions, and a protrusion for evaluating any pain-related TMD, Intra-articular disorders, Degenerative disorders, headache attributed to TMD [12].

A Study Evaluating the Multifactorial Pathophysiology of TMD utilized general terms instead of specific terms associated with the problem of the Temporomandibular joint and stated that dental occlusal factors like a posterior crossbite, skeletal malocclusion, and TMD association were not consistent [13].

The current study revealed that trauma from occlusion was associated with Class III skeletal malocclusion than Class II and Class I which indirectly affects the TMJ health.

An analysis of the relationship between skeletal malocclusion and TMD revealed that it was a contributing element, but not the cause. The data supporting these findings was insufficient, and most of the investigations failed to show a significant association between the skeletal pattern and TMD [14].

In a case report authors highlighted those patients with class III skeletal malocclusion had severe facial asymmetry, posterior crossbite on the unilateral side, and TMJ disorder [15]. A comparison of skeletal malocclusion with TMD in the present study shows a significant correlation. TMD was highest in skeletal class I compared to skeletal class II and III. Intra-articular TMD with the highest presence was observed in skeletal class I malocclusion compared to skeletal class II, and III.

Research on the connection between crossbite and masticatory muscle pain is still controversial. Alarcon et al state that samples with posterior cross bite on the left side had a higher activity of the temporalis muscle on the contralateral side and individuals with normal occlusion had the opposite pattern. Long-term effects of skeletal Class III malocclusion on the TMJ, jaw movement, and masticatory system result from an incorrect sagittal relationship between the maxilla and mandible. The condyle-fossa relationship may be compromised because of the TMJ's susceptibility to tension or compression forces from the surrounding tissues [16].

Muscular pain was observed greater in skeletal class III in the current study and deep bite was higher than posterior cross bite in causing muscle pain.

A study on the comparison of skeletal malocclusion and craniofacial structures revealed that before treatment for any craniofacial abnormalities, there is a need to find out the underlying condition related to TMD [17].

The perspective in the Pullinger et al studies states that overjet significantly correlates with pain-related TMD whereas the overbite was associated with internal derangement of TMJ, especially disc displacement with reduction. In opposition, Manifrendi et al manifested that there is no clinical interrelation between overjet, overbite, and midline shift and concluded that TMD is due to family inheritance (genetics) and psychological and psychosocial factors [18]. The present study revealed rather than abnormal overjet, overbite, and deep bite showed a strong association irrespective of the type of skeletal malocclusion.

De Luca et al stated that headache is a predisposing factor or the triggering factor that affects muscles of mastication in and around the joint which results in myofascial pain. Among the different types of headaches, tension-type headache has a higher incidence with TMD [19]. Evaluation of headache clinically also

plays a crucial role in TMD associated with headache. Headache-related TMD was observed in skeletal class I & II malocclusion patients when compared to skeletal class III malocclusion in the present study. Most of the participants did not show any palpable masticatory muscle tenderness.

The present study revealed that low mandibular plane angle and normal angle had an effect on TMJ than high angle patients irrespective of the type of skeletal malocclusion. This shows a significant relation with the existing studies where greater incidence is seen in normal and low-angle cases compared to high-angle classes which are mostly encountered in skeletal class III patients [20].

Clicking sounds of the TMJ are identified as highest in class 1 skeletal malocclusion patients when compared to skeletal class II & III patients in our study. One participant had severe clicking sounds with restricted mouth opening and that is observed in skeletal class I patients.

Skeletal class II growth patterns individuals had higher overjet the reason is due to loss of guidance in the incisal area which resulted in excessive pressure on TMJ and further led to TMD and supporting to this patient with retruded mandible and TMD symptoms had higher facial plane and convex profile revealed an irreversible displacement of the disc with reciprocal click [21].

Vertical growth of skeletal variables had a higher impact when compared to horizontal growth pattern individuals because the variance in height of the mandible results in chances of application of more pressure on condyle and excessive vertical expansion of the mandibular ramus may be the cause of change in condylar width and height and further leading to TMD problem [22].

Final discussion summary:

This study found a strong association between skeletal malocclusion and TMD. Class I malocclusion had the highest intra-articular TMD, while Class III showed more muscle pain. Headache-related TMD was more frequent in Class I & II, with deep bites contributing more to muscle pain than crossbites. Low and normal mandibular plane angles had a greater impact on TMJ dysfunction. TMJ clicking was most common in Class I, while Class II patients exhibited disc displacement. Vertical growth patterns posed a higher TMD risk due to increased condylar stress and mandibular changes.

Scientific novelty:

- Provides detailed evidence between the different skeletal malocclusions with particular types of TMD.
- It highlights that Class 1 malocclusion is more strongly associated with intraarticular TMD, which contrasts with the previous studies.
- Mandibular plane angle has a stronger impact on TMJ dysfunction, which implies a mechanical foundation and understanding of TMJ biomechanics.

- According to the findings, vertical growth patterns represent a greater risk factor for TMD than horizontal patterns, offering fresh insights on joint loading and mandibular adaptation.

Practical Significance:

1. Early diagnosis and intervention
2. Preventive Strategies for TMD
3. Enhanced treatment protocol

CONCLUSION

The occlusal factors were categorically described as predisposing, triggering, or perpetuating factors but were denied to be confirmed as etiological factors for causing TMD owing to the existing evidence. However, the studies published in recent times agree with the reality and the existing evidence is still inadequate between skeletal malocclusion and TMJ. Our study definitively provides proof of skeletal malocclusion and TMJ, but further randomized multicentered trials are needed to strengthen the evidence.

DECLARATIONS

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RESEARCH ARTICLE

FABRICATION OF TRIPLE ANTIBIOTIC AND CISSUS QUADRANGULARIS CONJUGATED LYOPHILIZED GUIDED TISSUE REGENERATION MEMBRANE AND ITS CHARACTERISATION

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ABSTRACT

Background: Periodontitis is a severe gum infection that damages the soft tissue and, if untreated, can destroy the bone that supports your teeth.Cissus quadrangularis, a medicinal plant known for its bone healing properties, has been used in traditional medicine for centuries. Antibiotic-laden membranes have gained significant attention due to their ability to prevent infections, which are a major challenge in orthopedic surgeries and bone grafting procedures. The main aim of the study is to fabricate and characterize the triple antibiotic and Cissus quadrangularis conjugated lyophilized membrane for periodontal bone regeneration.

Materials and Methods: 10mg of Cissus quadrangularis was weighed and dispersed in 100 ml of deionised waste and heated at 50 degree centigrade for 24 hrs then the extract was collected using filtration method with wattmann filter paper. 1 g of PEG and Carrageenan (2 g) was dissolved in 100 mL of cissus quadrangularis extract at 60 ◦C using magnetic stirrer under 400 rpm . Triple antibiotics were also added. Following a 24-hour period, the petri plate was dried for 12 hours at 50°C. The membrane was then removed from the petri dish and placed in a desiccator. Following membrane formation FTIR, contact angle, Antimicrobial activity were compared between Cissus quadrangularis membrane and carrageenan (control group) and Group 2 consisted of Cissus quadrangularis membrane, triple antibiotic, and carrageenan (test group).

Results: Tensile strength of the control group is 14.62 N and Test group is 32.13 N. Hence the control group membrane has more tensile strength than the test group membrane. The Test group membrane showed an contact angle average of 75.27 degree and Control group membrane showed an contact angle average of 46.51degree. Hence the control group membrane indicates the better hydrophilic nature of the material. The control group membrane showed better antimicrobial activity than the test group membrane against the tested bacteria (Streptococcus mutans).

Conclusion: The present study results showed a higher tensile strength, good antimicrobial property and enhanced wettability in the prepared novel membrane when compared to the control group. By addressing both the prevention of infection and the promotion of bone healing, this novel membrane builds on existing research and offers a promising solution for use in guided tissue regeneration.

Keywords: Triple antibiotics, Cissus quadrangularis, Guided tissue regeneration, Membrane, Periodontal regeneration

INTRODUCTION

Periodontitis is a severe gum infection that damages the soft tissue and, if untreated, can destroy the bone that supports your teeth.[1] It can lead to tooth loss or increased risk of other serious health complications. Periodontitis is usually caused by poor oral hygiene. It begins with plaque, a sticky film of bacteria that forms on the teeth.[2] If plaque is not removed, it can harden into tartar, which is harder to remove. This can cause the gums to become inflamed, leading to gingivitis, the earliest stage of gum disease. Without treatment, gingivitis can progress into periodontitis.[3]Symptoms of periodontitis are swollen, tender, or bleeding gums, receding gums and persistent bad breath.[4] Periodontal bone regeneration is a treatment approach aimed at restoring lost bone and supporting structures in the jaw that have been destroyed due to advanced periodontal disease, like periodontitis.[5] When periodontitis progresses, the bone that supports teeth deteriorates, leading to tooth mobility and, ultimately, tooth loss if not treated. Bone regeneration aims to reverse this damage and rebuild the lost bone. In periodontal bone regeneration, various materials are used to stimulate and support the growth of new bone and tissue. These materials typically act as scaffolds for natural bone regeneration or directly encourage tissue repair. The main categories of materials include bone grafts, barrier membranes, and biologics/ growth factors.[6] Bone regeneration is a complex process that involves the restoration of bone tissue lost due to trauma, disease, or surgical interventions.[7] The search for effective materials and strategies to enhance bone healing has led to the development of innovative biomaterials.[8] Among these, guided tissue regeneration membranes play a crucial role by providing a barrier that protects the site of injury, promotes bone regeneration, and minimizes complications such as infection and delayed healing. One emerging approach is the use of lyophilized membranes, which are dehydrated at low temperatures to preserve the bioactive components and enhance the stability of the material.

Antibiotic-laden membranes have gained significant attention due to their ability to prevent infections, which are a major challenge in orthopedic surgeries and bone grafting procedures. The use of broad-spectrum antibiotics, such as triple antibiotic combinations, has proven effective in reducing the risk of bacterial contamination and post-operative infections.[9] However, beyond infection control, promoting the actual regeneration of bone tissue remains a critical goal.[10] In this regard, herbal and natural compounds have shown potential as bioactive agents that can stimulate bone growth and repair. Cissus quadrangularis, a medicinal plant known for its bone healing properties, has been used in traditional medicine for centuries. Recent scientific studies have demonstrated its ability to promote bone formation and enhance the healing process.[11] The bioactive compounds in Cissus quadrangularis, such as flavonoids and triterpenoids, have been shown to stimulate osteoblast differentiation and mineralization, key processes in bone regeneration.[12] In this context, the combination of a triple antibiotic formulation with Cissus quadrangularis in a lyophilized membrane presents a novel strategy for bone regeneration. This conjugated membrane offers dual functionality: protection against bacterial infection and enhance bone healing.[13] By integrating these two therapeutic agents into a single scaffold, the membrane can provide an ideal environment for bone repair while mitigating the risk of infection- a critical factor in the success of bone grafts and implants. This study aims to fabricate and characterize the triple antibiotic and Cissus quadrangularis conjugated lyophilized membrane for periodontal bone regeneration.

MATERIALS AND METHODS

PREPARATION OF CQ EXTRACT AND MEMBRANE

10mg of Cissus quadrangularis was weighed and dispersed in 100ml od deionised waste and heated at 50 degree centigrade for 24 hrs then the extract was collected using filtration method with wattmann filter paper. 1 g of PEG and Carrageenan (2 g) was dissolved in 100 mL of cissus quadrangularis extract at 60 ◦C using magnetic stirrer under 400 rpm . This pure biopolymer solution is kept as control. Triple antibiotics (Erythromycin, Penicillin, and Amoxicillin) were infused into the biopolymers to create the corresponding membranes in order to fabricate drug-infused membranes. Before being added to the biopolymer solution for membrane casting, a triple antibiotic was combined with double-distilled water. A polystyrene petri dish (90 mm in diameter) was filled with drug solutions (25 mL) in a biopolymer matrix, and the dish was left to dry at room temperature. Following a 24-hour period, the petri plate was dried for 12 hours at 50°C. The membrane was then removed from the petri dish and placed in a desiccator. Group 1 consisted of Cissus quadrangularis membrane and carrageenan (control group); Group 2 consisted of Cissus quadrangularis membrane, triple antibiotic, and carrageenan (test group).

CHARACTERIZATION-STRUCTURAL PROPERTY

Fourier transform infrared (FTIR) spectroscopy

The functional group analysis for the prepared group 1 and group 2 lyophilized guided tissue regeneration

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membrane was done using FTIR spectroscopy (Perkin elmer, USA). The guided tissue regeneration membrane samples were placed to an attenuated total reflectance crystal surface and the spectra were captured within the range of 400-4000 cm-1. Scans were performed across different areas of the sample to investigate the presence of various elements within it.

Field emission Scanning electron microscopy (FE-SEM) analysis

A JEOL JSM-IT800 SEM was used to perform FE-SEM analysis on the newly prepared group 1 and group 2 lyophilized guided tissue regeneration membranes in order to investigate the structural morphology of the membrane. Samples were made after the membranes were cut into tiny pieces with scissors. Following a vacuum-sprayed application of platinum to the sections, FE-SEM analysis was performed at a 500× magnification.

PHYSICAL PROPERTY

Tensile strength

The universal testing machine (Instron Electroplus E3000, USA) was used to evaluate the tensile strength of the newly prepared group 1 and group 2 lyophilized guided tissue regeneration membranes. 10 × 15 mm test samples were used for analysis, with a crosshead speed of 5 mm/min. Forces were applied to the specimens in groups 1 and 2 after the membranes were fitted onto the analyzer. The breaking force was defined as the force at which the scaffold broke. Tensile stress was calculated by multiplying this breaking force by the specimens' area, and tensile strain was defined as the deformation at the breaking force.

Swelling Analysis

To measure the degree of swelling and fluid absorption, the samples from groups 1 and 2 were dry weighed, submerged in 5 mL of 10% PBS solution for an hour, and then wet weighed. After blotting the membrane with tissue paper to get rid of extra PBS solution, it was weighed. Swelling Ratio (SR) = ((W - W0) / W0) × 100% was the formula used to determine the swelling ratios, where W stands for the initial wet weight and W0 for the initial dry weight.

Contact angle analysis

The wettability of the guided tissue regeneration membranes in groups 1 and 2 was assessed using the sessile drop method. Using a micropipette, a droplet of distilled water (2 µL) was applied horizontally to the guided tissue regeneration membrane. The contact angle of the water drop that formed at the interface with the guided tissue regeneration membrane sample was measured using an Ossila goniometer.

ANTIMICROBIAL PROPERTY

Minimum inhibitory concentration

The extract of Cissus quadrangularis was tested for minimum inhibitory concentrations (MICs) using the broth microdilution method. In the assay, the MIC of the Cissus quadrangularis extract containing carageenan and triple antibiotic against S. mutans was evaluated at a distinct concentration level. By adding 2,3,5-triphenyl tetrazolium chloride (TTC) salt, which serves as an indicator, the growth of the bacteria was seen.

RESULTS

Carrageenan is extracted from Seaweeds that contain repeatable units of anhydro galactose and sulfated or non-sulfated galactose repeating units, which is a high molecular weight polymer. Together, carboxylic and sulfate vibrations in the FTIR spectra are reported to validate the formation of carrageenan. Peak indicated in 1100 in carrageenan and 1200 in triple antibiotic.(Figure 1) FE-SEM parameters included 300 X magnification, scale bar was 10 micrometer, working distance was 4.2mm and KV value was set to 3.00KV.

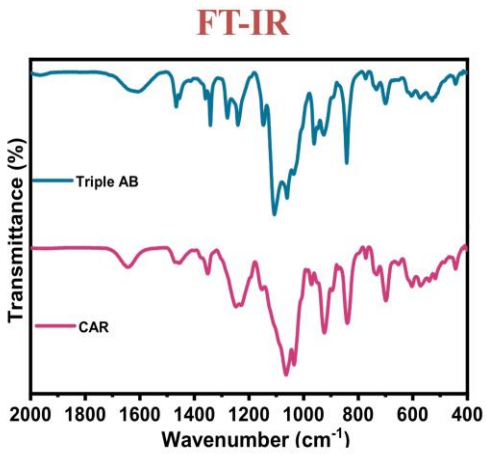


Figure 1. FTIR analysis

The group 1 sample image displays a rough, uneven surface texture with interconnected structures.(Figure 2) The group 2 sample image displays a rough, variable surface texture with interconnected structures.

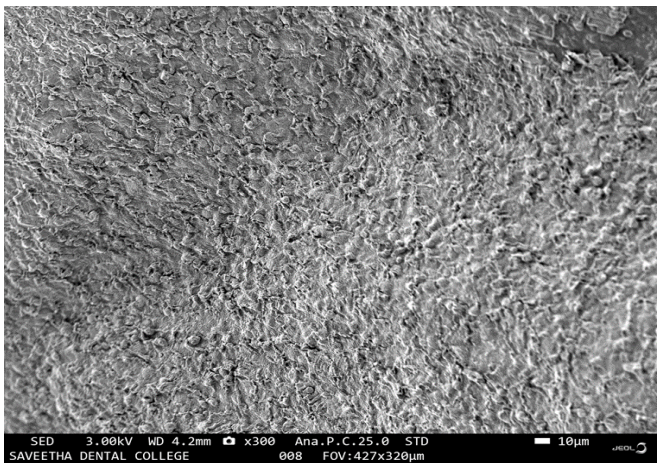


Figure 2. FE SEM image of group 1 membrane

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(Figure 3) Contact angle measurement was used to express the guided tissue regeneration membrane's wettability.

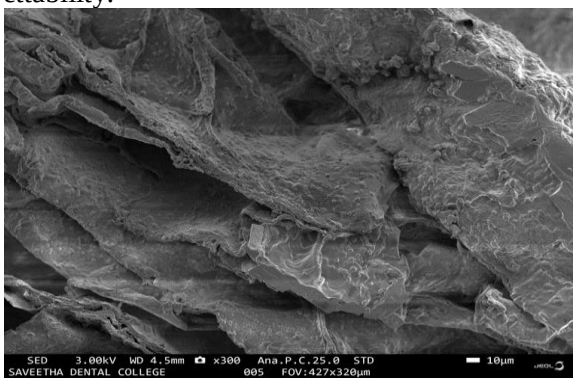


Figure 3. FE SEM image of group 2 membrane

The material's hydrophilicity or hydrophobicity is determined by the contact angle. A lower contact angle suggests that the material is more hydrophilic. In this study, the group 1 membrane showed an contact angle average of 75.27 degree and group 2 membrane showed an contact angle average of 46.51degree. (Figure 4)

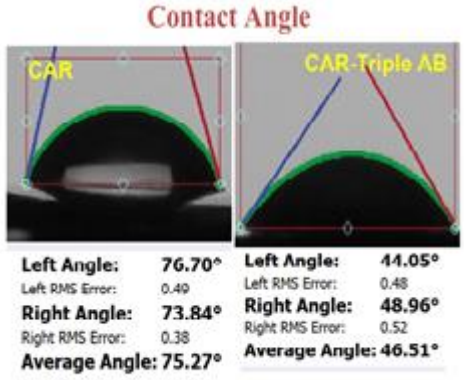
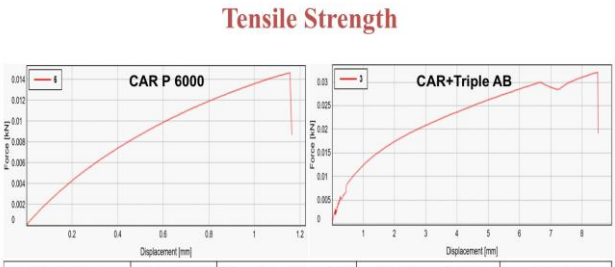


Figure 4. Contact angle analysis

Tensile strength of the group 1 was 14.62 N and group 2 was 32.13 N. Hence the group 2 membrane has more tensile strength than the group 1 membrane.(Figure 5).



Materials Name	Maximum Force [N]	Tensile stress at Tensile strength [MPa]	Tensile strain (Displacement) at Break (Standard) [%]	Tensile stress at Break (Standard) [MPa]
CAR	14.62	10.44	2.89	2.89
CAR+Triple AB	32.13	22.93	21.24	21.24

Figure 5 Tensile strength analysis

The group 2 membrane showed a good antimicrobial activity against the tested bacteria (Streptococcus mutans). Zone of inhibition was found to be an average of 0.358, 0.312, 0.264 at different concentrations of 25µl, 50µl and 100µl; compared to the average value of 0.192 of the control group. (Figure 6&7) This shows an enhanced antimicrobial activity that can be attributed to the triple antibiotic component in the membrane.



Figure 6. The anti-microbial activity

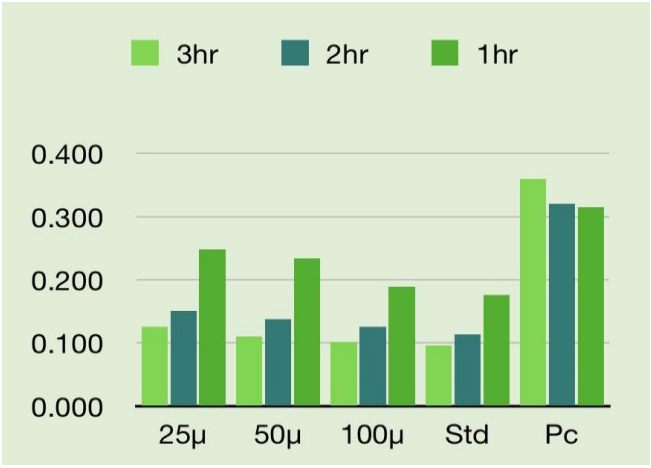


Figure 7. The anti-microbial activity with MIC assay

DISCUSSION

The development of a triple antibiotic and Cissus quadrangularis conjugated lyophilized membrane for bone regeneration is a promising strategy that integrates both periodontal infection control and enhanced bone healing. To fully appreciate the potential impact of this approach, it is important to analyze how it compares and builds upon findings from other research studies in related fields. Several studies have focused on the use of antibiotic-loaded membranes for infection prevention in bone regeneration applications. Wu et al. (2021) explored membranes impregnated with vancomycin and

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gentamicin for bone regeneration and infection control, showing a dual-functionality membrane similar to the one proposed in this study.[5] However, while these studies focus on bacterial control, they often lack an osteogenic component, limiting their direct role in enhancing bone regeneration.[14] The current study addresses this gap by introducing Cissus quadrangularis, a bioactive plant extract known for its bone healing properties, into the membrane matrix. This dual-action approach could prove superior, offering not only infection control but also active stimulation of bone formation.The use of herbal extracts like Cissus quadrangularis for bone regeneration has gained interest due to their osteogenic potential. Few Studies have explored the efficacy of Cissus quadrangularis in bone fracture healing, demonstrating accelerated bone formation and increased mineralization in experimental models.[15] The flavonoids and triterpenoids present in Cissus quadrangularis have been shown to enhance the proliferation and differentiation of osteoblasts, the cells responsible for bone formation.[16] The inclusion of Cissus quadrangularis in the lyophilized membrane developed in this study adds a unique bioactive component that enhances its bone regeneration capabilities, distinquishing it from purely antibiotic or synthetic membranes.[17]

Tetracycline or amoxicillin significantly decreased the adherence of S. mutans or A. actinomycetemcomitans on the ePTFE, glycolide fiber, or collagen membranes, according to the earlier paper by Chi-Fang Cheng et al. This result suggests that using antibiotic-loaded GTR membranes for periodontal regeneration therapy is beneficial and efficient.[18] Gabriel et al concluded that Four bacterial strains were used to assess the antibacterial activity of the AMX-loaded membranes, which varied depending on the drug and nAp concentrations. SEM/EDX examination revealed extensive mineralization in the simulated bodily fluid (SBF) after 21 days. For dentistry and tissue engineering applications, the investigated electrospun nanocomposite membranes coated with amoxicillin may be a viable fibrous-based antibiotic delivery system.[19] Kalluri et al concluded that The maximum tensile strength of 3.1 MPa and modulus of 39.9 MPa could be achieved at membrane compositions of 8.9 weight percent PLGA, 7.2 weight percent SEP, and 2 weight percent HAp, according to optimization utilizing response surface approach. HPLF proliferative activity was higher in optimized PLGA/SEP/HAp membrane specimens electrospun on a static collector than in tissue culture polystyrene and commercial collagen membrane.[20] Naoyuki kaga et al concluded that the PLGA membranes with pillar and hole patterns had a greater contact angle, which suggested that they were more hydrophobic. However, compared to the non-patterned PLGA membrane, a considerably greater number of MC3T3-E1 cells attached to the grooves, pillars, and holes of the patterned PLGA membranes.[21]

Lyophilization is a widely used technique in tissue engineering for developing scaffolds with enhanced stability and preservation of bioactivity. The process allows for the incorporation of both natural and synthetic materials into a highly porous structure, providing an ideal environment for cell infiltration and tissue growth. The conjugation of antibiotics and Cissus quadrangularis in a lyophilized membrane leverages these advantages.[22] The porous structure created by lyophilization facilitates the controled release of antibiotics, ensuring sustained antimicrobial activity, while also providing a scaffold conducive to cell attachment and proliferation. This dual-functionality membrane draws on earlier work that developed lyophilized chitosan- based membranes for wound healing, noting their success in creating an environment that promotes both tissue growth and infection control.[23]

The concept of a dual-action membrane that addresses both infection control and bone regeneration is supported by recent research trends. Few authors investigated the combination of bioactive molecules and antibiotics in scaffolds for bone repair, noting that a synergistic approach could significantly improve outcomes in bone regeneration, especially in infection-prone environments.[24] Their findings align with the current study's hypothesis that combining a triple antibiotic with a bone-promoting agent like Cissus quadrangularis could enhance both the speed and quality of bone healing. Comparatively, other dual-action scaffolds, such as those developed by Srinivasan et al. (2020) using antibiotic and growth factor combinations, emphasize infection control but lack the natural osteogenic stimulation provided by Cissus quadrangularis.[25] By integrating both antimicrobial and osteogenic agents in one membrane, the proposed lyophilized membrane may offer a more holistic solution for bone regeneration, particularly in clinical scenarios where infection risk is high, such as in osteomyelitis or post-traumatic bone defects. It is important to acknowledge potential limitations in our study which includes only characterization of the membrane; further nano ct and animal trials are necessary to know the efficacy of the membranes. Future research should also focus on optimizing the concentration of both antibiotics and Cissus quadrangularis to balance antimicrobial action with osteogenic promotion. Moreover, clinical trials will be necessary to confirm the membrane's safety and effectiveness in humans, as much of the current evidence is based on in vitro or animal models.

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CONCLUSION

In conclusion, the integration of a triple antibiotic combination with Cissus quadrangularis in a lyophilized membrane represents a novel and potentially superior approach. Our study results showed a higher tensile strength, good antimicrobial property and enhanced wettability in the prepared novel membrane when compared to the control group. By addressing both the prevention of infection and the promotion of bone healing, this novel membrane builds on existing research and offers a promising solution for use in guided tissue regeneration.

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CONFLICT OF INTEREST

The authors have none to declare.

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RESEARCH ARTICLE

EFFECT OF TETRACYCLINE, DOXYCYCLINE AND TRIPHALA ON COLLAGEN MEMBRANE DEGRADATION: AN IN VITRO STUDY

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ABSTRACT

Background: Collagen membranes are widely used in periodontal regeneration. However, strategies to enhance membrane stability include the incorporation of antimicrobial agents or natural inhibitors of collagenase activity. Hence, present study aimed to evaluate and compare the efficacy of tetracycline hydrochloride, doxycycline hyclate, and Triphala in delaying collagen membrane degradation at varying concentrations in an in vitro model.

Methods: Present invitro analysis used Healiguide collagen membranes. They were treated with tetracycline (TTC), doxycycline (DOX), and Triphala (TRI) at concentrations of 25 mg/ml, 50 mg/ml, 75 mg/ml, and 100 mg/ml. Collagenase enzyme (Clostridial collagenase, HiMedia Laboratories, India) was used to induce degradation. A total of 48 specimens were used, with collagen degradation assessed at 4-time intervals (Days 2, 7, 14, and 21) using spectrophotometric analysis. Statistical analysis was performed using ANOVA with post hoc Tukey's tests to compare among the groups and $p < 0.05$ considered significant.

Results: All tested agents significantly reduced collagen degradation compared to the control group ($p < 0.05$). Doxycycline at 25 mg/ml and 75 mg/ml exhibited the most pronounced inhibition of collagen breakdown, showing statistically significant differences from the control. Tetracycline was also effective, particularly at 75 mg/ml, while Triphala at 50 mg/ml and 75 mg/ml demonstrated moderate inhibition but did not surpass the efficacy of doxycycline.

Conclusion: Present study concludes doxycycline as the most effective in delaying collagen membrane degradation, followed by tetracycline and Triphala. The given growing concern over antibiotic resistance, Triphala may serve as a promising natural alternative for membrane stabilization in periodontal applications.

Keywords: Collagen Membrane, Doxycycline, Matrix Metalloproteinases, Periodontal Regeneration, Tetracycline, Triphala

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INTRODUCTION

Achieving periodontal regeneration involves restoring lost structures in the periodontium using specialized materials like grafts, growth factors, and barrier membranes. In periodontal therapy, both non-resorbable and resorbable membranes have been employed to aid in healing. Expanded polytetrafluoroethylene (e-PTFE), a widely used non-resorbable membrane, requires surgical removal, which poses drawbacks such as the need for a secondary surgery. To address this, resorbable membranes made from poly-lactic acid, polyglycolic acid, polyurethane, and collagen are designed to naturally break down in the body over time, enhancing convenience and reducing surgical intervention.[1, 2] Collagen-based membranes are widely preferred because they are biocompatible, have low immunogenicity, and support the migration and differentiation of cells needed for healing.[3]

Collagen membranes can degrade prematurely due to enzymes like matrix metalloproteinases (MMPs), which are released at the surgical site during healing. To increase membrane longevity and improve outcomes, various methods like stabilizing collagen structure through cross linking or adding antimicrobial agents to membranes have been used. These techniques aim to control MMP activity and maintain membrane integrity.[4, 5] For example, tetracycline hydrochloride (TTC) has shown effectiveness in slowing down membrane degradation, extending the healing process and supporting regeneration.[6] Doxycycline hyclate, a derivative of tetracycline, has shown significant antimicrobial effects against periodontal pathogens and inhibits collagenase, a factor that otherwise contributes to rapid degradation of collagen membranes.[7]

However, concerns about antibiotic resistance due to long-term antibiotic use have led researchers to explore safer alternatives like herbal medicine. Triphala, a combination of Emblica officinalis, Terminalia chebula, and Terminalia belerica, is a traditional Ayurvedic formulation known for its collagenase-inhibitory properties.[8, 9] Each component of Triphala has shown promising results in reducing collagenase activity, with Terminalia chebula identified as a potent collagenase inhibitor. Thus, present study aimed to evaluate the impact of tetracycline hydrochloride, doxycycline hyclate, and Triphala at various concentrations on the degradation of collagen membranes by collagenase enzyme in a laboratory setting.

MATERIALS AND METHODS

Sample Size Estimation

As there are no previous studies guiding for calculating sample size, we have taken a sample of 12 per group with total sample size of 48.[10] This sample size ensured adequate statistical power to detect significant differences in collagen membrane degradation among the study groups across multiple time intervals. THIS study was conducted at GITAM Dental College and Hospital, Visakhapatnam, following approval from the Institutional review board.

Preparation of Collagen Membranes:

5 mm diameter discs were created from bioabsorbable Healiguide collagen membrane for testing.

Preparation of Phosphate Buffer Solution (PBS) and Drug Solutions:

Phosphate buffer solution was prepared by mixing sodium chloride, potassium chloride, disodium phosphate, and monopotassium phosphate in distilled water, then adjusting the pH to 7.4. Tetracycline hydrochloride, doxycycline hyclate, and Triphala powder were dissolved in PBS to create solutions at 25 mg/ml, 50 mg/ml, 75 mg/ml, and 100 mg/ml concentrations.



Figure 1. Depict the armamentarium of the current study that shows the Healiguide membrane, doxycycline (Yellow tablets), Tetracycline (pink tablets), Triphala Churna box, collagenase enzyme, photo-colorimeter, micropipette (green colour), cuvettes etc.

Collagenase Enzyme Preparation:

Collagenase enzyme from Hi-Media laboratories was used. A solution with 1 mg of collagenase (containing 125 collagen digestion units) in PBS was prepared, and 120 µl of this solution was applied to each test sample.

Study Groups:

- Group 1: Control group
- Group 2 (Test Group 1): Tetracycline-treated group
- Group 3 (Test Group 2): Doxycycline-treated group
- Group 4 (Test Group 3): Triphala-treated group

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Each test group received collagenase enzyme, and discs were incubated with PBS, tetracycline, doxycycline, or Triphala at four concentrations (25 mg/ml, 50 mg/ml, 75 mg/ml, 100 mg/ml) at 4°C for 24 hours.



Figure 2. Depict the 5mm collagen membrane discs that were used in the study

Incubation of Collagen Membrane in Collagenase

After initial incubation, collagenase enzyme solution (15 collagen digestion units per 1 ml PBS) was added to samples. Degradation of the collagen membrane was assessed at four time points: days 2, 7, 14, and 21. Using spectrophotometric analysis, protein release at each interval was measured, reflecting the degree of collagen degradation over time.

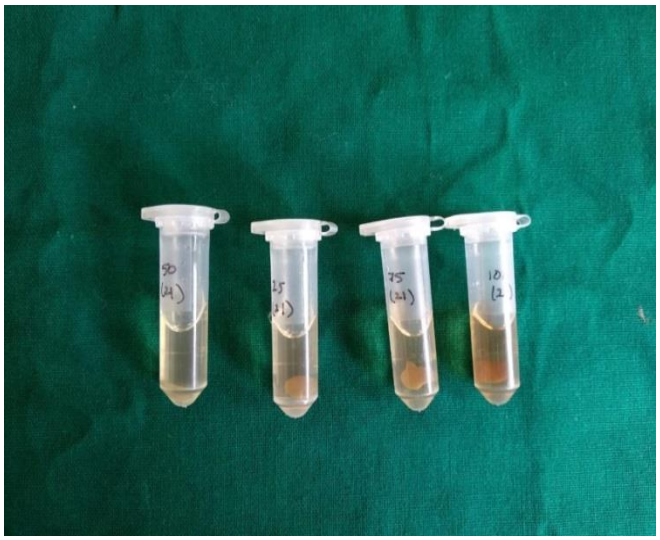


Figure 3. Depict the incubation of collagen membranes in the drugs

Spectrophotometric Analysis

A protein solution containing 10 to 100 µg protein in a volume of up to 1 ml was pipetted into a series of test tubes. The volume in the test tube was adjusted to 1 ml with acetate buffer. 5 ml of diluted reagent was added to the test tube and the contents were mixed by vortexing. The absorbance at 595 nm was measured against a reagent blank prepared from 1 ml of buffer and 5 ml of protein reagent. A calibration curve was constructed with a concentration of protein (µg) against the corresponding absorbance and the amount of protein in the unknown sample was determined.

The test samples to be examined were added to a separate test tube followed by the addition of 5.0 mL of Coomassie blue to each tube and mixed. The total solution was transferred into a cuvette for analysis of protein. The spectrophotometer was adjusted to a wavelength of 595 nm and the amount of protein in the medium was analyzed as changes in the optical density at 595nm wavelength (OD595). Protein concentrations of the test samples were calculated on days 2, 7, 14, and 21. The data collected were statistically analyzed.



Figure 4. Depict the photo-colorimeter that was used in the current study

Statistical Analysis

Study data were entered in Excel and analysis was done using SPSS version 20, with ANOVA and post hoc Tukey's tests for pair-wise comparisons. A p-value < 0.05 was considered significant. All test groups, including those treated with tetracycline, doxycycline, and Triphala, showed reduced protein release compared to the control.

RESULTS

All concentrations of tetracycline with collagenase significantly reduced protein release, indicating inhibited collagen degradation. Among doxycycline-treated samples, all concentrations similarly showed a decrease in protein release, particularly at 25 mg/ml and 75 mg/ml, where differences from the control were statistically significant.

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Table 1. Depict the mean collagen degradation/ protein release in Control and Tetracycline Groups at different time intervals and concentrations

Concentration (mg/ml)	Time Interval	Control Group (Mean Protein Release, SD)	Tetracycline Group (Mean Protein Release, SD)	p-value
25	Day 2	95.20 (±10.3)	38.75 (±7.27)	0.001*
	Day 7	102.30 (±9.7)	41.60 (±8.6)	0.001*
	Day 14	110.40 (±11.5)	42.30 (±7.8)	0.001*
	Day 21	118.10 (±10.2)	44.50 (±8.2)	0.001*
50	Day 2	95.20 (±10.3)	38.50 (±13.3)	0.044*
	Day 7	102.30 (±9.7)	40.20 (±12.4)	0.044*
	Day 14	110.40 (±11.5)	42.10 (±11.3)	0.044*
	Day 21	118.10 (±10.2)	43.70 (±10.2)	0.044*
75	Day 2	95.20 (±10.3)	47.50 (±9.1)	0.007*
	Day 7	102.30 (±9.7)	48.10 (±8.8)	0.007*
	Day 14	110.40 (±11.5)	49.00 (±9.0)	0.007*
	Day 21	118.10 (±10.2)	50.30 (±9.3)	0.007*
100	Day 2	95.20 (±10.3)	49.00 (±8.7)	0.024*
	Day 7	102.30 (±9.7)	50.20 (±8.4)	0.024*
	Day 14	110.40 (±11.5)	51.30 (±8.9)	0.024*
	Day 21	118.10 (±10.2)	52.00 (±8.7)	0.024*

mg/ml- milligrams/ millilitres, SD- standard deviation, * indicates significance, # indicates non-significant, p value <0.05 was considered statistically significant

For Triphala-treated samples, 50 mg/ml and 75 mg/ml concentrations significantly lowered protein release with collagenase. Comparisons between groups revealed that at 75 mg/ml, both tetracycline and Triphala were significantly different from the control (p = 0.046), with doxycycline at 75 mg/ml showing significant inhibition of degradation as well (p = 0.018).

Table 2. depict the significance regarding the protein release/ collagen degradation in Control and Doxycycline Groups at different time periods and concentration

Concentration (mg/ml)	Time Interval	Control Group (Mean Protein Release ±SD)	Doxycycline Group (Mean Protein Release ± SD)	p-value
25	Day 2	95.20 (±10.3)	52.00 (±16.7)	0.006*
	Day 7	102.30 (±9.7)	53.50 (±15.3)	0.006*
	Day 14	110.40 (±11.5)	54.20 (±14.7)	0.006*
	Day 21	118.10 (±10.2)	55.00 (±15.0)	0.006*
50	Day 2	95.20 (±10.3)	58.50 (±17.6)	0.008*
	Day 7	102.30 (±9.7)	59.30 (±16.4)	0.008*
	Day 14	110.40 (±11.5)	60.10 (±15.9)	0.008*
	Day 21	118.10 (±10.2)	61.00 (±16.2)	0.008*
75	Day 2	95.20 (±10.3)	63.00 (±14.1)	0.008*
	Day 7	102.30 (±9.7)	63.80 (±13.7)	0.008*
	Day 14	110.40 (±11.5)	64.60 (±13.9)	0.008*
	Day 21	118.10 (±10.2)	65.20 (±14.0)	0.008*
100	Day 2	95.20 (±10.3)	62.00 (±22.3)	0.003*
	Day 7	102.30 (±9.7)	63.50 (±21.1)	0.003*
	Day 14	110.40 (±11.5)	64.10 (±20.8)	0.003*
	Day 21	118.10 (±10.2)	65.30 (±20.4)	0.003*

mg/ml- milligrams/ millilitres, SD- standard deviation, * indicates significance, # indicates non-significant, p value <0.05 was considered statistically significant

Table 3. Depict the significant mean protein release/ collagen degradation in Control and Triphala Groups at different concentrations and time periods

Concentration (mg/ml)	Time Interval	Control Group (Mean Protein Release ±SD)	Triphala Group (Mean Protein Release±SD)	p-value
25	Day 2	95.20 (±10.3)	58.50 (±39.6)	0.138#
	Day 7	102.30 (±9.7)	60.10 (±37.2)	0.138#
	Day 14	110.40 (±11.5)	61.30 (±36.7)	0.138#
	Day 21	118.10 (±10.2)	62.50 (±37.8)	0.138#
50	Day 2	95.20 (±10.3)	58.50 (±31.5)	0.055
	Day 7	102.30 (±9.7)	59.70 (±30.1)	0.055
	Day 14	110.40 (±11.5)	60.80 (±30.8)	0.055
	Day 21	118.10 (±10.2)	61.70 (±31.4)	0.055
75	Day 2	95.20 (±10.3)	47.50 (±14.3)	0.003*
	Day 7	102.30 (±9.7)	48.70 (±13.7)	0.003*
	Day 14	110.40 (±11.5)	49.60 (±13.9)	0.003*
	Day 21	118.10 (±10.2)	50.50 (±14.0)	0.003*
100	Day 2	95.20 (±10.3)	65.50 (±29.4)	0.184#
	Day 7	102.30 (±9.7)	66.30 (±28.8)	0.184#
	Day 14	110.40 (±11.5)	67.00 (±28.2)	0.184#
	Day 21	118.10 (±10.2)	67.50 (±28.4)	0.184#

mg/ml- milligrams/ millilitres, SD- standard deviation, * indicates significance, # indicates non significant, p value <0.05 was considered statistically significant

Collagen Degradation over Time:

Day 2: Early collagen degradation was observed across all groups, with control showing the highest protein release, indicating active degradation. The test groups, particularly doxycycline and tetracycline, showed minimal protein release, indicating initial effectiveness in inhibiting collagenase activity. **Day 7:** By the seventh day, degradation in the control group continued to increase, while the test groups maintained relatively stable protein release levels, especially at higher concentrations (50 mg/ml and 75 mg/ml). Notably, Triphala at 50 mg/ml and 75 mg/ml showed strong inhibition by this stage.

Day 14: The control group experienced a steady increase in collagen breakdown, with a marked difference in protein release compared to all test groups. The tetracycline and doxycycline groups continued to show lower degradation, and Triphala's effects were sustained at 50 mg/ml and 75 mg/ml, reflecting its mid-term stability.

Day 21: At this final time point, significant degradation was noted in the control group, whereas the doxycycline and tetracycline groups displayed the most sustained inhibition of degradation. Triphala continued to show effectiveness, particularly at 75 mg/ml, supporting its potential for longer-term collagenase inhibition. (Tables 1 to 4)

Table 4. Depict the significant inter group Comparison among Triphala, Doxycycline, and Tetracycline Groups at different concentrations

Concentration (mg/ml)	Group Comparison	Mean Difference	p-value
25 mg/ml	Tetracycline vs. Doxycycline	-13.25	0.922#
	Tetracycline vs. Triphala	-19.75	0.790#
	Doxycycline vs. Triphala	-6.50	0.990#
50 mg/ml	Tetracycline vs. Doxycycline	-20.00	0.752#
	Tetracycline vs. Triphala	-20.00	0.752#
	Doxycycline vs. Triphala	0.00	1.000#
75 mg/ml	Tetracycline vs. Doxycycline	-15.50	0.787#
	Tetracycline vs. Triphala	0.00	1.000#
	Doxycycline vs. Triphala	15.50	0.787#
100 mg/ml	Tetracycline vs. Doxycycline	-13.00	0.912#
	Tetracycline vs. Triphala	-16.50	0.839#
	Doxycycline vs. Triphala	-3.50	0.998#

mg/ml- milligrams/ millilitres, SD- standard deviation, * indicates significance, # indicates non-significant, p value <0.05 was considered statistically significant

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DISCUSSION

An effective periodontal regeneration membrane should be durable, biocompatible, and facilitate healing with minimal complications. Collagen membranes, commonly used in this role, degrade under the influence of collagenase enzymes, potentially shortening their functional period. Studies by Hutmacher and Hyder demonstrate that these membranes perform best when degradation is controlled, making collagenase inhibition a key focus in improving regeneration.[11]

In this study, the use of tetracycline in varying concentrations (especially at 75 mg/ml) effectively slowed membrane degradation, corroborating findings from previous studies. Moses et al. and Zohar et al. found that collagen membranes treated with tetracycline at 50 mg/ml resisted degradation, providing a protective effect through MMP inhibition.[12, 13]

Doxycycline is another widely used antibiotic for periodontal treatment, where it is known to inhibit collagenase and MMPs. Studies by Kutan et al. highlight doxycycline's osteogenic and antimicrobial properties when applied to collagen membranes, supporting healing and protecting against infections.[14] In the current study, doxycycline at 25 mg/ml and 75 mg/ml significantly delayed degradation, aligning with Chang et al.'s findings on its anti-collagenase efficacy.[15] Our results indicate that 75 mg/ml doxycycline concentration is particularly effective in stabilizing collagen membranes.

Triphala, a natural compound used traditionally in Ayurveda, has shown promising anti-collagenase properties. Zaveri et al. and Nair et al. demonstrated Triphala's role in enhancing periodontal healing by inhibiting collagenase activity.[16,17] The tannins in Triphala, particularly from Terminalia chebula, possess strong anti-collagenolytic properties, which contribute to delayed membrane degradation.[18] The presence of bioactive compounds, such as phenols and flavonoids, further supports its potential as a natural inhibitor of collagen breakdown.

In the current study, Triphala at 50 mg/ml and 75 mg/ml significantly reduced membrane degradation in the presence of collagenase. The findings align with Nair et al., emphasizing Triphala's potential in periodontal healing. Although Triphala was effective, it did not surpass the performance of tetracycline or doxycycline at any concentration, possibly due to differences in the mechanism of action and bio-availability of its active components.

Triphala offers an appealing alternative to antibiotics, particularly for patients concerned about resistance or side effects.

Future studies could explore lower concentrations of Triphala and its use in combination with other bioactive agents to optimize its effects on collagen membranes.

This study's limitations include its restriction to a single type of bioresorbable membrane and the testing of only four concentrations. Further research on various membrane types and Triphala's efficacy at different doses would provide broader insights. Additionally, examining collagen membrane structure under different treatments microscopically could add valuable details about degradation patterns.

CONCLUSION

Present study concludes that tetracycline, doxycycline, and Triphala in various concentrations effectively slow down collagen membrane degradation when collagenase is present. Doxycycline exhibited the highest efficacy, particularly at 25 mg/ml and 75 mg/ml concentrations, followed by tetracycline and Triphala. Triphala's results support its potential as a natural, antibiotic-free option in periodontal therapy, though additional studies are needed to maximize its clinical application.

DECLARATIONS

Acknowledgments

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Ethical approval

Approval for the conduction of the study was obtained from the Institutional Review Board GITAM Dental College and Hospital, Visakhapatnam, Andhra Pradesh, India.

Informed Consent

Not Applicable

Sukanya Ampili,Naveen Vital Kumar Gidijala,Tejaswin Polepalle,Shiva Shankar Gummaluri,Trinath Kishore Damera, Bhargavi Devi Vedula. Effect of tetracycline, doxycycline and triphala on collagen memebrane degradation: An in vitro study. Bulletin of Stomatology and Maxillofacial Surgery.2025;21(3).284-290.doi:10.58240/1829006X-2025.3-284

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RESEARCH ARTICLE

DEVELOPMENT AND CHARACTERIZATION OF SELENIUM CONVERSION COATING ON TITANIUM DENTAL IMPLANTS FOR IMPROVED OSSEOINTEGRATION

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ABSTRACT

Background: With the increase in the incidence of peri-implant complications, the development of implant surface coatings focused on improving osseointegration is the need of the hour. The aim of this study was the development and characterization of selenium conversion coatings on titanium dental implants, representing an innovative approach to enhancing osseointegration.

Materials and methods: A 0.1 M solution of sodium selenite was prepared by dissolving it in deionized water, with the pH adjusted to 4 using phosphoric acid. Titanium implants were cleaned, etched in 10% sodium hydroxide, and immersed in the selenium solution for 1 hour with agitation. The coated implants were rinsed, dried, and characterized with Scanning Electron Microscope (SEM), Energy Dispersive X-ray (EDX), Fourier Transform Infrared Spectroscopy (FTIR), biocompatibility, and corrosion analysis. Student's independent t-test was performed to compare the biocompatibility of the selenium conversion coating-based titanium implants (Group A) versus bare titanium implants (Group B) at 24, 48, 72, 96, and 120 hours.

Results: Material characterization revealed successful development of selenium conversion coating on titanium implant surface that appeared as a generalized rough surface with spherical agglomerates. The coating proved its biocompatibility with greatest percentage of cell viability noted at 24 hours with a p-value of 0.653. No statistically significant difference was noted among both Groups A and B in relation to the biocompatibility. Corrosion analysis revealed the coating to be thermodynamically stable with good corrosion resistance properties. **Conclusion:** The developed selenium conversion coating has proven its potential for exploration as a dependable implant surface coating. Additionally, extended in vivo studies are needed to validate its clinical effectiveness.

Keywords: Selenium; peri-implantitis; osseointegration; dental implants

INTRODUCTION

Edentulism remains a prevalent condition globally, with significant implications on an individual's health and well-being.[1] The rehabilitation of edentulous patients is essential for improving their quality of life and reducing the burden of oral diseases on public health systems. As the global population ages, addressing edentulism and enhancing access to rehabilitative dental care will be increasingly important in promoting healthy aging and overall well-being.

Dental implants have revolutionized the field of restorative dentistry, emerging as the preferred treatment option for edentulism.[2] Over the past few decades, dental implants have increasingly supplanted traditional options, such as removable dentures and fixed bridges, due to their superior functionality, longevity, and ability to enhance the quality of life for patients. This shift reflects advances in implant technology, surgical techniques, and a growing body of evidence supporting the efficacy of implants in providing long-term oral health benefits. Advances in implant technology have also made the procedure more accessible to a broader range of patients. Techniques such as guided implant surgery, immediate loading, and the development of mini-implants have expanded the indications for implants, allowing even those with limited bone density or other complicating factors to benefit from this treatment.[3]

However, with the increase in dental implant placements worldwide, there is also an increase in the prevalence of peri-implant diseases and complications, which also greatly impacts the long-term success of the implants.[4] The focus has now shifted towards understanding and managing the complications around dental implants. Peri-implant diseases arise in the peri-implant space as a reaction to the peri-implant bacteria, leading to increased probing depths, suppuration, progressive bone loss, and mobility of the implant.[5] The primary etiologic factor for these biologic complications is the formation of gram-negative anaerobic pathogenic bacteria-based biofilm over the implant surface.[6] The rate of bacterial biofilm formation over the implant surface is further compounded by various factors such as the implant design, implant surface characteristics, poor oral hygiene maintenance by the patient, previous history of periodontal disease, a hyperactive host immune response, presence of systemic risk factors like diabetes and bone disorders, smoking.[7] Moreover, the inadequately planned implant placements leading to increased cases of mechanical failures and advanced diagnostic abilities for early detection of per-implant diseases may also have contributed to the rise in the cases of per-implant diseases.

Recent developments in material science have enabled the creation of dental implant surface coatings that not only enhance osseointegration but also exhibit antimicrobial properties and promote healing.[8] The various mechanisms that have been developed to enhance osseointegration can be broadly divided into enhancing surface roughness, increasing wettability, providing chemical bioactivity, and antimicrobial coatings.[9,10] From sandblasted and acid-etched surfaces, nanostructured implant surfaces, and bioactive glass coatings to antimicrobial peptide coatings and drug-eluting, coatings are being explored to provide a more stable osseointegration with minimal post-operative complications.[11]

Recently, conversion coatings have gained significant attention in biomedical engineering.[12] These coatings chemically modify the surface of a metal through a reaction between the metal substrate and a chemical solution, resulting in the formation of a protective layer. They have been shown to improve their performance by enhancing corrosion resistance, wear protection, and adhesion properties. They have been broadly classified as phosphate conversion coatings, chromate conversion coatings, anodized coatings, oxide coatings, and elemental conversion coatings. Various elements like gadolinium, and strontium have been explored as elemental conversion coatings owing to their inherent antimicrobial and antioxidant properties. Multiple strategies like chemical immersion technique, electrochemical deposition, and plasma-assisted techniques are available for the development of conversion techniques. They have been postulated to provide improved corrosion resistance and enhanced osseointegration with dental implants.

In the present study, a selenium conversion coating was developed on the titanium implant surface. Studies have proven the antimicrobial, antibiofilm, antioxidant, and anti-inflammatory properties of selenium in various biomedical applications.[13-15] Though selenium has been explored extensively for its antimicrobial and antioxidant properties, selenium as a conversion coating on implants has not yet been explored. This coating may provide a mechanically strong implant surface coating along with antimicrobial and osseoinductive properties. The objective of present study was to develop, characterize, and analyze the surface, chemical. biocompatibility, and corrosion-resistant properties of selenium conversion coatings on titanium dental implants.

MATERIALS AND METHODS

Approval from the Institute's Scientific Review Board was obtained for conduction of the study. Commercially pure titanium implants of grade 2 variety of size 20*15*2 mm were procured from Ti Anode Fabricators Pvt Ltd, Chennai, India. The MG-63 cell line was obtained from the National Centre for Cell Science, Pune, India. All media, supplements, and cell staining reagents were sourced from HiMedia Laboratories Private Limited, Thane, India.

Preparation of selenium solution: To prepare the selenium conversion coating solution, sodium selenite was dissolved in deionized water to achieve a concentration of 0.1 M, the pH of which was arrived at 4 using a small amount of phosphoric acid. The low pH enhances the deposition process by ensuring the titanium surface interacts effectively with selenium ions.

Development of selenium conversion coating on titanium implants: To begin with, the titanium implants were thoroughly cleaned and degreased by ultrasonication in ethanol for 10 minutes to remove oils or contaminants, following which they were rinsed with deionized water and air-dried. The implants were then immersed in 10% sodium hydroxide for 30 minutes at room temperature. This step was done to acid etch the implant surface for the production of a rough surface that would be conducive to the adhesion of the coating. The implants were then thoroughly rinsed with distilled water to remove the etchant and were ultrasonicated in distilled water for 10 minutes to remove residual chemicals.

For the deposition of selenium conversion coating, the treated titanium implants were immersed in the prepared selenium solution for a duration of 1 hour. The solution was allowed for gentle continuous agitation with a magnetic stirrer at 40 rpm to ensure uniform exposure to selenium ions. Following the coating process, the implants were withdrawn from the solution and washed with deionized water to remove any surplus selenium ions. They were then allowed to air dry in a clean environment. The developed selenium conversion coated titanium implant surfaces were then characterized to analyze the surface, chemical composition, biocompatibility, antimicrobial activity, and corrosion analysis.

Material characterization: The surface properties of a titanium coating were meticulously examined using advanced characterization techniques to ensure a comprehensive analysis. A Field Emission Scanning Electron Microscope (FE-SEM, JSM IT800, JEOL, Tokyo, Japan) was employed to evaluate surface morphology and structural details. A thin platinum layer was sputter-coated onto the sample for 30 seconds to enhance conductivity and prevent charging issues during imaging. The FE-SEM was operated at an accelerating voltage of 3 kV, achieving high-resolution images with a spatial resolution of 10 micrometers.

To complement the structural analysis, Energy-dispersive X-ray (EDX) spectroscopy was utilized to determine the chemical composition of the material. This approach provided detailed insights into the elemental distribution on the titanium-coated surface.

Additionally, the vibrational properties of the material were characterized using Fourier Transform Infrared Spectroscopy (FTIR) with a Bruker Alpha II instrument. This technique enabled the identification of functional groups and vibrational modes, shedding light on the chemical interactions and material properties.

These combined methodologies offered a robust and multidimensional understanding of the titanium coating's physical and chemical attributes, ensuring a detailed and reliable evaluation for further applications.

Biocompatibility analysis:

The study assessed the biocompatibility, cell morphology, and growth patterns of implant coatings using MG-63 cells. Selenium conversion coated titanium implants comprised Group A while uncoated titanium implants were used as the control group comprised Group B. Dulbecco's Modified Eagle's Medium (DMEM) was prepared and supplemented with essential growth factors and nutrients. Cryopreserved MG-63 cells were cultured until they reached 80% confluency, then seeded onto coated titanium implants at a density of 10,000cells/cm².

Fluorescence staining techniques were employed to analyze cellular behaviour, including attachment, spreading, and proliferation. Rhodamine B and Acridine Orange were used for single-dye staining, while combined staining methods were applied for more comprehensive insights. The imaging process preserved the morphology of the stained cells, allowing for accurate evaluation.

For detailed visualization, fixed and stained cells were transferred onto glass slides, with DAPI mounting medium used for nuclear counterstaining. Image J software was used to process the acquired images and

analyze cellular activity. The number of living and dead cells was counted to compute cell viability, a critical indicator of biocompatibility. Assessments were conducted at various time points to monitor changes in cell viability over time.

Corrosion analysis: The coated titanium implants were placed in the electrochemical cell as the working electrode, ensuring that the implant surface area was exposed to the electrolyte, phosphate buffer saline with a pH of 7.4. Under open circuit potential, the titanium implants were allowed to stabilize in the electrolyte for 30–60 minutes, with no external current applied. Impedance measurements were conducted using an amplitude of ±10 mV. Impedance spectra were taken at frequencies ranging from 0.01 Hz to 200 kHz. The potentiodynamic polarization was scanned at a rate of 1 mV/s.

Statistical analysis: The data was analyzed using IBM SPSS Statistics for Windows, Version 23.0 (released in 2015 by IBM Corp., Armonk, NY, USA). An unpaired t-test was utilized to compare the biocompatibility of selenium-coated titanium implants to the bare titanium implants, with p-values < 0.05 indicating statistical significance.

RESULTS

Material characterization: The SEM analysis (Figure 1) revealed a surface that showed a generalized rough surface with selenium nanoparticles as spherical aggregates. The coating appeared as a uniformly spread layer of selenium with no agglutination of selenium ions which showed the optimized nature of the coating. No significant defects or contaminants were observed, indicating a well-prepared surface that is likely to enhance the implant's corrosion resistance and biocompatibility. The rough uneven coating on the implant surface created by the conversion coating would aid in adhesion, differentiation and migration of cells over the coating. This would ultimately decrease the osseointegrative period allowing for enhance osteoblast attachment and bone deposition. EDX analysis (Figure 2) revealed titanium and oxygen as the predominant component at 83.6wt% and 13.9% respectively. It also showed the presence of selenium at 2.5wt% which proved the successful coating of selenium conversion coating on titanium. FTIR analysis (Figure 3 and Table 1) revealed predominant waves at 860, 1419 which depict the presence of selenium ions and the interaction between selenium and titanium respectively. The waves ranging between 3500-3000 depicted the stretching vibrations of hydroxyl groups. They along with the wave at 1625 depicted the presence of water molecules in the interlayers. A recent study that charcaterized the selenium and hyaluronic acid coating on titanium dental implants reported characteristic waves that were in accordance with the waves observed in our study.[16]

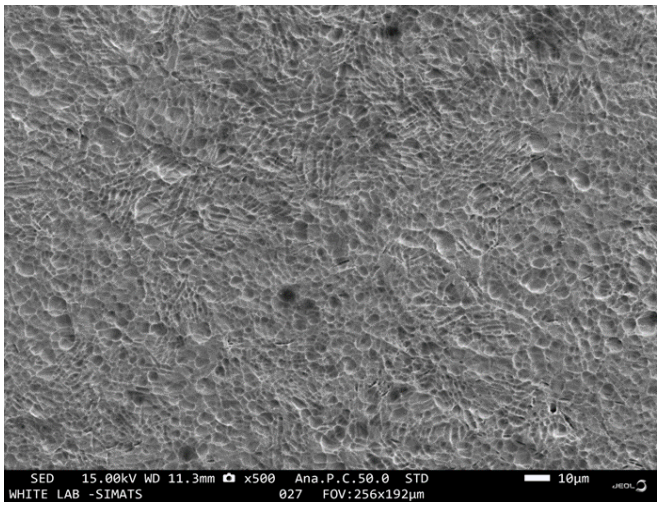


Figure 1. SEM image of the developed selenium conversion coating on titanium implant

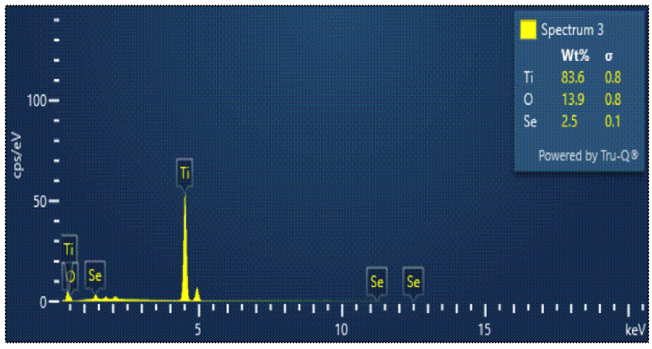


Figure 2. EDX analysis of the developed selenium conversion coating on titanium implant

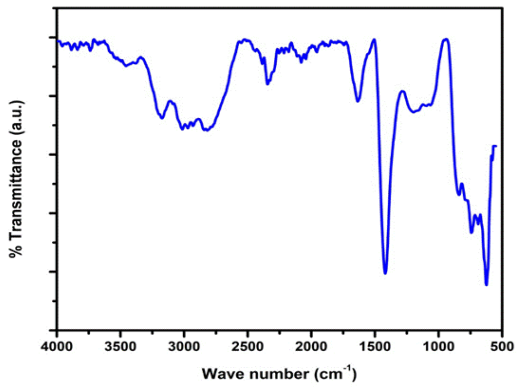


Figure 3. FT-IR analysis of the developed selenium conversion coating on titanium implant

Table 1. The absorbance peaks observed with the selenium conversion coating on titanium implant on FTIR analysis.

WAVE NUMBER (CM^{-1})	BOND	FUNCTIONAL GROUP	INTERPRETATION
3500-3000	Hydrogen bonded – OH	Hydroxyl group	stretching vibration of hydrogen-bonded hydroxyl groups
1625	H-O-H	-	Bending vibrations of water molecules
1419	Ti-O	Titanium oxides	interaction between Ti-O and Se
860, 800-550	Se-O	Selenite functional group	Stretching vibration of selenite ion

Biocompatibility analysis

The biocompatibility of selenium-coated titanium implants was assessed using MG-63 cells and staining techniques. The cells showed spindle-shaped morphology and well-defined organelles, with filopodial extensions and nuclear material (Figure 4). No statistically significant difference was found between groups, suggesting the selenium-coated titanium was as biocompatible as the bare titanium implant surface (Figure 5 and Table 2). A study on selenium-doped hydroxyapatite-coated titanium implants also showed excellent biocompatibility with the proliferation of pre-osteoblasts.[17]

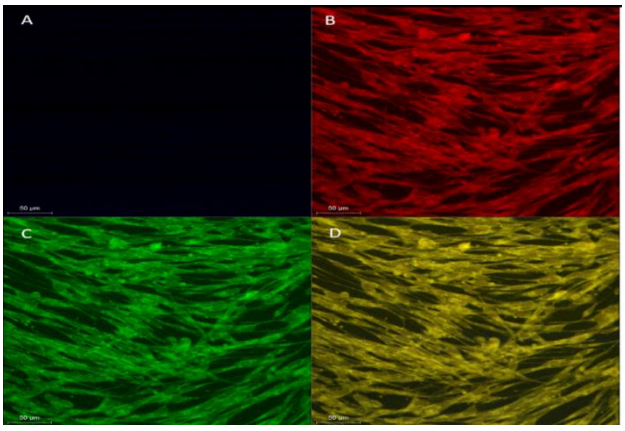


Figure 4. The biocompatibility analysis of a selenium conversion coating on a titanium implant was conducted using a confocal microscope, using various staining methods. A - blank; B - rhodamine b staining; C - Acridine orange staining; D - combination staining

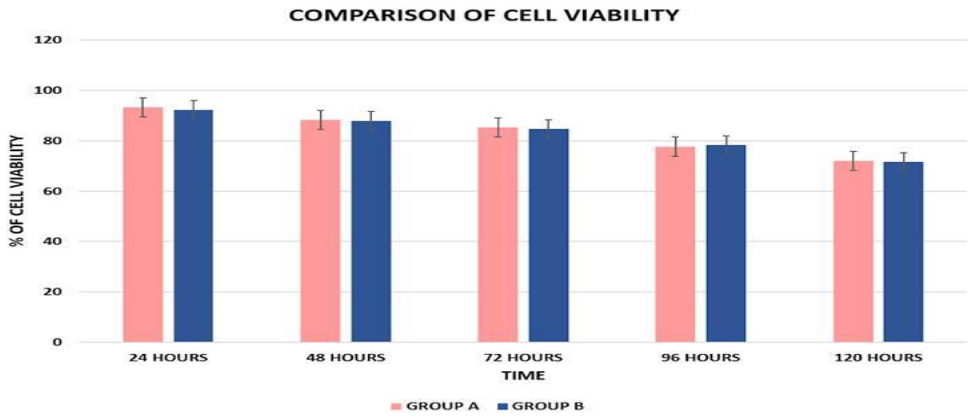


Figure 5. Comparison of biocompatibility of selenium conversion coating on titanium implants versus bare titanium implants at different time points

TABLE 2. Comparison of biocompatibility of selenium conversion coating on titanium implant (group A) and bare titanium implant (Group B) - unpaired t test

TIME	SAMPLE	N	MEAN (% OF CELL VIABILITY)	STANDARD DEVIATION	P VALUE
24 HOURS	Group A	3	93.33	1.154	0.653
	Group B	3	92.33	1.527	
48 HOURS	Group A	3	88.33	1.527	0.275
	Group B	3	88.00	2.645	
72 HOURS	Group A	3	85.33	1.643	1.000
	Group B	3	84.67	1.943	
96 HOURS	Group A	3	77.67	2.516	0.632
	Group B	3	78.32	3.511	
120 HOURS	Group A	3	72.00	2.645	0.833
	Group B	3	71.56	3.055	

Corrosion analysis: The corrosion analysis of coated and bare implant surfaces was performed and the results are depicted in Figure 6. According to potentiodynamic data, the coated surface demonstrated greater corrosion resistance by having a lower corrosion current (Icorr) and a higher positive corrosion potential (Ecorr) than the bare metal. Electrochemical impedance spectroscopy, represented by the Nyquist plot, revealed a larger impedance arc for the coated surface, signifying higher resistance to current flow. Bode impedance analysis at low frequencies showed a two-fold increase in impedance for the coated surface, confirming enhanced corrosion resistance. The Bode phase angle at mid-frequency levels indicated near-ideal capacitive behavior for the coated surface. Overall, the coating demonstrated excellent electrochemical performance and corrosion resistance, which could improve the long-term in vivo stability of implants. These findings align with previous research on selenium conversion coatings for magnesium alloys, which showed minimal corrosion rates.[18]

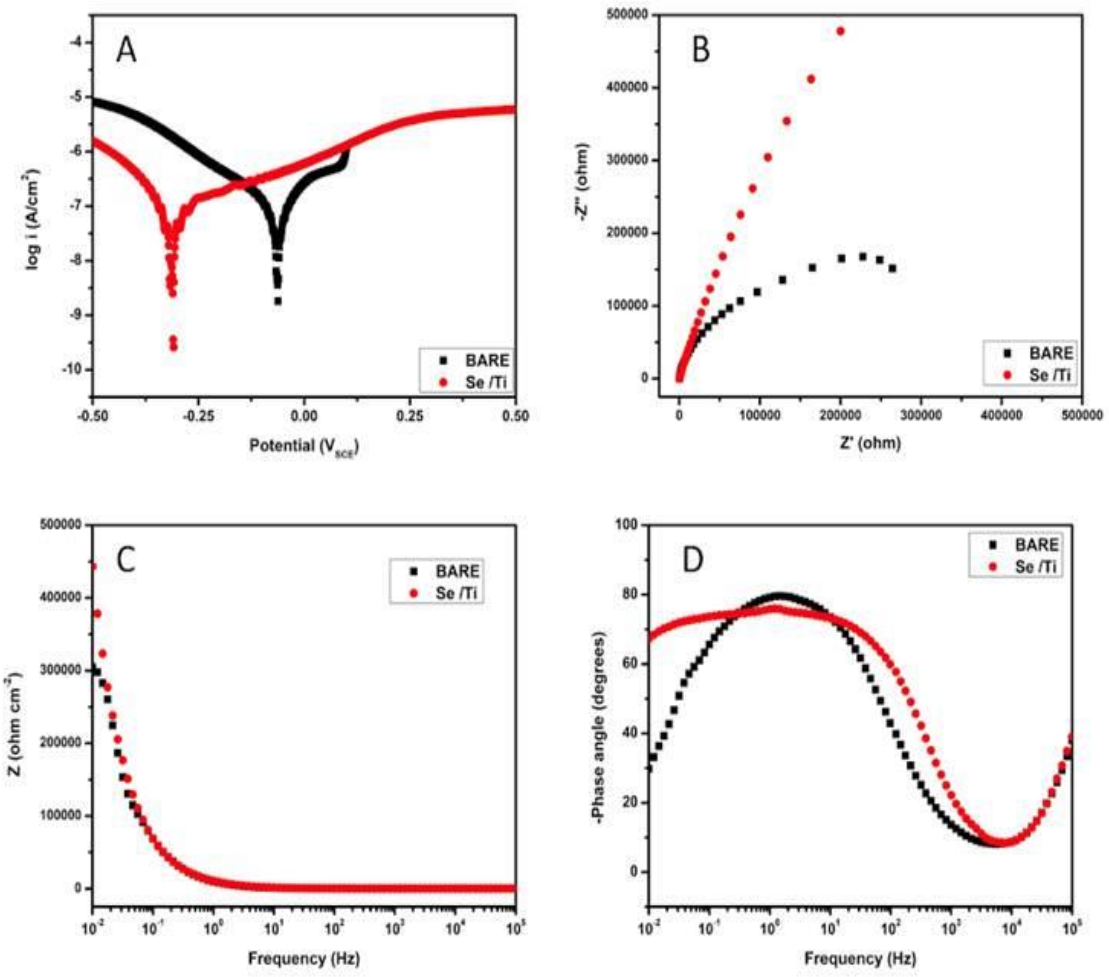


Figure 6. Corrosion analysis of the developed selenium conversion coating on titanium implant. A - potentiodynamic polarization, B - Nyquist plot, C - bode impedance, D - bode phase angle

DISCUSSION

Attaining a stable osseointegrated implant with healthy well-integrated per-implant soft tissue is one of the prerequisites for the long-term success of the implant with minimal biologic complications. The aim of this study was to develop and characterize selenium conversion coating on titanium dental implants. Conversion coatings have gained meaningful notice, due to their advantages such as improved corrosion resistance, enhanced surface roughness, and cell adhesive properties with the added benefits of customization and biocompatibility.[19] They can be broadly classified as chromate, phosphate, oxide, zinc, silicate, and ceramic coatings. Each type of conversion coating has unique properties that make it suitable for specific applications.

In this study, a selenium conversion coating was applied to titanium dental implants using a chemical process and dip coating method. SEM analysis (Figure 1) revealed a uniform layer of selenium nanoparticles in spherical aggregates, with no clumping, indicating an even distribution. The rough surface of the coating would support osteoblastic cell attachment, proliferation, and differentiation. Elemental mapping (Figure 2) confirmed a 2.5% selenium coating. FTIR analysis (Figure 3 and Table 1) identified characteristic peaks for selenite, selenium-titanium interactions, hydroxyl groups, and water molecules. These findings align with previous research showing that increased surface roughness enhances apatite formation on magnesium implants.[18]

Confocal analysis of selenium-coated implants cultured with MG63 cells showed similar cell viability to bare titanium implants at various time points, indicating comparable biocompatibility (Figure 5 and Table 2). Spindle-shaped viable cells arranged in stacked layers with filopodial extensions suggested intercellular communication and migration (Figure 4). The nucleus and organelles were clearly visible. These results demonstrate that the coating supports cell adhesion and proliferation. Similar findings were reported in a study on selenium coatings for orthopedic implants, showing no inhibition of osteoblast growth.[20] They also noted a decreased bacterial adherence of *Staphylococcus aureus (S. aureus)* and *Staphylococcus epidermidis (S. epidermidis)* which they attributed to the presence of selenium. Another study that assessed the selenium doped hydroxyapatite coated titanium implants showed excelled biocompatibility and also proved antibacterial properties over the biofilms of *Pseudomonas aeruginosa* (*P. aeruginosa)* and *S. aureus*.[17] The corrosion analysis (Figure 6) further proved that the developed coating had greater thermodynamic stability and corrosion resistance in comparison to the bare titanium implants. Congruent with our findings were the results of another study which stated that selenium and hyaluronic acid-based implant surface coating showed a decreased rate of corrosion along with an increase in the polarization resistance.[16] Improved corrosion resistance properties would ultimately increase the longevity of the implants owing to the reduced wear and tear, effective barrier function leading to enhanced material integrity and improved mechanical properties. These properties would ultimately increase the biocompatibility, osseointegration and the functional longevity of the implants.

Metalloid nanoparticle implant coatings have gained favor owing to their increased surface area, bioactivity, antimicrobial properties and wear resistance.[21]

Silicon, Boron, and strontium-based coatings have been explored as coatings for orthopedic, dental, cardiovascular and neural implants. One such metalloid nanoparticle that is essential to many biological processes in the human body as a trace element is selenium. The second highest selenium concentration remains in the bone among all human tissues.[22] Selenium as a component of selenoproteins also exhibits antioxidant, antimicrobial, anti-inflammatory, and anticancer properties.[23-25] The antimicrobial mechanism of selenium has been postulated to have a multifactorial mode. Protein degradation, loss of cell membrane integrity, photocatalytic action, prevention of bacterial adhesion and microcolony formation, interference with enzymatic functions and transport mechanisms, reactive oxygen species (ROS) production have been suggested to be the various mechanisms of antimicrobial activity.[21, 26, 27] Studies have proven antimicrobial activity of selenium nanoparticles against common oral pathogens such as *Streptococcus mutans (S. mutans)*, *S. aureus*, *Porphyromonas gingivalis (P. gingivalis)*, *Escherichia coli (E. coli), and Candida albicans (C. albicans)* with reactive oxygen damage, cell membrane damage and protein leakage stated as the main mechanism of antimicrobial action.[28, 29] Selenium has also been proven to exert antioxidant and anti-inflammatory properties owing to its presence in the form of selenoproteins. Various selenoproteins such as the selenoprotein P, glutathione peroxidases (GPx), thioredoxin reductases (TrxR) have shown to exert a protective effect against free radicals induced oxidative stress among cells and tissues thereby maintaining a redox homeostasis.[30, 31] Research has shown that selenoproteins play a significant role in immunoregulation, thereby preventing the emergence of hyper-responsive phenotypes that lead to chronic inflammatory states.[32]

Selenium influences osteoblast differentiation, the process by which precursor cells develop into mature bone-forming cells. According to research, selenium may encourage mesenchymal stem cells to differentiate into osteoblasts, aiding osteogenesis.[33, 34] Selenoprotein-dependent signaling pathways in osteoblasts have been shown to enhance the synthesis of bone matrix proteins, including collagen and osteocalcin, both of which are essential for bone structure and mineralization.[35] Several genes related to selenoproteins and at least nine genes involved in selenoprotein biosynthesis have been identified in osteoblasts and osteoclasts.[34] Thioredoxin reductase 1 (TrxR1), a selenoprotein, is upregulated by vitamin D and is expressed early in the osteoblast differentiation process, illustrating part of the mechanism through which selenium supports bone

formation.[36, 37] Glutathione peroxidase 1 (GPx1), an important antioxidant enzyme in osteoclasts, plays a role in inhibiting osteoclastogenesis when activated.[36]

In bone marrow matrix cells cultured under low selenium conditions, there was reduced expression of GPx and TrxR, along with signs of chromosomal damage.[38] These issues were corrected with selenium supplementation, which restored normal selenoprotein activity. Supplementing with higher-than-normal levels of selenium has been shown to influence specific pathological changes through various mechanisms, such as the suppression of Nuclear Factor Kappa B (NF-κB) activation and its associated inflammatory responses by GPx and TrxR. Selenium has also been found to protect bone marrow stromal cells from the inhibitory effects of hydrogen peroxide on osteoblastic differentiation by reducing oxidative stress and blocking Extracellular Signal-Regulated Kinase (ERK) activation.[39, 40]

Moreover, the noteworthy aspect of the implant coating developed in this study is that it is a conversion coating, a thin protective layer of metals formulated through a chemical conversion process, where the metal reacts with a chemical solution to create a stable, typically corrosion-resistant coating. Typically, these chemical reactions transform the metal surface into a new chemical compound, often an oxide or phosphate, which adheres strongly to the substrate. The key characteristics of these coatings that prove their superiority over other physical coatings are due to the production of a thin layer of coatings that chemically bonds to the surface materials, thereby preventing delamination. They also improve the corrosion resistance and material durability, ultimately increasing the longevity of the material. Nonetheless, this study is not without its limitations, which lie in the fact that mechanical testing, antimicrobial analysis, and cell line studies to analyze the mineralization potential of the developed coating was not performed.

Based on the scientific evidence available, we postulate that selenium conversion coating as a dental implant coating would provide an antimicrobial, antibiofilm, anti-inflammatory, anti-oxidant, and ossoeinductive effect owing to the presence of selenium while also improving the corrosion resistance and material durability owing to the presence of the coating as a conversion coating. This would enhance the osseointegrative outcomes while minimizing the biologic complications. The results of this study prove the successful development of selenium conversion coating with optimal biocompatibility and good corrosion resistance. However, preliminary results are insufficient to claim clinical superiority over other standards available currently. Further long-term in vivo and clinical studies should be performed to analyze the antimicrobial, anti-inflammatory, antioxidant, osseoinductive, mechanical, and genetic expression properties and the clinical efficacy of this coating.

CONCLUSION

In conclusion, the developed selenium conversion coating on titanium implants exhibited good surface, chemical, and corrosion resistance properties with optimal biocompatibility, presenting a promising approach to enhance the performance of titanium dental implants. By leveraging selenium's antioxidant, antimicrobial, biocompatible, and osseoinductive properties, these coatings can significantly improve osseointegration, reduce inflammation, and provide corrosion resistance, which are essential factors for the long-term success of dental implants. Overall, selenium conversion coatings offer a cost-effective and sustainable solution for enhancing the functionality and longevity of dental implants, making them a valuable advancement in dental implant technology.

DECLARATIONS

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Ethical approval: Approval for the conduction of the study was obtained from the Institutional Scientific Review Board (SRB/SDC/UG-1931/23/PERIO/188).

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RESEARCH ARTICLE

ASSESSMENT OF THE POTENTIALLY TOXIC EFFECT OF 2% AND 4% ARTICAINE ON THE PATIENTS' CARDIOVASCULAR SYSTEM DURING TOOTH EXTRACTION

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ABSTRACT

Aim: The purpose of this study was to conduct a comparative analysis of the potential toxicity of 2% and 4% articaine on the cardiovascualar system of patients during tooth extraction, as well as to assess the risk of complications associated with the use of anesthetics. As part of the study, the tasks were set to assess the toxicity of articaine on cell cultures and analyze hemodynamic changes in patients during the tooth extraction procedure.
Materials and Methods: The determination of the half-life concentration (LC50) in an in vitro study was used to assess cytotoxicity.Monitoring of the cardiovascular and respiratory systems was carried out using Mindray ePM 10.
Results: A cell culture study confirmed that the 2% drug has significantly lower cytotoxicity than the 4% drug. The monitoring results showed that systolic blood pressure was normal before surgery, with no statistically significant differences between the groups ($p>0.05$). 2 minutes after the injection of the anesthetic, the pressure remained normal, but after 4 minutes it increased, while in the 2% articaine group it was 3 mmHg lower than in the 4% group ($p<0.05$). By 8 minutes, the pressure returned to normal values. Dynamics analysis showed a significant increase in systolic blood pressure in both groups by 2 and 4 minutes, followed by a decrease by 8 minutes ($p<0.001$). Diastolic pressure and pulse also did not show significant differences between the groups, but their dynamics increased in both groups by 4 minutes, followed by a decrease by 8 minutes ($p<0.001$). Saturation remained normal, but it decreased by 4 minutes with recovery by 8 minutes ($p<0.001$).
Conclusion: The revealed results confirm that 2% articaine is a safe alternative to 4% articaine, especially for patients with concomitant pathologies. The study highlights the need for careful choice of anesthetics in clinical practice to minimize the risk of complications.

Keywords: Articaine; Cardiovascular system; Cytotoxicity; Tooth extraction; Dentistry

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INTRODUCTION

One of the most common operations in surgical dentistry is dental extraction. This manipulation requires knowledge from the doctor not only in the field of manual skills, but also an understanding of the risks of life-threatening complications that occur during the anesthesia stage. Undesirable reactions may occur due to violations of the anesthesia technique and may also be associated with the anesthetic itself in case of incorrect choice of its dosage and not careful collection of medical history.[1]

In addition, the lack of consideration of individual anatomical features of patients can also lead to complications during the work of a dental surgeon.[2] Increasingly, dentists prefer articaine-based local anesthetics. Articaine is a local anesthetic belonging to the amide group of anesthetics. Good solubility in water leads to rapid penetration through the membrane of a nerve cell or axon and creates intracellularly high concentrations, which is due to a short latency period of 1-3 minutes. Systemic toxicity is prevented by the high value of binding to plasma proteins[3]

Articaine does not cross the placental and blood-brain barriers, unlike other amide anesthetics. This is due to the relatively poor permeability of the vascular endothelium to arrhythmia, therefore the drug penetrates poorly into the bloodstream and lingers longer at the injection site .[4,5]

Adverse reactions occur quite rarely, which allows it to be used in pregnant women and children.[6]

Therefore, articaine is the optimal anesthetic for anesthesia in pregnant and lactating women due to its high degree of binding to plasma proteins – 95%.[7] Studies prove that articaine-based anesthetics are recommended for use in the treatment of pregnant women who have not been diagnosed with extragenital and obstetric pathology .[8]

Articaine-based drugs are the recommended choice of local anesthesia in children from 5 years of age due to their low toxicity and wide range of therapeutic effects [9]. And according to O.V. Gulenko's research, articaine can also be used in the treatment of children under 4 years of age [10]

Articaine, among other things, is a suitable alternative for patients with a true allergy to lidocaine. No cross-reactivity has yet been reported between lidocaine and articaine .[11]

For various somatic diseases, you also need to know how to choose the right anesthetic. For example, lidocaine and mepivacaine have a sedative effect, therefore, if a patient has central nervous system diseases, it is recommended to use articaine, since it does not affect the central nervous system.[12]

Articaine is used in dental practice more and more often, and previously it was compared with the "gold standard" in the form of lidocaine. But research does not stand still, the effectiveness of articaine has been proven, and now the topic of reduced concentration of local anesthetics in solution is relevant.

The novelty of the proposed topic on literary sources and patent documentation is determined by the fact that there are currently no comprehensive studies in the domestic and foreign scientific literature devoted to the study of indications for the use of 2% articaine.

The aim of the study was to compare the use of 2% and 4% articaine during tooth extraction and reduce the risk of complications with the use of anesthetics.

To achieve this goal, the following tasks were set:

1. To evaluate the potential toxicity of 2% and 4% articaine on human cell culture (epitheliocytes).
2. To evaluate the stress response of the patient's body during tooth extraction and indirectly the toxic effect of the anesthetic by recording changes in hemodynamics (blood pressure, pulse).

MATERIALS AND METHODS

Assessment of the potential toxicity of 2% and 4% articaine in human cell culture (epitheliocytes)

To substantiate the reason for the use of 2% articaine in clinical practice, especially in patients with concomitant pathology, children and the elderly, a comparative analysis of the cytotoxicity of 2% and 4% articaine in an in vitro study was performed, namely, the determination of the half–life concentration (LC_{50}).

The study was conducted based on the Laboratory of Molecular and Cellular Technologies and Experimental Research of the Institute of Physics and Technology of the National Research Nuclear University MEPhI.

The experiment was conducted on the primary culture of epithelial cells of the human oral cavity (Human oral epithelial primary cell culture, Celprogen Inc., USA). This cell culture was chosen due to its proximity to the conditions during infiltration anesthesia.

Subcultivation protocol:

1. The vial was thawed, stirring gently, in a water bath at 37°C.
2. The contents of the vial were transferred to a sterile centrifuge tube with a volume of 15 ml and carefully preheated medium was added for the primary culture of epithelial cells of the human oral cavity. The cells were centrifuged at 100 g for 5 minutes. The supernatant was removed, and the cellular precipitate was re-suspended in 500 µl of medium.
3. 500 µl of cell suspension was transferred to a 25 cm^2 culture vial with 10 ml of medium.
4. The cells were incubated at 37°C in a humidified incubator in an atmosphere of 5% CO2. The medium was changed every 2-3 days, cultured to a confluent of

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70%, and then the cells were transplanted for the experiment.

The cells were planted in 96-well plates with a density of $5*10^3$ cells/well. The cells were counted and viability was determined by staining with trypan blue (Invitrogen, USA) in a dye–suspension ratio of 1:1, with a total volume of 20 µl and measuring in a LUNA II Brightfield Cell Counter (Biolab, USA). After reaching 100% confluence, a cytotoxicity study was performed. The cells were washed with a phosphate buffer and the studied preparations dissolved in the culture medium in the following concentrations were added: 100, 50, 25, 12,5, 6,25, 3,13, 1,56 and 0.78%. A medium without the addition of a drug was used as a control.

The study was carried out in three repetitions. After 7 days, the culture reaches a confluent of 100%.

After 3 hours of incubation, cell viability was determined according to the MTT protocol:

1. The medium was removed from the wells of the tablet and washed with phosphate buffer.
2. 50 ml of serum-free medium and 50 ml of MTT (dimethyl sulfoxide) solution were added to each well.
3. The tablet was incubated at 37°C for 3.5 hours.
4. After incubation, 150 µl of MTT solvent was added to each well.
5. The tablet was wrapped in foil and shaken in an orbital shaker for 15 minutes until the MTT formazane was completely dissolved.
6. Light absorption was measured at a wavelength of 590 nm in an XMark spectrophotometer (Bio-Rad Laboratories, USA).

Visual control of proliferation was performed in an inverted Nexcope NIB600-FL microscope.

According to the obtained optical density values, cell viability was determined with normalization to control values (wells without the addition of the drug were taken for viability of 100%), graphs of the dependence of viability on the concentration of drugs were plotted, and the half-year concentration (LC_{50}) was determined.

Assessment of the stress response of the patient's body by recording changes in hemodynamics

To assess the stress level for the body and the potential reaction to the anesthetic, the cardiovascular and respiratory systems were monitored.

These measures were carried out using the Mindray ePM 10 device, a medical device designed to monitor the patient's condition during dental procedures. This compact and lightweight monitor provides reliable and accurate display of vital signs of the patient, such as blood pressure, pulse rate, oxygen saturation in the blood and respiratory function.

During the monitoring, blood pressure (systolic and diastolic), pulse, and saturation were measured before surgery, 2, 4, and 8 minutes after administration of the anesthetic.

Pulse oxygen saturation (SpO2) monitoring is a non–invasive method used to measure the amount of oxygenated hemoglobin and pulse rate by measuring the absorption of selected wavelengths of light. The light generated on the side of the probe emitter is partially absorbed when passing through the controlled tissue.

The amount of transmitted light is determined on the detector side of the probe. When examining the pulsating part of the light signal, the amount of light absorbed by hemoglobin is measured, and pulse oxygen saturation can be calculated. This device is calibrated to display functional oxygen saturation.

The monitor uses an oscillometric method to measure noninvasive blood pressure (NIBP). The measurement of NIBP is based on the principle that the pulsating blood flow through an artery creates fluctuations in the arterial wall. An oscillometric device uses a blood pressure cuff to sense these fluctuations, which manifest as tiny pulsations of cuff pressure. Oscillometric devices measure the amplitude of pressure changes in the occlusal cuff when the cuff deflates from pressure above systolic. The amplitude suddenly increases when the pulse breaks through the occluded place in the artery. As the cuff pressure decreases further, the pulsations increase in amplitude, reach a maximum (which approaches the average pressure), and then decrease. The oscillometric method measures average blood pressure and determines systolic and diastolic blood pressure.

RESULTS

Assessment of the potential toxicity of 2% and 4% articaine in human cell culture (epitheliocytes)

As a result of the study, viability values were obtained (Table 1) and graphs of the dependence of viability on the concentration of the studied drugs in the medium were constructed (Fig. 1 and 2). The images of epitheliocytes after incubation with drugs (Fig. 7) show that the destruction of the monolayer under the action of the drug is observed at lower concentrations for the drug of 4% than for 2%. Based on the experimental data on the effect of drug concentrations on cell viability, the half-life concentration (LC_{50}) values were determined, which for drugs of 2% and 4% were 65.7% and 17.5%, respectively.

The results obtained allow us to conclude that the 2% drug has significantly lower cytotoxicity than the 4% drug.

Table 1. The average values of epithelial cell viability for different concentrations of drugs are 2% and 4%.

The concentration of the drug in the medium, %	Viability, %	
	2%	4%
100,00	9,6%	10,3%
50,00	77,5%	7,2%
25,00	91,3%	8,7%
12,50	91,6%	86,0%
6,25	99,8%	96,8%
3,13	88,9%	92,0%
1,56	101,6%	96,0%
0,78	101,7%	99,8%

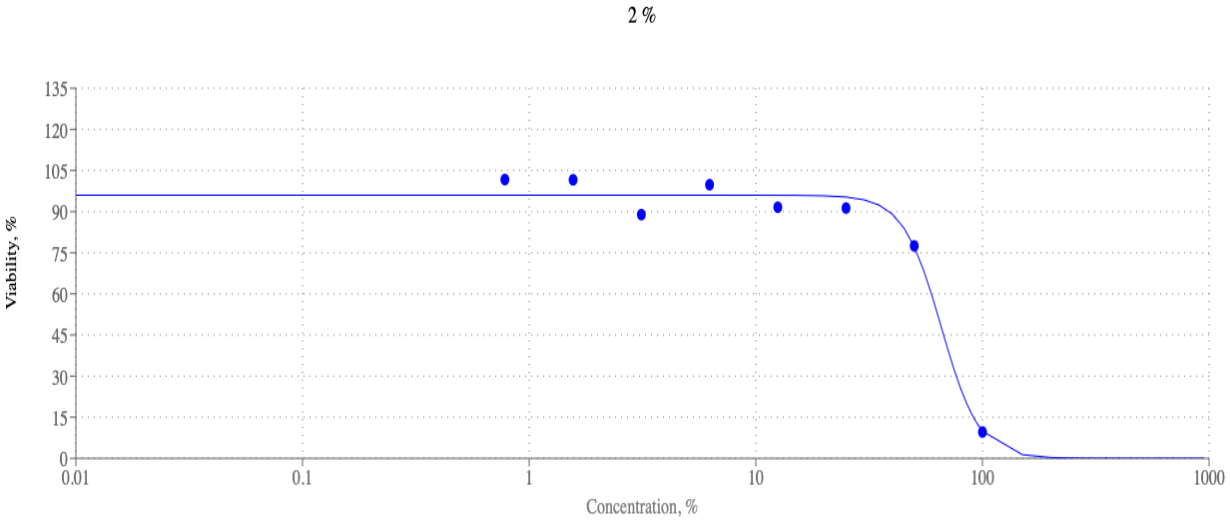


Figure 1. The dependence of epithelial cell viability on the concentration of the drug 2% in the medium.

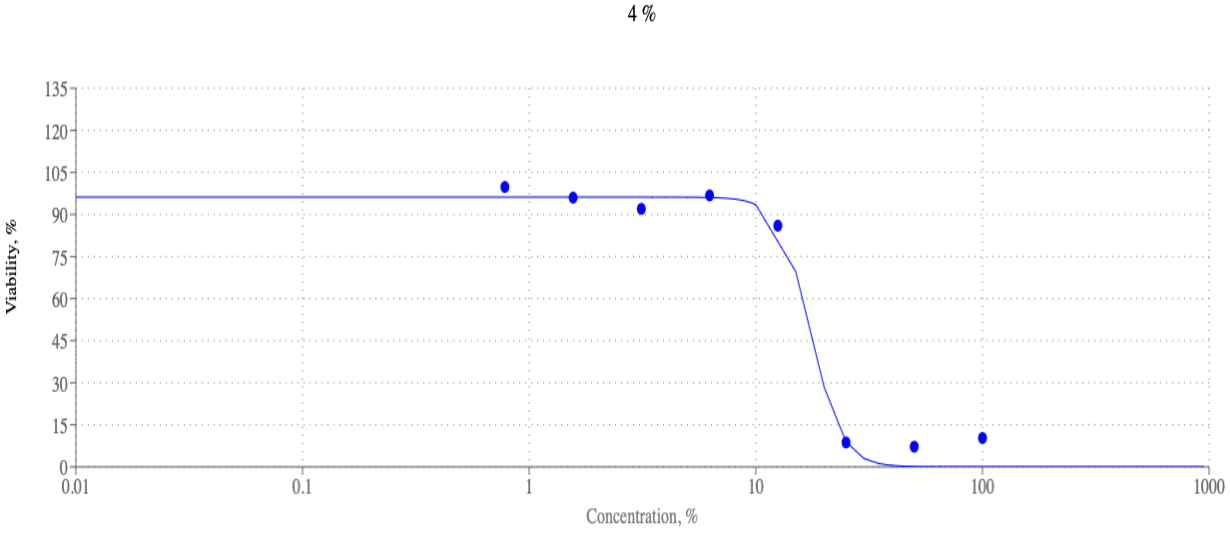


Figure 2. The dependence of epithelial cell viability on the concentration of the drug 4% in the medium.

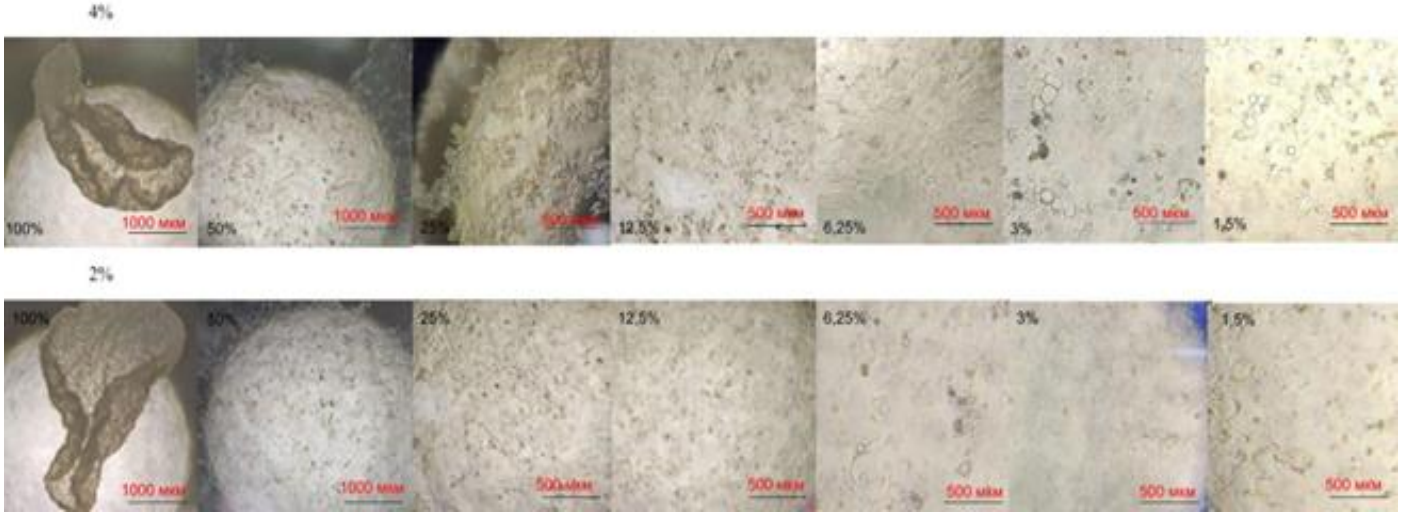


Figure 3. Images of the cell monolayer of epitheliocytes after incubation for 3 hours with different concentrations of 2% and 4% preparations.

Assessment of the stress response of the patient's body by recording changes in hemodynamics

According to the standards of the European Society for the Study of Hypertension and the recommendations of the Ministry of Health of the Russian Federation of 2020, the following ranges of figures are set for blood pressure (BP) [13]:

- Normal –120-129/80-85 mmHg;
- High normal – 130-139/85-89 mmHg;
- 1st degree – 140-159/90-99 mmHg;
- 2nd degree – 160-179/100-109 mmHg;
- 3rd degree – more than 180/110 mmHg.

For the pulse of an adult, the norm is 60-80 beats per minute, but under certain conditions, the norm can be 50 beats (for trained people) and 90 (for women and young people).

When measuring the average oxygen saturation rate in adults, when measuring with a pulse oximeter, the normal oxygen saturation level in an adult is more than 95%. Saturation from 94% to 90% indicates respiratory failure of the 1st degree. With respiratory failure of the 2nd degree, saturation decreases to 89%-75%, and hypoxemic coma is less than 60% .[14]

The results of monitoring are given in Tables 2-5.

Table 2. The results of monitoring systolic blood pressure in the study groups in dynamics (n=56). Each cell contains: mean±the standard error of the mean, the median, and the range from minimum to maximum values.

Measurement period	Group 1 (n=28) Articaine 2%	Group 2 (n=28) Articaine 4%	p
Before the operation	112,4±10,8 123,5 99-140	124,2±7,7 123,5 110-138	>0,05
2 minutes after the injection of the anesthetic	126,6±11 129 99-145	127,1±8,3 127 110-140	>0,05
4 minutes after the injection of the anesthetic	125,3±7,2 126,5 110-140	130,6±5,6 130 114-145	**<0,05 (0,028)**
8 minutes after the injection of the anesthetic	119±8,8 120 100-131	119,3±6,5 121 109-132	>0,05
p	**<0,001**	**<0,001**	

Before surgery, on average, all patients had normal systolic blood pressure, and there were no statistically significant differences between the groups (p>0.05).
After 2 minutes in both groups, blood pressure remained normal without statistically significant differences (p>0.05).
4 minutes after administration of the anesthetic, systolic blood pressure increased, but remained within the normal or high normal range, and 3 mmHg less in the 2% articaine group compared to 4% (p<0.05).
After 8 minutes, the pressure decreased by 5-6 mmHg and reached normal values in both groups. There were no statistically significant differences (p>0.05).
When assessing the dynamics of systolic blood pressure within each group, there was a significant increase by 2 minutes with a gradual decrease by 8 minutes with the administration of 2% articaine and a significant increase by 4 minutes with a decrease by 8 minutes with the use of 4% articaine (p<0.001).

Table 3. The results of monitoring diastolic blood pressure in the study groups in dynamics (n=56). Each cell contains: mean±the standard error of the mean, the median, and the range from minimum to maximum values.

Measurement period	Group 1 (n=28) Articaine 2%	Group 2 (n=28) Articaine 4%	p
Before the operation	70,25±7,3 70 56-89	71,1±7,8 71 56-88	>0,05
2 minutes after the injection of the anesthetic	73,71±5,9 74 62-88	72,18±7,6 72 60-90	>0,05
4 minutes after the injection of the anesthetic	72,9±5,6 71,5 60-88	73,9±6,2 74 62-91	>0,05
8 minutes after the injection of the anesthetic	67,5±5,3 69 58-80	67,9±4,7 68 60-78	>0,05
P	**<0,001**	**<0,001**	

When comparing diastolic blood pressure between the 2% and 4% articaine groups, no statistically significant differences were found at all registration periods (p>0.05). However, when assessing the dynamics of the indicator within the groups, a statistically significant increase in blood pressure was revealed by 4 minutes with a decrease by the eighth in both groups (p<0.001).

Table 4. The results of pulse monitoring in the study groups in dynamics (n=56). Each cell contains: mean±the standard error of the mean, the median, and the range from minimum to maximum values.

Measurement period	Group 1 (n=28) Articaine 2%	Group 2 (n=28) Articaine 4%	p
Before the operation	70,2±7,8 70 49-88	69,7±8,1 72 48-81	>0.05
2 minutes after the injection of the anesthetic	74,6±8,2 75,5 56-90	71,6±7,6 74 50-82	>0,05
4 minutes after the injection of the anesthetic	76,1±7,4 76 60-90	75,7±6,4 78 60-85	>0,05
8 minutes after the injection of the anesthetic	67,7±6,7 68 51-86	67,6±8,3 70 50-80	>0,05
p	**<0,001**	**<0,001**	

When comparing the pulse rate between the 2% and 4% articaine groups, no statistically significant differences were found at all registration periods (p>0.05). However, when assessing the dynamics of the indicator within the groups, a statistically significant increase in heart rate was revealed by 4 minutes with a decrease by the eighth in both groups (p<0.001). At all registration dates, the indicator remained normal.

Table 5. The results of saturation monitoring in the study groups in dynamics (n=56). Each cell contains: mean±the standard error of the mean, the median, and the range from minimum to maximum values.

Measurement period	Group 1 (n=28) Articaine 2%	Group 2 (n=28) Articaine 4%	P
Before the operation	96,1±2,1 96 92-99	95,9±2 96 92-99	>0,05
2 minutes after the injection of the anesthetic	95±1,9 95 90-98	94,3±1,2 94 92-97	**<0,05 (0,049)**
4 minutes after the injection of the anesthetic	94,1±1,8 94 90-98	93,5±1,9 94 90-96	>0,05
8 minutes after the injection of the anesthetic	97,1±1,6 98 94-99	97,3±1,4 98 94-99	>0,05
p	**<0,001**	**<0,001**	

When comparing saturation between the 2% and 4% articaine groups, no statistically significant differences were found at all registration periods (p>0.05). However, when assessing the dynamics of the indicator within the groups, a statistically significant decrease was revealed by 4 minutes with a return to normal by the eighth minute in both groups (p<0.001).

Other correlations may also be indicative, for example, saturation with blood pressure and pulse, since its decrease by even 1% can lead to noticeable changes in hemodynamics (Table 6).

Table 6. The relationship of saturation with hemodynamic parameters in the study groups.

Relationship of indicators	Group 1 (n=28) Articaine 2%	Group 2 (n=28) Articaine 4%
Saturation/Systolic blood pressure	**r= (-)0,27, p<0,05**	**r=(-)0,39, p<0,001**
Saturation/Diastolic blood pressure	**r=(-)0,23, p<0,05**	**r=(-)0,27, p<0,05**
Saturation/Pulse	**r=(-)0,29, p<0,05**	**r=(-)0,27, p<0,05**

Weak reliable inverse relationships were found between the saturation level in both groups and hemodynamic parameters – with a decrease in saturation, a reversible increase in blood pressure and pulse occurred in both study groups (p<0.05).

DISCUSSION

The results of the study showed that at the initial stages of the procedure (before and 2 minutes after the injection of the anesthetic), systolic blood pressure remained within the normal range, which indicates the stability of hemodynamic parameters in both groups. However, 4 minutes after the injection of the anesthetic, a significant increase in systolic blood pressure was recorded, which was more pronounced in the 4% articaine group. This indicates that a higher concentration of anesthetic may have a more pronounced effect on the cardiovascular system, which requires special attention when choosing anesthetics for patients with a predisposition to cardiovascular diseases.

Analysis of the dynamics of blood pressure, pulse, and saturation also confirmed that when using 2% articaine, a less pronounced hemodynamic reaction is observed, which makes it the preferred choice for patients requiring anesthesia. Even though temporary fluctuations in the parameters were noted in both groups, they did not exceed the normal values, which indicates that the anesthetics used in the studied concentrations do not lead to serious disorders.

In addition, the results of the study emphasize the importance of monitoring hemodynamic parameters during anesthesia, which can help in the timely detection of possible complications and reduce the risk of adverse outcomes. The inverse relationship between saturation level and hemodynamic parameters also indicates the need for an integrated approach to assessing the patient's condition during anesthesia.

Articaine, produced in concentrations of 2% and 4%, is widely used due to its effectiveness and rapid action. However, the potential toxic effects on the cardiovascular system, especially in patients with concomitant somatic diseases, remains a serious problem that requires careful study.

Studies have shown that various classes of drugs, including local anesthetics such as articaine, can cause cardiovascular toxicity.[15] Cardiovascular toxicity can manifest itself in different ways, including changes in blood pressure, heart rate, and heart contractility. In a detailed review by Mladěnka et al. The need for careful monitoring of cardiovascular parameters during procedures using local anesthetics is emphasized.

Despite the results obtained in the course of current research, significant knowledge gaps remain. There is a lack of comprehensive studies on the effect of 2% and 4% articaine solution on the cardiovascular system in patients with various somatic diseases. Most existing studies either study a healthy population or insufficiently represent patients with concomitant diseases, which limits the applicability of the results to risk groups.[16]

According to Anisimova et al., the use of a 2% articaine solution with 1:200,000 epinephrine administered by infiltration or a modified periodontal method registered an increase in the threshold of electrical excitability of the pulp by 95.93% and 93.58%, respectively. There were no statistically significant hemodynamic changes in the studied patient groups,[17] which is consistent with our data.

The purpose of the study is Kämmerer et al. The anesthetic efficacy of 2% and 4% articaine solution was compared in case of blockage of the inferior alveolar nerve for the extraction of mandibular teeth. The local anesthetic effect of a 4% articaine solution is not significantly better than that of a 2% solution . [18]

When studying the pharmacokinetics of articaine, it was proved that the maximum concentrations of articaine in the blood serum when using a 2% solution of articaine are lower. It was also noted that the plasma half-life of a 2% solution of articaine is shorter than that of a 4% solution: 18.54 minutes versus 23.62 minutes, which is beneficial if the patient has a concomitant pathology. [19, 20]

Future research should aim to fill in these gaps by conducting studies focusing on the effects of 2% and 4% articaine on the cardiovascular system in various patient groups, especially those with pre-existing cardiovascular diseases. Randomized controlled trials evaluating hemodynamic changes in these patients can provide valuable information about the safety of these anesthetics.

In addition, it would be useful to conduct long-term studies that would monitor cardiovascular parameters in patients receiving multiple doses of articaine. This study could help develop recommendations for safe use in patients with cardiovascular diseases, which would ultimately improve patient safety and the quality of medical care in dental clinics.

CONCLUSION

In the course of the study, a comprehensive assessment of the potential toxicity of 2% and 4% articaine on the cardiovascular system of patients undergoing tooth extraction was carried out. Taking into account the results obtained, indicating adequate anesthesia of the surgical area up to and including the 8th minute, 2% articaine demonstrated no less effectiveness than 4% articaine.

Thus, the data obtained confirm that 2% articaine can be a safe alternative to 4% articaine, which is especially important for patients with concomitant diseases, children and the elderly, who may have a higher risk of complications.

Articaine shows good anesthetic efficacy and low toxicity, so it can be recommended for use with short-term tooth extraction, especially in patients with

Alexey Kuznetsov, Aglaya Kazumova, Natalia Ivanova, Yuriy L. Vasil'ev. Assessment of the potentially toxic effect of 2% and 4% ARTICAINE on the patients' cardiovascular system during tooth extraction.Bulletin of Stomatology and Maxillofacial Surgery.2025;21(3).303-312.doi:10.58240/1829006X-2025.3-303

concomitant diseases or conditions such as pregnancy.

The study highlights the need for careful choice of anesthetics in clinical practice. It is important to take into account the individual characteristics of the patient, including the presence of concomitant diseases, age and general physical fitness. Recommendations for the use of 2% articaine can help reduce the risk of complications and improve the quality of medical care, which is an important aspect of modern dental practice.

In the future, it is advisable to conduct additional studies aimed at studying the long-term effects of using different concentrations of articaine, as well as their effect on different groups of patients. This will make it possible to more accurately determine the optimal approaches to anesthesia and improve the safety of dental procedures.

DECLARATION

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CASE REPORT

HISTOPATHOLOGYC AND DERMOSCOPIC FINDINGS OF SUBACUTE CUTANEOUS LUPUS ERYTHEMATOSUS

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ABSTRACT

The relevance, pathogenesis, classification, and clinical characteristics of a rare disease-subacute cutaneous lupus erythematosus-are presented.
A clinical case in a 60-year-old woman without signs of organ pathology is also described.
A dermatoscopic examination was performed. In the affected areas on the facial skin, twisted, short linear, occasionally branching, unfocused vessels, predominantly bright red in color, forming incomplete polygonal structures, were identified. In the affected areas on the skin of the extremities, irregularly arranged dotted-granular and twisted short linear vessels of dull red color were visualized, along with structureless zones of varying degrees of discoloration, exhibiting whitish-orange shades.
Pathomorphologically, the process is characterized by mild focal hyperkeratosis, vacuolar degeneration of the basal layer of the epidermis, a mildly expressed perivascular lymphohistiocytic inflammatory infiltrate, as well as a moderately expressed periadnexal (perieccrine) lymphohistiocytic inflammatory infiltrate.
Based on the medical history, clinical presentation, and the results of dermoscopic and histological examinations, the diagnosis of psoriasiform subacute cutaneous lupus erythematosus was established.

Keywords: Psoriasiform Subacute Cutaneous Lupus Erythematosus dermoscopyc features, pathomorphological features.

INTRODUCTION

Cutaneous lupus erythematosus (CLE) is a chronic autoimmune inflammatory disease affecting the connective tissue of the skin. It is characterized by a relapsing course, immune dysregulation, and a heterogeneous clinical presentations.
CLE can manifest as an isolated skin condition or as part of systemic lupus erythematosus (SLE). The annual incidence of CLE and SLE in the United States and European countries is approximately 4 and 3 cases per 100,000 people, respectively. Notably, without therapeutic intervention, 5–18% of CLE patients develop SLE within 3–5 years.[1–4]
The diagnosis of CLE relies on a comprehensive evaluation of patient history, clinical manifestations, biochemical and immunoserological findings. Dermatoscopic and histopathological examinations also play a crucial role in differential diagnosis.[1,5]

PATHOGENESIS

CLE is a multifactorial disease in which genetic, environmental and immunological factors play a significant role in pathogenesis.
The key genetic mechanisms contributing to the disease include:

- initiation and enhancement of antibody formation, associated with HLA gene variants (HLA-DR3, HLA-DR2, HLA-DRw52, HLA-DRw6, HLA-DQ1, HLA-DQ2, etc.);
- increased interferon-α (IFN-α) activity, which has a proinflammatory effect due to genetic variations in the *IRF5* and *STAT4* genes;
- accumulation of cellular debris, triggering an autoimmune response due to mutations in genes responsible for apoptotic cell clearance (*C1q, TREX1, DNASE1*);
- polymorphisms in the *PTPN22* (T-cell signal regulator) and *TNFAIP3* (inflammatory inhibitor) genes, contributing to immune dysregulation. [6, 7]

The key environmental triggers include ultraviolet UV-radiation (UVR), smoking, and certain medications.[3, 5, 11-14]

UV exposure can initiate and exacerbate skin lesions by:

- inducing keratinocyte apoptosis and increasing the expression of nuclear Ro/SSA and La/SSB autoantigens;
- triggering IFN-α hyperproduction.[11, 16]

Smoking negatively impacts SCLE through several mechanisms:

- oxidative stress, which damages cell membranes, promotes Ro/SSA and La/SSB autoantigen release, and amplifies autoimmune inflammatory responses;
- immune dysregulation, activating TNF-α, IL-6, IFN-γ, and other proinflammatory cytokines;
- increased photosensitivity. [4, 8]

Several medications have been linked to drug-induced SCLE, including:

- terbinafine;
- TNF-α inhibitors;
- anticonvulsants;
- calcium channel blockers;
- β-blockers; among others.[1, 2, 15]

CLINICS

CLE is classified into three clinical and histological subtypes:

- acute cutaneous lupus erythematosus (ACLE);
- subacute cutaneous lupus erythematosus (SCLE);
- chronic cutaneous lupus erythematosus (CCLE).[1–4, 8]

The following forms of ACLE are recognized:

- localized: presents with the characteristic "butterfly rash" over the nose and cheeks;
- widespread: manifests as maculopapular rashes;
- vesicular: characterized by the formation of subepidermal blisters .[2, 5]

SCLE typically presents as lesions on sun exposed skin areas, predominantly on the upper third of the trunk and upper limbs. [4]:

- annular or polycyclic form: non-scarring annular or polycyclic rashes;
- papulosquamous form: erythematous-squamous or psoriasiform plaques with tightly adherent scales and mild hyperkeratosis;
- target lesions (Rowell's syndrome): resembling erythema multiforme, necrotizing vasculitis, and other conditions.

CCLE manifests in various clinical forms:

- chronic discoid form: cicatricial localized or disseminated lesions;
- hypertrophic or warty form: characterized by extensive hyperkeratosis;
- deep form (lupus panniculitis): presents with subcutaneous nodules;
- acral form: resembles frostbite, affecting distal skin areas;
- mucocutaneous form: includes ulcers and plaques in the oral cavity. [2, 5]

Non-specific clinical signs of cutaneous lupus erythematosus include vascular manifestations (vasculopathy, leukocytoclastic vasculitis, urticaria, Raynaud's phenomenon, thrombophlebitis, fingertip necrosis) and follicular abnormalities (alopecia areata, telogen effluvium).[5, 9, 10]

HISTOPATHOLOGY

The histopathological pattern of CLE (ACLE, SCLE, CCLE) is primarily characterized by interface dermatitis — manifested mainly by a lymphocytic inflammatory infiltrate (cytotoxic CXCR3+ lymphocytes) localized predominantly in the perivascular and periadnexal areas. Notably, apoptotic or necroptotic keratinocytes are present. This pattern also includes the expression of

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interferon-regulated cytokines and chemokines, epidermal and follicular hyperkeratosis, the presence of mucin in the dermis, and other associated features. [2, 3, 12, 17]

CLINICAL CASE

Patient M., a 60-year-old female of Yerevan, Armenia, sought consultation in November 2024 at the National Center for Burns and Dermatology of the Ministry of Health of the Republic of Armenia. She presented with complaints of rashes on the skin of her face, upper, and lower extremities.

The patient reports having had the condition for 20 years. Previously, she underwent multiple treatments for presumed diagnoses, including psoriasis and Devergie's disease, among others. She has periodically received Diprospan injections, with varying degrees of effectiveness. There is no known family history of skin diseases. No concomitant conditions have been identified.

The patient is primarily concerned about the rash on her face and extremities, accompanied by mild itching.

Objective findings: slightly infiltrated erythematous-squamous lesions are observed on the cheeks, periorbital region, eyebrows, and nasal root. Similar morphological elements are present on the upper and lower extremities, with additional follicular lesions (Figures 1–3)



Figure 1. Clinical images of skin lesions on the face



Figure 2. Clinical images of skin lesions on the face and extremities



Figure 3. Clinical images of skin lesions on the face and extremities

Laboratory findings:

- general clinical and biochemical blood tests, as well as general urine analysis, show no abnormalities;
- blood cortisol level (9 a.m.): 135.7 nmol/l;
- antibodies to phospholipids (IgM/IgG): negative;
- antibodies to cardiolipin (IgG, IgM, IgA): negative;
- antinuclear IgG antibodies: Positive.

DERMOSCOPIC FINDINGS

These dermoscopic findings describe different structural characteristics of skin lesions on the face and extremities.

Facial Lesions:

- background: erythematous;
- vascular pattern: varying diameters, predominantly bright red, forming incomplete polygonal structures tortuous, short-linear, and branching vessels;

- additional features: small, irregularly placed white superficial scales, whitish-yellowish structureless areas.

Extremity Lesions:

- background: yellowish-pinkish;
- vascular pattern: irregularly placed dull red in color punctate-granular and short-linear tortuous vessels;
- additional features: whitish-orange discolored structureless zones, indistinct scaly structures, focal hyperpigmentation(figure,4,5).



Figure 4.



Figure 5. Dermatoscopic images of facial skin lesions: bright red polymorphic vessels, small irregularly distributed white scaly structures, and whitish-yellow structureless zones are visualized against an erythematous background.



Figure 6



Figure 6,7.Dermatoscopic images of skin lesions on the extremities: irregularly distributed punctate-granular and tortuous short-linear vessels of a dull red color, along with whitish-orange structureless zones, are visualized against a yellowish-pink background.

PATHOMORPHOLOGICAL RESULTS

The main morphological changes are represented by: mild focal hyperkeratosis and vacuolar degeneration of the basal layer of the epidermis (Figure 8 and 9); perivascular lymphohistiocytic inflammatory infiltrate (Figure 10);periadnexal (perieccrine) lymphohistiocytic inflammatory infiltrate(Figure 11).

Conclusion: based on the above data, the diagnosis was: psoriasiform (papular-squamous) subacute cutaneous lupus erythematosus.

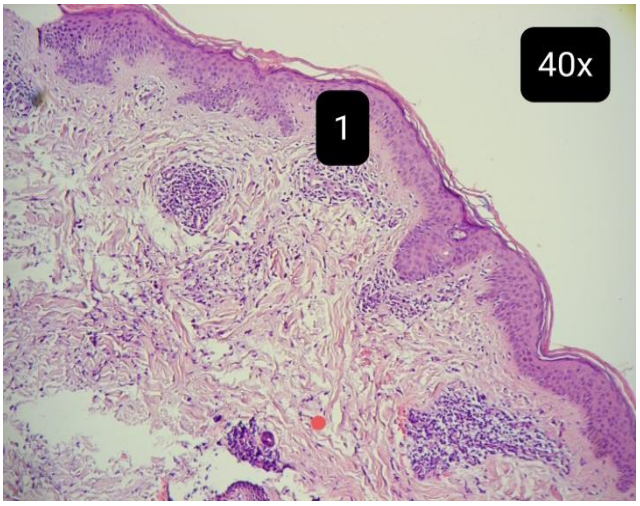


Figure 8.

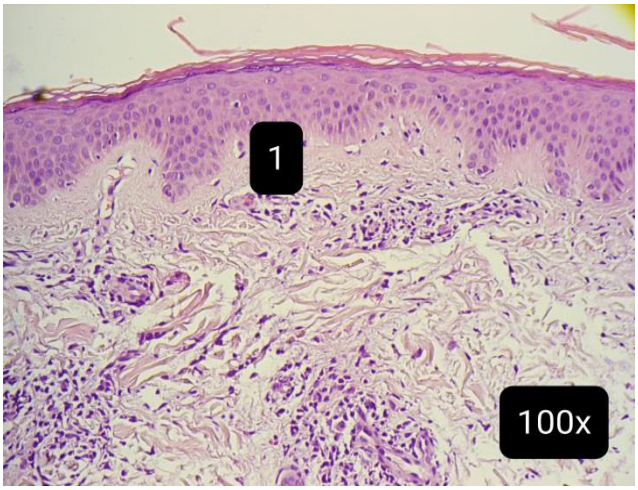
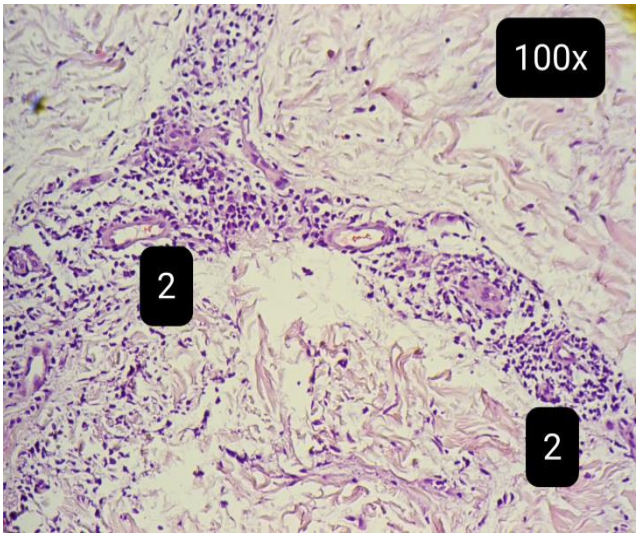


Figure 9.



Fugure 10.

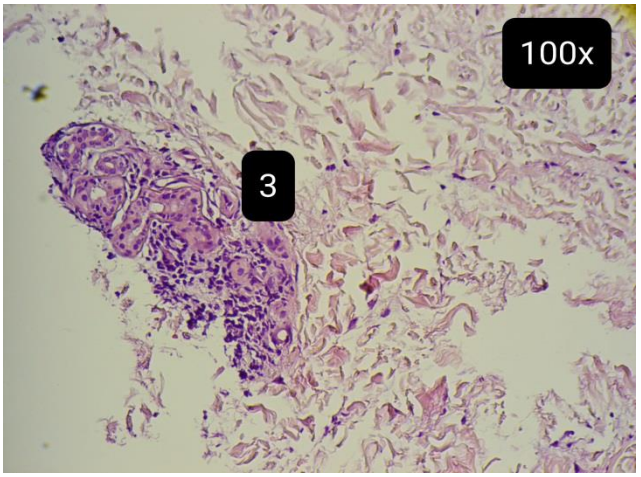


Figure 11.

Figure 8-11. Histological images of skin biopsy specimens from lesions (hematoxylin and eosin staining, ×40, x100): Fig. 8-9 show focal hyperkeratosis and vacuolar degeneration of the basal layer of the epidermis; Fig. 10 shows a perivascular lymphohistiocytic inflammatory infiltrate; Fig. 11 shows a periadnexal (perieccrine) lymphohistiocytic inflammatory infiltrate

CONCLUSIONS

The presented clinical case demonstrates the importance of dermatological and histological examinations in the diagnosis of subacute psoriasiform cutaneous lupus erythematosus.

DECLARATIONS

Conflicts of Interest

The authors declare no conflicts of interest.

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