



ORIGINAL ARTICLE

GASEOUS OZONE-TREATED HUMAN DENTAL PULP STEM CELLS SECRETOME ENHANCES ANGIOGENESIS IN A CHICK EMBRYO MODEL

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Abstract

Background: Ozone has become more common in medicine across the globe as an adjuvant treatment method for a variety of illnesses. The combination of ozone and treated human dental pulp stem cell-conditioned media hDPSCs-CM can enhance angiogenesis due to their synergistic action, resulting in increased growth factor expression.

Objectives: The aim of the study was to evaluate the angiogenic potential of ozone-treated human dental pulp stem cell-conditioned media (hDPSCs-CM).

Materials and Method: HDPSCs were isolated from the extracted tooth. Passaged four cells were characterized with flow cytometry and then exposed to 10µg/mL gaseous Ozone concentration. The conditioned media (CM) were obtained from the treated cells, and growth factor analysis was performed. The functionality of the Ozonated hDPSCs-CM was assessed by the Chick Yolk Sac Membrane (YSM) assay.

Results: Ozonated hDPSCs-CM had significantly higher ($p < 0.01$) expression of angiopoietin-2 (Ang-2), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), macrophage colony stimulating factor (M-CSF), and vascular endothelial growth factor (VEGF). The in-ovo YSM assay revealed a notably greater pro-angiogenic potential associated with Ozonated hDPSC-CM.

Conclusion: Ozonated hDPSCs-CM appear to be the most effective source for inducing angiogenesis.

Keywords: Angiogenesis; conditioned media; dental pulp stem cells; secretome; growth factors; yolk sack model.

Introduction

The term ozone, which means "odor" in Greek, was first used in 1840 by the German chemist Christian Friedrich Schonbein, who is recognized as the father of ozone therapy.¹ Ozone is a versatile gas with a variety of uses, including bioenergetics, immunostimulants, detoxicants, antihypnotics, analgesics, and biosynthetics. Over the last few years, O₃ has become more and more popular in medicine across the globe as an adjuvant treatment method for a variety of illnesses.²

Various studies related to medicine showed that ulcers, cutaneous infections, acute and chronic viral diseases, neoplasia, and vascular issues like venous insufficiency, obstructive arteriopathies, and vascular degenerative diseases have all been treated with ozone.³ Ozone therapy is frequently utilized as an adjuvant to several conventional dental treatment methods for oral mucosal lesions, dental caries, periodontal disease, and peri-implantitis because of its conservative and non-invasive nature.⁴

The gold standard of care for severe bony lesions has been autologous bone grafting; however, the procedure is associated with certain disadvantages, such as donor site morbidity and a shortage of appropriate graft volume. Using stem cells and growth factors in such conditions as dental implants or periodontal defect repair can overcome these disadvantages.⁵ Stem cells can be found in significant quantities in teeth, and the cells obtained from them are known as human dental pulp stem cells (hDPSCs). The best source of hDPSCs is young adults' extracted wisdom teeth or exfoliated primary teeth, which are otherwise disposed of as medical waste. Its popularity in the field of regenerative medicine and dentistry over the past ten years has been attributed to its ease of accessibility and capacity to differentiate into many phenotypes.⁶ They demonstrated their therapeutic actions through processes of paracrine secretion and produced a novel by-product known as the conditioned medium (CM) or secretome, which is known to secrete growth factors that support angiogenesis and aid in the processes involved in tissue healing.⁷

The process of angiogenesis, which forms new blood vessels from the pre-existing vascular network, especially capillaries, is essential to normal postnatal growth and development.⁸ Angiogenesis is a well-coordinated process consisting of a series of

successive steps.⁹ This pathway includes several enzymes and regulatory components. The angiogenic factor (ANG), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF), and numerous others are among them. They all play various roles in angiogenesis: induction, initiation, cell multiplication, migration, cell stability, wound healing, inflammatory response, and inhibiting angiogenesis.^{9,10}

In clinical dentistry, endodontic therapies like apexification and apexogenesis, healing of the extraction socket, and tissue regeneration during the post-surgical phase for implant placement cannot be successful without sufficient tissue vascularization.¹¹ Revascularization of irradiated tissues is also necessary, as it increases the fibroblastic cellular density and reduces the amount of nonviable tissue that needs to be surgically removed.¹² Thus, angiogenesis plays a crucial role in the final success of these therapies. Currently, recommending angiogenic growth factors as a therapy is a popular practice for improving angiogenesis. Although therapeutically effective, it has certain drawbacks, such as leaky vasculature arising from a lack of spatiotemporal control over the release of these hormones. Hence, the development of stem-cell-based therapeutic applications as possible targets for disordered angiogenesis is a race in progress.¹³

Studies on ozone therapy have shown that the number of fibroblasts and angiogenesis in the rat buccal mucosa were both enhanced, thus strengthening the idea that ozonization will increase the potential of hDPSCs to proliferate and thus increase their angiogenesis capability.^{2,14} Taking into consideration these studies, it can be hypothesized that ozonated hDPSCs-CM will exhibit a synergistic impact that will have better angiogenesis compared to untreated hDPSCs-CM. Conventional in vitro, in vivo, and ex-vivo models of angiogenesis are laborious and time-consuming, requiring complex infrastructure for the cultivation of embryos. In terms of time and expense, the well-established Ovo chick embryo yolk sac membrane (YSM) methodology is straightforward, repeatable, and very cost-effective.¹⁵ Therefore, the primary goal of this work is to evaluate the growth factor profile and pro-angiogenic potential of the Ozone-treated hDPSCs CM in the in-ovo chick embryo YSM.

Materials and Method

DPSC isolation and culture

The previously established explant culture procedure was used to isolate and expand hDPSCs *in vitro*.¹⁶ Bits of dental pulp tissue were broken up into tiny pieces and put on a plastic culture plate. To completely submerge the tissues, the required amount of fetal bovine serum (FBS) was added. In order to culture the explant tissue, the next step was to incubate the tissue for 24 hours at 37°C and 5% CO₂. The hDPSCs were then cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% FBS and a small amount of antibiotic-antimycotic solution added to help prevent the growth of contaminating bacteria, mycoplasma and fungi at the same temperature and CO₂ settings. After 6-7 days, every flask was examined for cell outgrowth using an inverted phase-contrast microscope (OLYMPUS). Each flask was filled with freshly prepared complete medium, and the process of incubation continued. Cells were trypsinized when they achieved 70–80% confluency; cell outgrowth encircling the tissue is seen at the same time. The medium in the flask was disposed of for trypsinization, and any remaining FBS was cleaned up using a PBS wash. In the flask, 4 ml of Gibco [0.25%] trypsin was added. A microscope was used to examine the flask following a minute of incubation. An equivalent volume of complete medium was added to the flask in order to halt the trypsin process. Additionally, this cell culture was put into a falcon tube, and it was centrifuged for five minutes at 1800 rpm. After the cells formed a pellet, the supernatant was taken off, and the pellet was suspended in the whole medium. The suspension was moved into a fresh, sterile T25 flask and given the name passage zero (P0). The flask's medium was shifted every two days. Passaging was repeated once the flask was 80–90% confluent with cells. The identical process was continued until the cells were in passage 4.

Characterization of cells

Flow cytometry was used to characterize MSCs utilizing particular cell surface markers. BD Biosciences supplied the panel of antibodies, which included CD90, CD73, CD105 (PE-tagged), and CD34, CD45, and HLADR (FITC Tagged). FACS analysis was performed on passage 4 cells. An

eppendorf tube was filled with around 100 µl of the cell suspension (1×10^6 cells/ml). Following that, cells were fixed at room temperature for up to 30 minutes using 4% paraformaldehyde. Following fixation, cells were rinsed in PBS containing 0.5% BSA (Gibco). For one hour, the cells were treated with certain antibodies. Following incubation, 500 µl of PBS was used to wash. After that, samples were collected using the FACS analyzer. Quest Pro software was used for data analysis.

Ozone treatment

hDPSCs were exposed to 10 µg/mL ozone gas by putting the ozone gas into the culture medium within the closed beaker and connecting a thin silicon tube to the Ozone machine. At a density of 1×10^4 cells per well, cells were allowed to seed in 96-well plates. Similarly, Ozone is introduced to only culture media without hDPSCs to make Ozonated media (Figure 1).



Figure 1. Ozone machine with thin silicon tube

Preparing the Conditioned Medium (CM)

In order to collect CM, hDPSCs were seeded at a density of 1×10^4 cells into the T75 cell culture flask. Additionally, cells were allowed to reach a confluency of 80–90%. Subsequently, the culture medium was separated, and the cells underwent a sterile PBS wash to eliminate any remaining serum in the flask. Moreover, the culture flask was filled with fresh MEM- α supplemented with a 1% antibiotic-antimycotic solution without serum, and it was incubated for 48 hours. Culture media were filtered using 0.22 µm syringe filters and kept at -80°C until additional analysis was performed. Similarly, Ozonated CM media were prepared.

Control Group Preparation

hDPSCs were added to normal culture media as explained in cell isolation and culture, without exposing them to gaseous Ozone. The conditioned media formed then collected as described above and used as control group for growth factor analysis. For YSM analysis 5 groups were taken, Phosphate buffered saline (PBS), Media control containing culture media without hDPSCs without gaseous Ozone, Ozonated Media exposed to gaseous Ozone but without hDPSCs, CM conditioned media containing hDPSCs without gaseous Ozone, Ozonated CM containing hDPSCs and exposed to gaseous Ozone

Growth factor analysis

Following the manufacturer's instructions, the growth factors in the hDPSCs-CM and Ozonated hDPSCs-CM were assessed using the LEGEND plex multi-analyte flow assay kit (Human Growth Factor Panel (13-plex); Biolegend, San Diego, CA, USA; Cat. No. 740180). angiopoietin-2 (Ang-2), fibroblast growth factor (FGF), macrophage colony stimulating factor (M-CSF), Hepatocyte Growth Factor (HGF), and vascular endothelial growth factor (VEGF) were among the growth factors evaluated. Firstly, 25 µl of CM were incubated with the microbeads for two hours. After adding the detecting antibodies, the mixture was incubated for 30 minutes. After incubation, samples were cleaned using a buffer solution. To retrieve the pellet, the materials were centrifuged for five minutes at 2000 rpm. Next, the pellet was again formed in 200 µl of sheath fluid. A Thermo Fisher Scientific, Waltham, Massachusetts, USA, Attune NxT flow cytometer was used to collect the samples. Utilizing LEGEND plex Data Analysis Software (BioLegend, San Diego, CA, USA), the data were examined.¹⁷

Yolk sac membrane (YSM) assay for conditioned medium functional evaluation

As previously mentioned,¹⁸ the YSM test was used to evaluate the conditioned medium's functioning. Day 0 chick eggs were purchased from Venkateshwara Hatcheries Pvt. Ltd. in Pune, India, and were kept at 37°C for 48 hours in a humidified incubator. Following incubation, a tiny hole was made at the blunt end of the eggs, and 3-5 milliliters of albumin were extracted. Additionally, 200 µl of ozonated conditioned media from the hDPSCs was introduced to the YSM's top. The control group used hDPSCs-CM that had not been preconditioned with ozone. After using transparent tape to seal the aperture, the eggs were left in the humidified incubator for a full day. The Wim CAM online program (Wimasis) was used to quantitatively evaluate images of the blood vessel sprouting in order to determine the overall vascular network length, vessel density, and total segments.

Statistical analysis

The comparative evaluation of the expression of growth factors in hDPSCs-CM and Ozonated hDPSCs-CM were done using t-test. Similarly, statistical analysis for YSM were carried out using SPSS software version 19-SPSS Inc. Chicago, IL, USA. $p < 0.05$ was considered statistically significant and $p < 0.01$ considered highly statistically significant.

Results

MSC isolation depicted the spindle-shaped morphology of the isolated MSCs obtained from dental pulp. Flow cytometry was used to investigate the expression of CD markers by assessing immunophenotypic features. The hDPSCs exhibited negative expression of HLA-DR, CD45, and CD34 and positive expression of CD90, CD105, and CD73 (Figure 2).

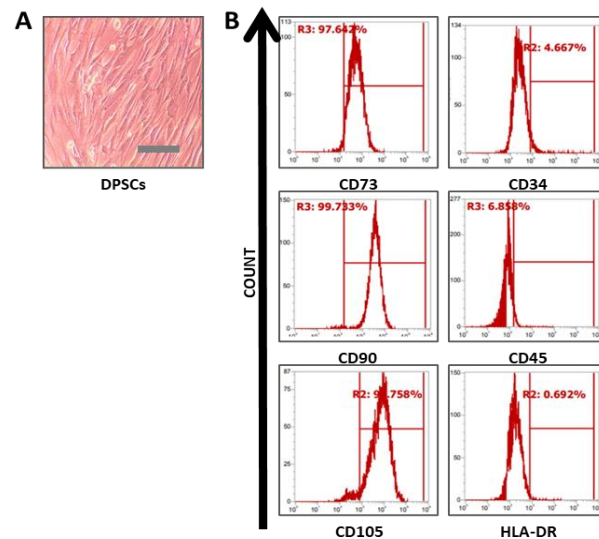


Figure 2. Isolation and characterization of DPSCs. (A) morphological features of DPSCs. Scale bar, 100 μm (B) flow cytometry analysis of cell-surface markers in human DPSCs. Cells were labelled with antibodies against MSC markers (CD90, CD105, CD73) and hematopoietic antigens (CD34, CD45, HLA-DR). DPSCs, dental pulp stem cells

Growth factor analysis of CM

Expression of angiopoietin-2 (Ang-2) and fibroblast growth factor (FGF-basic) was statistically significant ($p < 0.05$) in Ozonated hDPSCs-CM compared to only CM. Ozonated hDPSCs-CM

showed highly significant expression of hepatocyte growth factor (HGF), macrophage colony stimulating factor (M-CSF), and vascular endothelial growth factor (VEGF) compared to normal hDPSCs-CM. ($p < 0.01$) (Figure 3).

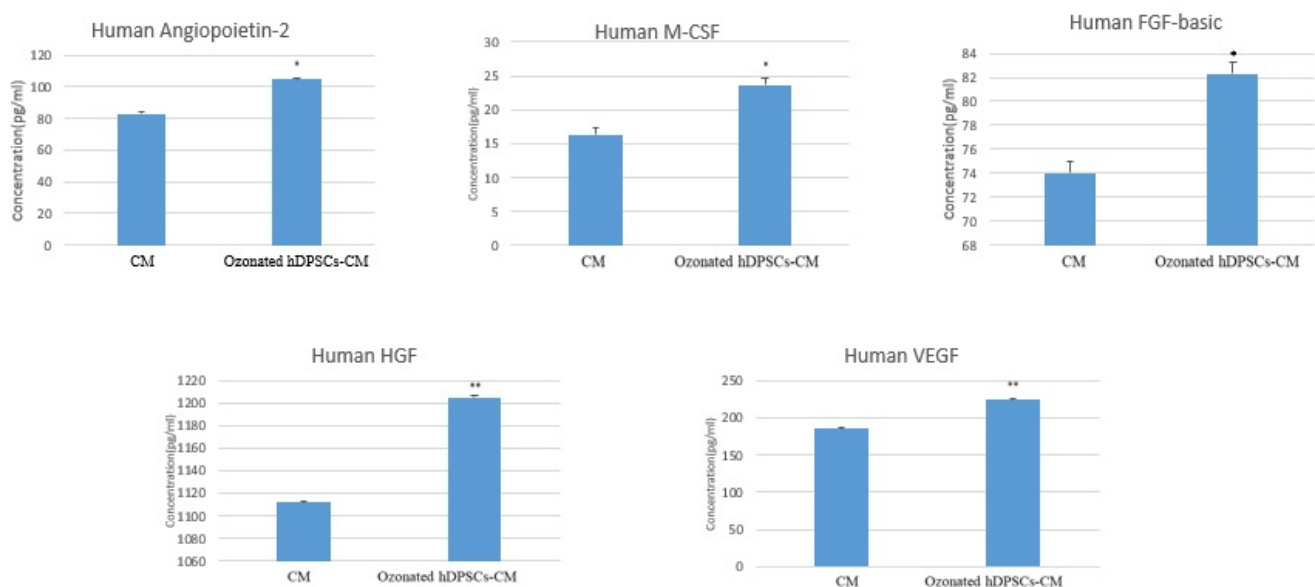


Figure 3. Quantitative growth factor analysis by cytometric bead array in the DPSC-derived conditioned media * $p < 0.05$ ** $p < 0.01$. Angiopoietin-2 (Ang-2), macrophage colony stimulating factor (M-CSF), fibroblast growth factor (FGF-basic), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF)

YSM assay for functional assessment of CM

YSM assays were used for functional assessment of ozonated hDPSCs in conditioned medium to evaluate their angiogenic potential. Ozonated hDPSCs-CM promotes blood vessel development. According to a quantitative evaluation of angiogenesis, Ozonated hDPSCs-CM considerably increased the length, density, and total segments of the

vascular network. Statistically the vascular network length and total segments were found to be highly significant in Ozonated hDPSCs-CM compare untreated hDPSCs-CM. ($p < 0.01$) Thus angiogenesis may be enhanced by hDPSCs-CM, the YSM assay's quantitative evaluation showed that the Ozonated hDPSCs-CM had a greater pro-angiogenic effect than other groups (Figure 4).

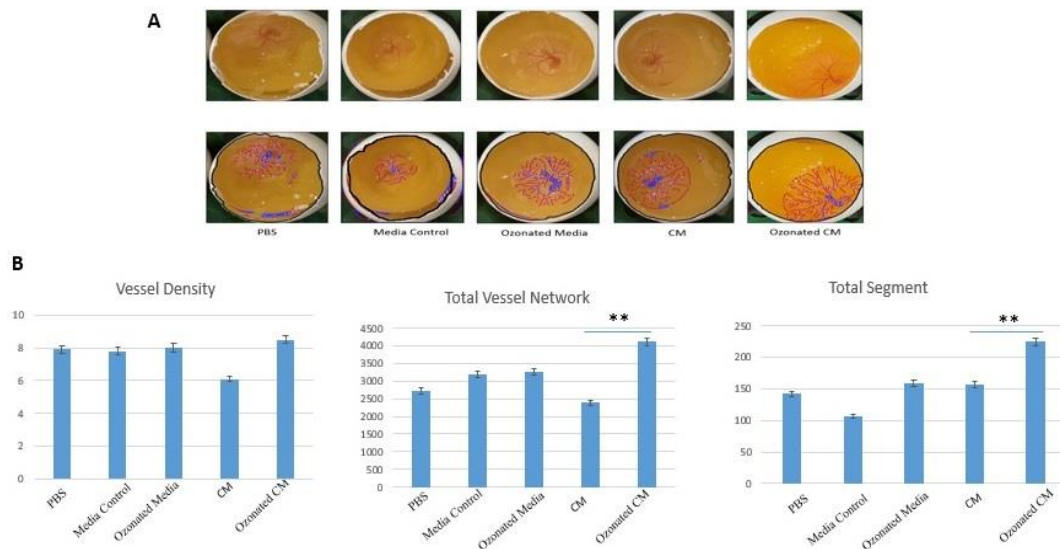


Figure 4. Screening of angiogenesis performed by utilizing the in ovo YSM model. (A) Photographs signifying the areas which were compared to quantify the angiogenesis. (B) Comparative angiogenesis in terms of vessel density, total vessel network, total segments in hDPSC-CM and Ozonated hDPSC-CM. $**p < 0.01$

Discussion

Many investigations have been conducted over the past ten years in an effort to determine the true participants in angiogenesis. Many studies are now being conducted to determine therapeutic angiogenesis. These include the chemical components known as growth factors, which are administered and play a crucial part in the process of angiogenesis.^{19,20} The growth factors examined in this work are thought to be important targets for angiogenesis manipulation since they are necessary for angiogenesis.²¹

Human dental pulp stem cells (hDPSCs) are regarded as a promising possibility for therapeutic uses in the field of regenerative medicine. Because of their paracrine capabilities, they can be employed as a substitute tool in place of a cell-based treatment strategy.²² One potential drawback of cell-based therapy—graft rejection being the most well-known to date—may be addressed by using cell-free therapy in regenerative medicine. It has been noticed that

hyperbaric oxygen tension in the graft is low prior to angiogenesis, and ischemia-induced prolonged low oxygen tension is a common cause of graft damage.²³ Oxygen is the most effective way to treat a compromised graft because it encourages the creation of new vessels.²⁴ Low O_3 concentrations induce the alleged eustress in terms of cellular proliferation, which activates cell antioxidant response pathways² and provides the therapeutic effects necessary for clinical practice. Ozone therapy used in clinical settings for jaw osteoradionecrosis and wound healing has demonstrated encouraging outcomes.²⁵

Clinical research has also documented the possible therapeutic benefits of conditioned medium obtained from stem cells. A prior study by Martínez et al.²⁴ examined the growth factors of CM produced from hDPSCs and found that CM from hDPSCs secreted pro-angiogenic growth factors such as VEGF, PDGF-BB, and HGF. There have been cases reported where bone regeneration was enhanced by the

transplantation of scaffolds soaked in conditioned media produced from MSCs during sinus lift surgeries for the maxilla. Following engraftment, there have been no local or systemic issues.²⁶

42 patients who underwent ablative and non-ablative LASER procedures were included in a split-face clinical evaluation of CM generated from embryonic cells. The use of soluble CM produced from embryonic cells to accelerate wound healing after LASER resurfacing procedures is supported by the trial's findings. CM promoted quicker, less noticeable wound healing, and more normal skin recovery.²⁷ The regeneration process in rats with critical-size bone defects was positively impacted by conditioned medium derived from human dental pulp mesenchymal stem cell cultures. The conditioned medium induced both increased vascularization and bone growth.²⁸ Researchers also looked at the endothelial proliferation of DPSC in vitro, and they discovered that endothelial markers, including von Willebrand factor, were overexpressed. This led them to conclude that hDPSC had angiogenic potential.²⁴ Dental stem cell-derived CM with growth factors and their potential in different physiologic and pathologic processes have been the subject of numerous investigations.²² The impact of ozone on tissue regeneration has not yet been investigated. In hDPSCs, there is no significant data. In order to examine the changes in hDPSCs' growth factor secretion and proliferation at the molecular level in vitro, as well as to evaluate these changes in ovo using a chick embryo model, we conditioned the cells with low O₃ concentrations in this study. The pro-angiogenic potential of ozonated hDPSC-derived CM is investigated, along with the levels of pro-angiogenic factors like VEGF, FGF, and PDGF. As of yet, no research has shown that treating hDPSCs with ozone can enhance their angiogenic potential.

VEGF, FGF, and HGF were found to be expressed at notably greater levels in ozonated hDPSC-CM in the current investigation when compared to untreated hDPSC-CM. From the recruitment of endothelial cells to the development of blood vessels, all three of these variables play a major role in encouraging angiogenesis. In this study, we screened the effect of ozonated hDPSCs-CM using the YSM model. To determine which source has the most potential for promoting angiogenesis, we assessed three distinct factors. When supplemented with ozonated hDPSCs-

CM, our data showed an increase in total vessel network length, vessel density, and total segments on the YSM assay, which was substantially greater than the remainder of the group.

The benefit of employing ozone to enhance the angiogenic potential of hDPSCs as a source of cell-free cultured medium with superior therapeutic and translational utility was therefore highlighted in the current work. Ozonated hDPSCs-CM is one of the best sources of angiogenic potential since it exhibits the highest concentration of the essential components for angiogenesis. The YSM test used in this investigation provides additional evidence that the results of the growth factor analysis have translational significance.

Conclusion

The five main factors that drive angiogenesis are VEGF, FGF, M-CSF, Ang-2, and HGF. These factors support the process of angiogenesis, from the stimulation of endothelial cells to the maturation of blood vessels. Ozonated hDPSCs-CM is one of the best sources of angiogenic potential since it exhibits the highest concentration of the essential components for angiogenesis. The YSM test used in this investigation provides additional evidence that the results of the growth factor analysis have translational significance.

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.

Ethical approval

The study was approved by the University 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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ԳԱԶԱՅԻՆ ՕԶՈՆՈՎ ՄՇԱԿՎԱԾ ՄԱՐԴՈՒ ԱՏԱՄԻ ՊՈՒԼՊԱՅԻ ՑՈՂՈՒՆԱՅԻՆ ԲԶԻԶՆԵՐԻ ՍԵԿՐԵՑՈՒՄԸ ՈՒԺԵՂԱՑՆՈՒՄ Է ԱՆԳԻՈԳԵՆԵԶԸ ՀԱՎԻ ՍԱՂՄԻ ՄՈԴԵԼՈՒՄ

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Ամփոփում

Ներածություն. Օգոնն ավելի տարածված է դարձել բժշկության մեջ ամբողջ աշխարհում՝ որպես տարբեր հիվանդությունների բուժման օժանդակ մեթոդ: Օգոնի և hDPSCs-CM-ի համադրությունը կարող է ուժեղացնել անգիոգենեզը՝ իրենց սիներգետիկ գործողության շնորհիվ, ինչը հանգեցնում է աճի գործոնի արտահայտման ավելացման:

Նպատակը. Հետազոտության նպատակն էր գնահատել օգոնով մշակված մարդու ատամի պուլպայի ցողունային բջիջներով պայմանավորված միջավայրի անգիոգեն ներուժը (hDPSCs-CM):

Մեթոդներ. հեռացված ատամից մեկուսացվել են hDPSC-ները: Անցված չորս բջիջները բնութագրվել են հոսքի ցիտոմետրիայով, այնուհետև ենթարկվել են 10 մկգ/մլ գազային օգոնի կոնցերտացիայի: Պայմանավորված միջավայրը (CM) ստացվել է մշակված բջիջներից, և կատարվել է աճի գործոնի վերլուծություն: Օգոնացված hDPSCs-CM-ի ֆունկցիոնալությունը գնահատվել է Chick Yolk Sac Membrane (YSM) վերլուծությամբ:

Արդյունքներ. անոթային էնդոթելիային աճի գործոն (VEGF): In-ovo YSM փորձարկումը բացահայտեց զգալիորեն ավելի մեծ պրոանգիոգեն ներուժ՝ կապված օգոնացված hDPSC-CM-ի հետ:

Եզրակացություն. Օգոնացված hDPSCs-CM կարծես ամենաարդյունավետ աղբյուրն է անգիոգենեզի առաջացման համար:

СЕКРЕТОМ СТВОЛОВЫХ КЛЕТОК ПУЛПЫ ЗУБА ЧЕЛОВЕКА, ОБРАБОТАННЫЙ ГАЗООБРАЗНЫМ ОЗОНОМ, УСИЛИВАЕТ АНГИОГЕНЕЗ НА МОДЕЛИ КУРИНОГО ЭМБРИОНА

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Резюме

Введение: Озон стал более распространенным в медицине по всему миру в качестве вспомогательного метода лечения различных заболеваний. Комбинация озона и hDPSCs-CM может усиливать ангиогенез благодаря их синергическому действию, что приводит к увеличению экспрессии факторов роста.

Цель: Целью исследования было оценить ангиогенный потенциал обработанной озоном среды, кондиционированной стволовыми клетками пульпы зуба человека (hDPSCs-CM).

Методы: hDPSC были выделены из удаленного зуба. Пассированные четыре клетки были охарактеризованы с помощью проточной цитометрии, а затем подвергнуты воздействию 10 мкг/мл газообразного озона. Кондиционированную среду (КС) получали из обработанных клеток и проводили анализ факторов роста. Функциональность озонированных hDPSCs-CM оценивали с помощью анализа мембраны куриного желтка (YSM).

Результаты: Озонированные hDPSC-CM имели значительно более высокую ($p<0,01$) экспрессию ангиопоэтина-2 (Ang-2), фактора роста фибробластов (FGF), фактора роста гепатоцитов (HGF), фактора стимуляции колоний макрофагов (M-CSF) и фактор роста эндотелия сосудов (VEGF). Анализ YSM in-ovo выявил заметно больший проангиогенный потенциал, связанный с озонированным hDPSC-CM.

Заключение: Озонированные hDPSCs-CM являются наиболее эффективным источником индукции ангиогенеза.