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HEMOSTASIS ACHIEVEMENT REFLECTING THE INFLAMMATORY STATUS OF THE PULP BASED ON CYTOKINE LEVELS: A COHORT STUDY

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ABSTRACT

Aim: To investigate whether hemostasis at the exposure site reflect the level of inflammation at canal orifices of primary teeth with carious pulp exposures through measuring the cytokines levels at the exposure sites and canal orifices.

Materials and methods: This study was conducted on (30) children aged 5-7 years with deep carious lesions in their primary molars. It included 41 carious primary molars at which hemostasis at the exposure site was achieved within five minutes (group I), and 41 carious primary molars at which hemostasis at the exposure site was not achieved within five minutes but was achieved at the canal orifice within five minutes (group II). To assess inflammatory markers at the exposure site and canal orifice, a pulpal blood samples were analyzed for IL-2, IL-6 and IL-8 levels (pg/ml) using ELISA. Data were statistically analyzed.

Results: The levels of IL-2, IL-6 and IL-8 at the exposure site in group (I) revealed significantly difference between all interleukins (P<0.0001). The levels of IL-8 in group (I) at the canal orifice, group (II) at the exposure site and at the canal orifice showed significantly the lowest while it revealed insignificant difference between IL-2 and IL-6.

Conclusions:IL-2, IL-6 and IL-8 cytokine expression in primary molars increased in exposure sites and increases with caries progression at canal orifices.

Keywords: Biodentine, cytokines, hemostasis, primary molars, pulpotomy, stainless steel crowns

INTRODUCTION

Dental caries in primary teeth continues to be an important oral health problem that affects a large part of the worldwide child population, with an overall prevalence of 46.2%. It is considered a silent symptomatology due to inadequate dental education and limited dental care access resulting in delayed treatment at late stage often with pulp involvement[1, 2].

Pulpal hemorrhage control is a crucial step in pulpotomy procedures. Controlling the bleeding during pulp therapy was achieved by applying minimal mechanical pressure with a wet cotton pellet over the exposed pulp. Prolonged or uncontrolled bleeding after coronal pulp amputation may indicate pulpal inflammation which may affect the pulp healing capacity. The capping materials of pulp should not be placed over a clinically visible blood clot, as it can prevent profound contact between the pulpal tissue and the capping material and can be a medium for bacterial growth where macrophages can cause hemolysis of erythrocytes within the pulpal tissue to hemosiderin which is destructive to pulp vitality causing a chronic inflammatory response [3].

There has been an disagreement regarding the evaluation of the bleeding site; ***Fuks (2002);*** claims that "blood at the amputation site is the one that counts and reflects the degree of pulpal inflammation". ***Ferreira et al., (2009)*** disagreed with this opinion, stated that "blood at the exposure site highly reflects the degree of pulpal inflammation and that this is the only site that can image the biological status of the pulp". ***American academy of pediatric dentistry, (2022)*** declared that both sites should be taken into consideration[4, 5, 6].

Cytokines are numerous and play an important role in the pathogenesis of pulpitis where they are used as a guiding factor of inflammation and its progression to tissue necrosis. During irreversible pulpitis, IL-2 and

IL-8 dramatically increases while IL-6 is associated with edema and pulpal tissue destruction. The pulpal inflammatory level can be determined according to the presence of pro-inflammatory and anti-inflammatory cytokines [7].

Evaluating the degree of pulp inflammation adequately can aid in achieving a correct treatment plan. In response to harmful stimuli, dental pulp releases biological molecules like cytokines which serve as reliable inflammation markers[8, 9].

Cytokines are one of the inflammatory mediators of the immune system that contribute to pulpal defense mechanisms which can trigger inflammation and participate in immune cell trafficking, cell proliferation, inflammation, and tissue damage in pulp space. The cytokines analysis during different stages of inflammation aid in having a more comprehensive understanding of the inflammatory process through measuring their concentration by the enzyme-linked immunosorbent assay (ELISA) methodology[9,10].

Cytokines levels at the exposure site can give an accurate information about the degree of pulpal inflammation regardless of the type of exposure as the infected teeth can have a higher level of cytokines than healthy teeth. Measurable levels of cytokines molecules can be found in pulpal blood as well as dentinal fluid, periapical fluid, and gingival crevicular fluid, where can be collected non-invasively and analyzed without extirpating the pulpal tissue [11-13].

Interleukin-2 is considered as a part of the body's natural response to microbial infection and in discriminating between foreign ("non-self") and "self". It is recognized as a type of cytokine signaling molecule in the immune system which regulates the white blood cells activity (leukocytes and lymphocytes) that is responsible for immunity. It is considered as a potent stimulant which is released by T-helper cells. It is a major T-cell growth factor and plays a main role in pathogenesis and progression of disease [14].

Interleukin-6 is a classic type of pro-inflammatory cytokine. It is considered as a multifunctional cytokine with multiple biological activities such as regulation of immune response, inflammation and hematopoiesis**.** It is responsible for multiple inflammatory manifestations as a response to stimuli as inflammation and trauma caused by various cells including macrophages, neutrophils, keratinocytes, fibroblasts and endothelial cells**.** IL-6 corresponds with recruitment of neutrophils and irreversible tissue damage [15, 16, 17].

Interleukin-8 is considered as a potent chemoattractant cytokine which is produced by a multiple tissue and blood cells that is resistant to heat, acidic environments and proteolysis. Their bio-chemical characteristics considered it as an ideal candidate molecule to function in the acute inflammation site, where it withstand sub-optimal conditions as pus collection which is located within an abscess. It is actively produced after stimulation of the cells into the extracellular space. It is considered unique as it is produced early and persist a prolonged period for days or weeks in the inflammatory response [18].

Subjects and methods:

Trial registration and study design:

This study was a retrospective cohort study. The study was registered in (https://clinicaltrials.gov/) with identification number: NCT03737682 and was verified on July 15, 2019.

Steering committees:

This study was approved by the Ethical Committee for Research, Faculty of Dentistry, Cairo University, Egypt. Informed consent was obtained.

Sample size calculation:

The sample size estimation was designed to test the assumed null hypothesis, Sample size calculation was done using the comparison of serum IL-2 between cases that achieved hemostasis at exposure site within 5 min and matched cases who didn't achieve hemostasis within 5 min, as it was the primary outcome of our study. The calculated minimum proper sample size was 41 cases in each group to be able to reject the null hypothesis with 80% power at $\alpha = 0.05$ level using Student's t-test for independent samples.

Subject selection:

Apparently, healthy Egyptian children aged 5 to 7 years were recruited from the Outpatient Diagnostic Clinic of the Pediatric Dentistry and Dental Public Health Department at Cairo University, Egypt. The eligibility criteria encompassed the absence of clinical symptoms indicative of pulp degeneration, no radiographic evidence of internal or external resorption, lack of spontaneous pain, an enlarged periodontal ligament space, furcal or periapical radiolucency, and physiological root resorption restricted to a maximum of one-third of the root length. Medical histories were collected from all participants.

Intra-operative procedure:

Following administration of local anesthesia using a solution without vasoconstrictor (Mepivacaine HCL 3%), teeth were isolated with a rubber dam, Firstly, for both groups, the caries was removed with a high-speed round bur once exposure of pulp chamber was done, the first blood sample was collected by placing a sterile cotton pellet on the exposure site for 45 seconds. Then the cotton pellet was removed, immediately diluted and stored in a container containing 0.08 ml phosphate buffered saline, then a damp cotton pellet was then placed with slight pressure for five minutes to obtain hemostasis.

Primary molars in which hemostasis at the exposure site was achieved within five minutes were included in group I, and primary molars in which hemostasis at the exposure site could not be achieved in five minutes were

included in group II. Secondly, for both groups, pulp chamber was accessed, a second blood sample was collected by a sterile cotton pellet placed in the pulp chamber adjacent to the canal orifices for 45 seconds. Then it was removed, immediately diluted and stored in a container containing 0.08 ml phosphate buffered saline (PBS concentrate)., then the coronal pulpal tissue was excavated using a large excavator and a damp cotton pellet was placed at the orifice using slight pressure to control hemorrhage. In group II, teeth for which hemostasis at the canal orifice could not be achieved within 5 minutes were excluded from the study. Collected blood samples were stored at -80 degrees Celsius until analysis.

The molars were treated with vital pulpotomy using a calcium-silicate-based material and was covered with stainless steel crown.

Inflammatory markers measurement:

Collected blood samples were analyzed for IL-2, IL-6 and IL-8 levels (pg/ml) using Enzyme Linked Immuno-Sorbent Assays (ELISA). After thawing, the blood samples were centrifuged at 1,500 g for 10 minutes at four degrees Celsius then blood samples were extracted from the cotton pellets. Cytokine concentrations were measured using an Invitrogen Immunoassay Kit (Glory Immuno Assays, Glory Science Co.,Ltd., China). Glory immunoassay employed the quantitative sandwich enzyme immunoassay technique. Pulpal blood samples were analyzed for IL-2, IL-6, IL-8 levels (pg/ml) using (ELISA).

Statistical analysis:

Statistical analysis was performed with SPSS statistics 20, Microsoft Excel ® 2016 and Graph Pad Prism. In numerical data (Quantitative), data were explored for normality by checking the data distribution using Kolmogorov- Smirnov and Shapi0ro-Wilk tests. Parametric data were presented as mean and standard deviation. comparison between two different groups was performed by using independent t-test, while comparison between more than two groups was performed by using One Way ANOVA test, followed by Tukey`s Post Hoc test for multiple comparisons. In categorial data (Qualitative), data were presented as frequency and percentages. Comparisons were performed using Chi square test. Correlation between different variable scores was performed using Spearman's rho correlation test. The correlation coefficient was used to measure the strength of a linear association between two variables. All *p*-values are two-sided, and *p*-values ≤0.05 were considered significant.

RESULTS

A total number of 30 children (12 boys and 18 girls) aged from 5-7 years with primary molars at which hemostasis at the exposure site was achieved within five minutes (group I), and primary molars at which hemostasis at the exposure site was not achieved within five minutes but the hemostasis was achieved at the canal orifice within five minutes (group II).

Intergroup comparison of the levels of IL-2 between both groups at the exposure site revealed insignificant difference (P=0.99), while the levels of IL-2 at canal orifice revealed significant difference between both groups (P=0.001) where group I was significantly higher than group II, as presented in table (1) and figure (1).

Table 1. Mean and standard deviation of IL-2 in both groups and comparison

	IL-2			
	At exposure site		At canal orifice	
	Mean	Standard deviation	Mean	Standard deviation
Group I	0.69	0.34	0.72	0.36
Group II	0.69	0.19	0.52	0.12
P value	0.99		0.001	

P: probability level which is significant at P ≤ 0.05

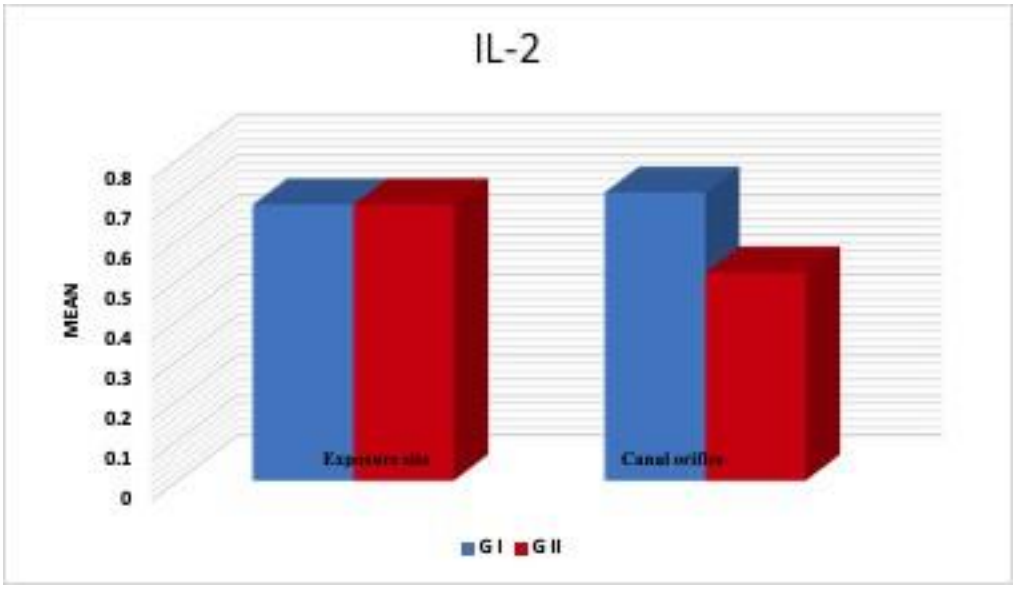

Figure 1. Bar chart showing mean of IL-2 in group I and II and comparison between them.

Intergroup comparison between levels of IL-6 revealed significant difference at exposure site (P=0.01) and at canal orifice (P=0.006) where group I was significantly higher than group II, as presented in table (2) and figure (2).

Table 2. Mean and standard deviation of IL-6 in both groups and comparison between them

	IL 6			
	At exposure site		At canal orifice	
	Mean	Standard deviation	Mean	Standard deviation
Group I	0.99	0.52	0.85	0.37
Group II	0.73	0.40	0.61	0.40
P value	0.01*		0.006*	

P: probability level which is significant at $P \leq 0.05$

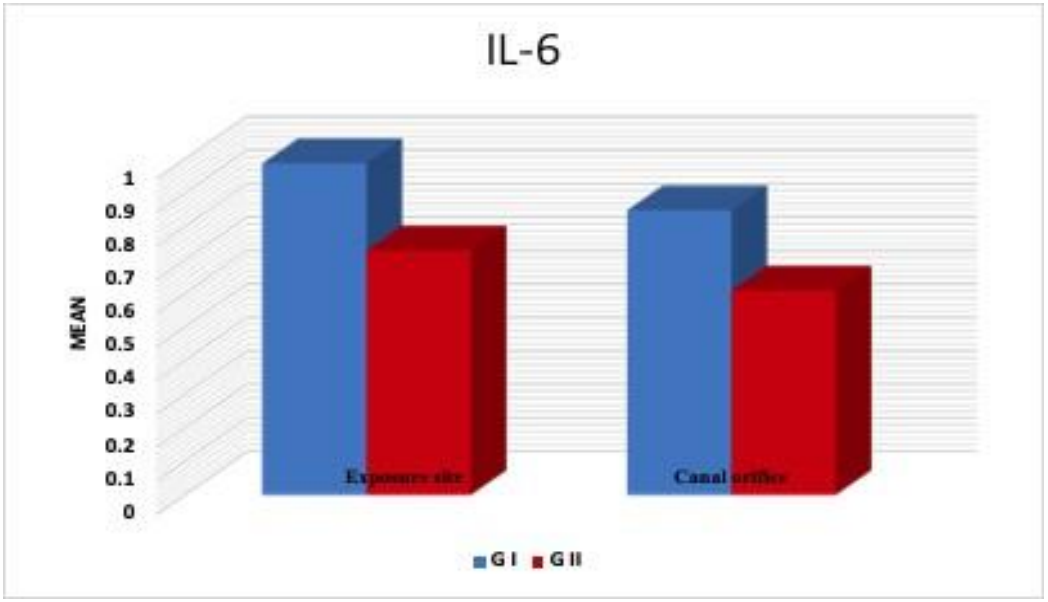

Figure 2. Bar chart showing mean of IL-6 in group I and II and comparison between them.

Intergroup comparison between the levels of IL-8 at exposure site revealed significant difference (P=0.01), while the levels of IL-8 at canal orifice revealed insignificant difference between both groups (P=0.66) where group I was significantly lower than group II, as presented in table (3) and figure (3).

Table 3. Mean and standard deviation of IL-8 in both groups and comparison between them

	IL-8			
	At exposure site		At canal orifice	
	Mean	Standard deviation	Mean	Standard deviation
Group I	0.28	0.05	0.35	0.10
Group II	0.35	0.17	0.34	0.11
P value	0.01*		0.66	

P: probability level which is significant at P ≤ 0.05

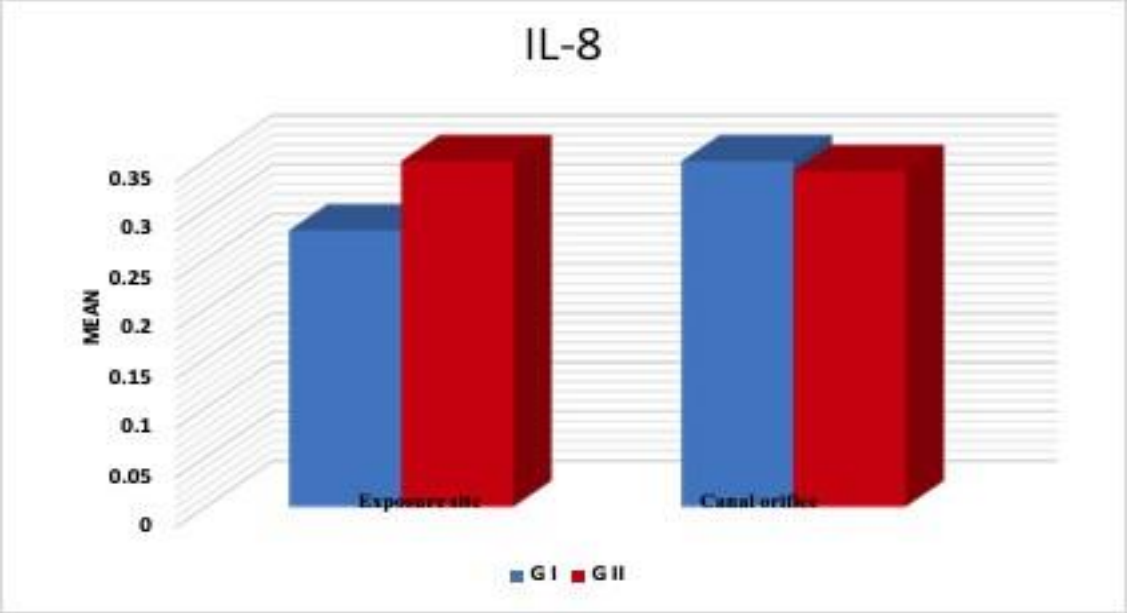

Figure 3. Bar chart showing mean of IL-8 in group I and II and comparison between them.

Interleukin levels at the exposure site in group I revealed significant difference between all interleukins (P<0.0001).Interleukin levels in group I at the canal orifice, group II at the exposure site and group II at the canal orifice showed that IL-8 levels was significantly the lowest where the mean of IL-2, IL-6 and IL-8 was 0.35, 0.35 and 0.34 respectively while it revealed insignificant difference between IL 2& IL 6 as presented in table (4) and figure (4).

Table 4. Comparison between all interleukins in both groups at exposure site and canal orifice

Site	Group	IL-2		IL-6		IL-8		P value
		Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation	
Exposure site	Group I	0.69 a	0.34	0.99 b	0.52	0.28 c	0.05	<0.0001
	Group II	0.69 a	0.19	0.73 a	0.4	0.35 b	0.17	<0.0001
Canal orifice	Group I	0.72 a	0.36	0.85 a	0.37	0.35 b	0.1	<0.0001
	Group II	0.52 a	0.12	0.61 a	0.4	0.34 b	0.11	<0.0001

P:probability level which is significant at P ≤ 0.05

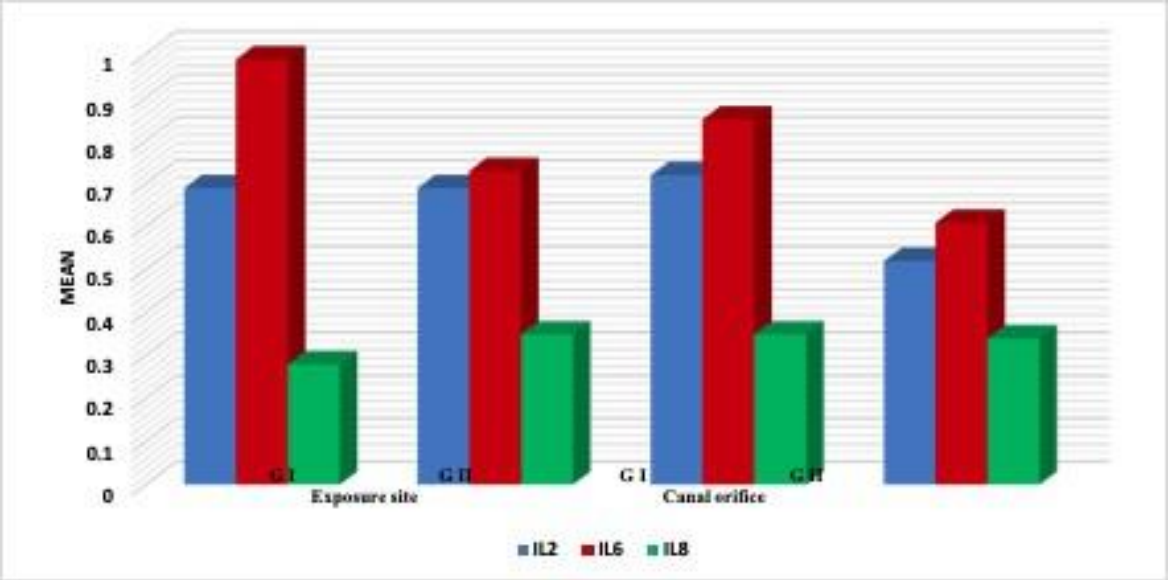

Figure 4. Bar chart showing comparison between all interleukins in both groups at exposure site and canal orifice.

DISCUSSION

Vital pulp therapy is typically the treatment of choice for deep dental caries in primary teeth. Pulpotomy is a recommended procedure for treating vital asymptomatic primary teeth with carious pulp exposures. conversely, direct pulp capping is considered a less invasive approach relying on the ability of dental pulp cells to differentiate and form reparative dentine. However, its use as a treatment modality in primary teeth remains a subject of debate [19, 20].

The degree of pulpal inflammation at the time of the exposure has a decisive impact on the treatment of primary tooth. The current modalities for assessing the pulpal condition are dependent on the patient's chief complaint, the history of symptoms, clinical and radiographic assessment, pulpal sensibility, and periapical tests have a poor correlation with the histopathological condition of the pulp where objective evaluation of the degree of pulpal inflammation at exposure site by measuring cytokines levels can detect the severity of pulpal tissue inflammation [21] .

Cytokines are critical mediators regulate immune and inflammatory responses through complex networks and serve as biomarkers for dental caries.

Quantification of cytokines has significant value in both clinical and biological aspects as their levels provide insights into physiological and pathological processes and can be used to aid diagnosis and treatment. Cytokines are classified according to the prospective of pro-inflammatory and anti-inflammatory effects [22].

The main motive of the present study was to question whether the hemostasis at the exposure site reflected pathological condition of the root pulp although only hemostasis at the canal orifices is used in the standard pulpotomy procedures. This could be due to inflammatory processes in the dental pulp result in vascular (e.g. vasodilatation) and cellular changes (e.g. in- creased numbers of immune cells). The molecular immune response releases inflammatory mediators, such as cytokines before activation of cellular immune response. The expression of these molecules in the early stage of pulp inflammation can be used as markers for the diagnosis of inflammatory changes within the pulp tissue. The increased levels of biomarkers, obtained from pulpal blood, are correlated with different stages of pulp inflammation. Diagnosis of the pulpal status should focus on either the extent of the microbial infection or the inflammatory reaction of the host tissue; however, current methods do neither [23-26].

Children in the present study were aged from five to seven years old as an inclusion criterion with a mean age 6.04 years. This could be due to this age range is the most favorable chronological age with considerable root length where resorption of the roots not yet started or may be minimal and to ensure patient cooperation. Dental problems are difficult to be managed in very young age group showing limited understanding and poor cognitive mechanism [27-29].

In the present study, the primary outcome was to measure the level of the Interleukin-2, because it has been detected in inflamed dental pulps and the level of IL-2 dramatically increase during irreversible pulpitis and could be used as a marker for inflammation [12].

In the current study, the comparison between levels of IL-6 in group I (tooth in which hemostasis was achieved at exposure site within 5 minutes) and group II (tooth in which hemostasis was not achieved at exposure site within 5 minutes but was achieved at canal orifice) have shown significant difference at exposure site (P=0.01)

this was in accordance with ***Mutluay et al., (2018)*** results. This could be explained by the presence of the chronic inflammation in the coronal pulp in which the structural characteristics of primary teeth initiate an inflammatory response before the caries lesion reaches the pulp then when the caries lesion reaches the pulp and bacterial invasion may provoke an acute response [7, 30].

On the other hand, the comparison between levels of IL-6 and IL-2 in both groups at canal orifice (p=0.006) (P=0.001) respectively and levels of IL-8 at exposure site (P=0.01) have shown significant difference. Unlike what was reported by ***Mutluay et al., (2018),*** the difference in the findings could be explained by higher levels of IL-2 at canal orifice may suggest that the pulp has initiated immunological repair by stimulating the expansion of helper T cells which are the predominant lymphocytes in inflammatory lesions while higher levels of IL-8 at exposure sites could be explained by being rapidly synthesized at local sites of inflammation where it fulfils its function of recruiting and activating acute inflammatory cells [7, 31, 32].

The levels of interleukin-6 at exposure site have shown higher levels in both groups. The findings of the current study were found opposite to ***Elsalhy et al., (2013)*** results, where the study samples were harvested from the exposure sites of permanent tooth. This could be explained by presence of difference in the biology of the pulp tissue between deciduous and permanent teeth. On the other hand, the odontoblastic layer in deciduous teeth is thinner than in permanent teeth, which may reflect a reduced regenerative capacity [33, 34].

Intergroup comparison in levels of IL-6 at canal orifice have shown significant difference between the two groups (P=0.006), these results were in agreement with ***Kabel et al., (2021)*** results where IL-6 have a prominent role during the inflammatory process in the pulp tissue of primary molars. While the levels of IL-8 at canal orifice have shown no significant difference between both groups (P=0.66), which is opposite to the previous study results. This could be explained by levels of IL-8 was correlated with the pulpal symptoms and have been observed in the late phase of inflammation [17, 35].

The levels of IL-6 in the current study have shown higher levels at canal orifice in both groups, these results were consistent with ***Ozdemir et al., (2015)*** results who found higher levels of IL-6 at canal orifice in cariously exposed primary molar. This could be due to IL-6 causes up- regulation of adhesion molecules and induces angiogenesis, leading to an increase in vascular permeability and inflammatory edema so it is associated with pulpal pathogenesis [36, 37].

In the current study, the comparison between levels of interleukin-2, interleukin-6 and interleukin-8 at exposure site and canal orifice were significantly difference which can reflect the degree of inflammation and pathological condition of pulp tissue at the canal orifices in carious exposed primary teeth so it can be used when deciding a pulpotomy or direct pulp capping indication.

Limitations of the study:

Limitations of this study include the generality of the results which was affected by the strict eligibility criteria. It is difficult to measure the cytokine levels from each canal orifice separately. There is no doubt that an increase in follow-up period would lead to more precise results.

CONCLUSIONS

1- Cytokines levels in exposure site might reflect the degree of inflammation of pulp at the canal orifices in carious primary teeth.
2- Direct pulp capping can be successfully done in primary teeth depending on the levels of cytokines at exposure site.
3- Measuring the proinflammatory cytokines levels could be considered as an accurate tool to detect degree of pulp inflammation and the prognostic outcome of vital pulp therapy.
4- Interleukin-2 and interleukin-6 were present in high concentrations and plays a role in the pathogenesis of pulpal diseases and could be used as a marker for determination of pulpal conditions.
5- The levels of interleukin-8 can be used as a potential indicator of pulp status and can be monitored to improve the reliability of prognosis of vital pulp therapy.

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