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Biological Behavior of Dental Pulp Stem Cells to MTA Mixture with either Diclofenac Sodium or i-PRF as Direct Pulp Capping Material

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Aim This study aims to investigate and compare the effect of MTA mixed with diclofenac sodium (DCS) or i-PRF as a direct pulp capping agent on the proliferation and mineralization potentials of human dental pulp stem cells (hDPSCs).
Materials and Methods hDPSCs were isolated from the pulp tissues of human-extracted wisdom teeth and then cultured. The characterization of hDPSCs was ensured by flow cytometric analysis. MTA powder was mixed with distilled water, i-PRF, or two different concentrations of commercially available DCS (Voltaren® 75mg/3mL and Epifenac® 1mg/mL). Extracts of the MTA mixtures were then prepared and cultured with hDPSCs. Cell viability and proliferation were examined by MTT assay, and the mineralization potential was evaluated using the alkaline phosphatase activity and alizarin red staining assays.
Results The MTA/Distilled group exhibited significantly the highest mean percentage of cell viability and proliferation, followed by the MTA/i-PRF group. Both DCS concentrations in the MTA mixtures expressed an increased mean percentage of cell viability and proliferation when compared to the positive control group; however, it was still less than that of the MTA/Distilled and the MTA/i-PRF groups. Regarding mineralization and calcium nodules formation, MTA/Voltaren revealed significantly superior results, followed by MTA/i-PRF. Moreover, MTA/Epifenac, when compared to MTA/Voltaren and MTA/i-PRF, showed significantly reduced cellular mineralization; however, it was relatively comparable to that of MTA/Distilled.
Conclusion MTA mixed with either Voltaren as DCS or i-PRF augmented the MTA cellular regenerative capability in terms of viability and mineralization potential, and these mixtures may be potentially used as direct pulp capping material for vital pulp therapy.

Keywords: Direct pulp capping, MTA, Diclofenac sodium, i-PRF, Human dental pulp stem cells.

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Introduction

Dental caries, with or without pulpitis, is a commonly spread dental disease among the world's population.[1] Treatment of such conditions ranges from just doing a coronal restoration to permanently devitalizing the tooth through making root canal therapy.[2] Pulp vitality is very critical to tooth health and long-term survivability; thus, preserving it is a priority in case pulpal exposure happens after dental caries or traumatic injuries.[3] Recently, evidence proved dental pulp regenerative potentials through the presence and help of fibrocytes, stem cells, and undifferentiated mesenchymal cells in the pulp tissue together with a variety of immune cells.[3,4] Consequently, vital pulp therapy, either in the form of pulp capping or pulpotomy procedures, should be considered first as a primary intervention for the management of carious and/or traumatic pulp exposures.[3]

Direct pulp capping (DPC) is a conservative pulpal treatment where a biomaterial is directly applied over the exposed pulp tissue, followed by immediate placement of properly sealing permanent coronal restoration.[4] This helps in hard-tissue formation and the protection of the remaining vital pulp tissues.[4] Ideally, bioactive DPC agents, besides their biocompatibility, should be able to stimulate the proliferation and differentiation of cells, incorporate, or at least promote inductive growth factors and control any existing inflammation.[5] Mineral Trioxide Aggregate (MTA), a tricalcium silicate-based material, has been widely used as a DPC material because of its biocompatibility, antibacterial activity, superior sealability, and stimulation of hard tissue formation.[4] However, studies revealed that the success rate of DPC intervention is higher when the cause of pulp exposure is mechanical rather than resulting from caries.[6] This is due to the fact that bacterial contamination of the pulp tissues occurs together with pulpal inflammation that actually remains even after removal of caries and biomaterial placement.[6,7] This most likely leads to chronic low-grade inflammation that hinders the ability of pulp tissue to maintain its vitality and heal.[7,8] This led to the search for agents that can control this unfavorable inflammatory process such as nonsteroidal anti-inflammatory drugs (NSAIDs) and platelet concentrates.

NSAIDs are commonly used in endodontics as therapeutics to alleviate pain and to control inflammation.[9] Diclofenac sodium (DCS) is one of the NSAIDs that has proven potent analgesic and anti-inflammatory properties.[10] Moreover, it was associated with calcium hydroxide paste and tricalcium silicate-based cements, with regard to its marked antimicrobial action.[11–13]

Platelet concentrates derived from peripheral blood and containing autologous growth factors have reported unique regenerative potential with remarkable biocompatibility, ease of accessibility, and affordability.[14,15] They have been proposed as a new biological approach for the management of clinical pulp exposures adopting pulp regeneration with new hard tissue formation.[16] Platelet-rich fibrin (PRF) has been developed as a purely autologous second-generation platelet concentrate. When compared to platelet-rich plasma, a first-generation platelet concentrate, PRF is relatively easy to prepare since it does not require using anticoagulant or external additives during its preparation.[15] Additionally, it contains a larger number of platelets and leukocytes which play a crucial role in immune defense enhancement in inflamed tissues together with hard tissue formation stimulation.[15] A liquid form of PRF, an injectable PRF (i-PRF), was created in 2017 using a lower speed and a shorter time of centrifugation, offering a more operable PRF version.[14]

Therefore, because of the advantageous properties of these agents as well as the paucity of literature regarding the usage of DCS and i-PRF as an incorporated component within MTA, that could present an innovative mix to be employed in vital pulp therapy. Thus, this in-vitro study aimed to investigate and compare the effect of MTA mixed with either DCS or i-PRF as a DPC agent on the proliferation and mineralization potentials of human dental pulp stem cells (hDPSCs). The null hypothesis was that there is no difference among the proposed MTA mixtures used as DPC material in terms of their effect on the proliferation and mineralization potential of hDPSCs.

Materials and Methods

Ethical approval for our research was obtained from the Research Ethics Committee, Suez University, SUEZ Med-IRB, with approval no. (23). Methods were conducted in compliance with relevant guidelines and regulations.

hDPSCs Collection, Isolation, and Culture:

Isolation of hDPSCs from the pulp tissue of extracted human impacted wisdom teeth (n = 5) of young, healthy dental patients between the ages of 18 to 25 years old was done. A written informed consent was signed by all donors to have their permission to use their extracted teeth in this research work. Immediately after extraction, teeth surfaces were properly cleaned with alcohol and then rinsed with sterilized distilled water. Next, the teeth were put in sterile falcon tubes containing 7.4 pH phosphate-buffered saline (PBS) (Sigma Aldrich, Saint Louis, USA), penicillin/streptomycin antibiotics, antimycotic, as well as 1% DMSO preservation media (Invitrogen Life Technologies, USA).

In the tissue culture laboratory, the teeth were decoronated at the cementoenamel junction with continuous sterile saline irrigation to avoid heat generation and pulp cells death. Then, the pulp was extirpated using a sterile barbed broach. Next, it was quickly put in another sterile falcon tube containing PBS. After that, the pulp tissue was immersed in 1% penicillin/streptomycin for 1 minute, then it was sliced into small fragments (approximately 1 x 1 x 1 mm).

The pulp tissue was enzymatically digested by placing the fragments in a solution containing collagenase type II 0.1 U/mL (Sigma Aldrich, Saint Louis, USA) for 1 hr at 37°C while shaking it well every 10 min until tissues were completely digested. The collagenase solution was inactivated by adding Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, ThermoScientific, Germany) supplemented with 10% fetal bovine serum (FBS) (Gibco, ThermoScientific, Germany). Centrifugation was then done at 1500 rpm for 5 min.

Digested pulp tissues were seeded in DMEM containing 10% FBS and 1% penicillin/streptomycin/amphotericin B cocktail (Invitrogen Life Technologies, USA), and then incubated at 37ºC in a 5% CO_2 humidified environment. The medium was changed every 2 days until adherent cells reached about 80% confluency after a 21-day period (passage zero). After that, the cells were expanded to passage 3 to be used in further assessments. During this time, a phase-contrast inverted microscope (Labomed, USA) and Vega Digital Camera (Labomed, USA) of magnification power 40x were employed to observe and examine hDPSCs daily.

hDPSCs Identification and Characterization:

The hDPSCs were examined under an inverted microscope to identify the change in cellular morphology, number, viability, and confluency. The characterization of isolated hDPSCs was assured using flow cytometric

analysis. The cells were suspended in PBS while adjusting the count at 1 x 10^6 cells/mL and centrifuged for 10 min at 800 x g. Then, the cells were stained with CD45-, CD105-, and CD90- fluorescein isothiocyanate (FITC) surface markers (eBioscience™, Thermofisher Scientific, USA) and incubated at 4°C for 45 min. Finally, the data was processed for flow cytometric analysis using the NAVIOS flow cytometer (Beckman Coulter Life Sciences, USA) and Navios software (Beckman Coulter, SM: BE14548).

Preparation of Different Mixtures of MTA DPC Material:

The DPC material used in this research was MTA powder (Angelus, Lodrina, Paraná Brazil) mixed with different liquids, including distilled water, i-PRF, or commercially available DCS solutions with two different concentrations (1mg/mL or 75 mg/3 mL).

The experimental groups of MTA pulp capping material mixtures were as follows:

- **MTA/Distilled Group**: MTA powder was mixed with distilled water (Pharmapack, Pharmaceutical Industries, Egypt).
- **MTA/i-PRF Group**: MTA powder was mixed with i-PRF prepared as follows: 10 mL of venous blood was drawn from a healthy young human donor, with an age range of 20-40 years, after signing an informed written consent. Blood investigations, such as bleeding time, clotting time, hemoglobin, and platelet count, were done before blood collection and found to be in the normal range. The collected blood was put in test tubes (Chixin Biotech, Wuhan, China) without anticoagulant and promptly centrifuged at 700 rpm for 3 min (60G) at room temperature [14]. The upper one milliliter yellow plasma layer just above the RBC layer was aspirated as i-PRF. MTA powder was immediately mixed with the freshly prepared i-PRF.
- **MTA/Epifenac Group**: MTA powder was mixed with 1 mg/mL commercially available DCS solution (Epifenac® 1mg/mL eye drops, EIPICO, Egypt).
- **MTA/Voltaren Group**: MTA powder was mixed with 75 mg/3mL commercially available DCS solution (Voltaren® 75mg/3mL ampoule, Novartis, Egypt).

Under complete aseptic conditions, the four tested MTA mixtures used in this study were prepared following the manufacturer's instructions. The MTA powder was mixed on a sterile glass slab with different liquids in a 3:1 powder-to-liquid ratio using a mixing spatula until a wet sand mixture consistency was reached.

To standardize the material volumes, freshly prepared materials were introduced in a Teflon mold with 5 mm diameter × 1 mm thickness standard holes to ensure dimensional accuracy. The tested materials were incubated for 48 hrs at 37°C and 100% humidity to allow for proper setting.

Preparation of Pulp Capping Material Mixtures Extracts:

To prepare the material extracts, the discs were removed from the mold after setting, grounded into powder, and placed in a sterile Eppendorf. The material was exposed to 365 nm ultraviolet light (UV Lamps #1706887; Bio Rad, CA, USA) overnight to ensure sterility. Then, a 1.5 mL DMEM culture media supplemented with penicillin/streptomycin was added to each Eppendorf and incubated at 37°C and 95% humidity for 24 hrs. Next, the Eppendorfs were fully mixed using a shaker and centrifuged at room temperature. The final supernatant was collected and filtered using sterile 0.45µm filters (Sartorius A.G. Goettingen, Germany) to be used as a material extract.

Randomization and Blinding:

Each MTA mixture extract was given a specific code and assigned into groups. Each group was placed in a well-sealed envelope. Generation of random sequence was achieved via computer software (http:// www. random. org/). The laboratory technician, the data collector, and the statistician were all blinded to the capping mixture used in each group. Only the operator knew the type of MTA mixture used at the time of application.

Outcome Measurements:

I. Evaluation of hDPSCs Viability and Proliferation:

Cellular viability and proliferation with different mixtures of MTA pulp capping material extracts were assessed using the methyl-thiazoldiphenyl-tetrazolium (MTT) proliferation assay (Vybrant® MTT Cell Proliferation Assay Kit, cat no: M6494, Thermo Fisher, Germany) at days 1, 3, and 7 of culture.

One day before the experiment, the hDPSCs were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin/amphotericin B in a 96-well plate at a density equal to 1 x 10^4 cells/well for 24 hrs to attach. On the day of the experiment, the culture medium was replaced by a new one, and the cells were exposed to each MTA mixture extract. MTT assay was executed in triplicate with three independent experiments performed for each group. The positive controls were the hDPSCs cultured in the growth medium without experimental material extract. Cell viability was assessed by measuring the absorbance at 570 nm using a spectrophotometer (ELx 800; Bio-Tek Instruments Inc., Winooski, VT, USA).

The percentage of viable cells was calculated using the following formula = $(A/B)\ x100$, where A is the viable cells in the experimental well, and B is the viable cells in the control well.

II. Evaluation of hDPSCs Odontogenic Differentiation (Mineralization) Potential:

hDPSCs mineralization potential with different experimental MTA extracts was evaluated using the alkaline phosphatase (ALP) activity assay and the alizarin red staining (ARS) assay. hDPSCs placed in an odontogenic differentiation medium served as a positive control group while hDPSCs added to DMEM with no odontogenic differentiation medium served as a negative control group.

Odontogenic Differentiation Medium (OM) Preparation:

The OM was prepared by supplementing DMEM with FBS (10%), dexamethasone (0.01 µM), ascorbic acid (50 µg/mL), sodium β-glycerophosphate (10 mM), and penicillin/streptomycin (10,000 U/mL) (Gibco, ThermoScientific, Germany).

A. Determination of ALP Activity:

The hDPSCs mineralization potential in the pulp capping material extract was assessed using the ALP assay kit, cat no: MAK447 (Sigma Aldrich, Saint Louis, USA). In 24-well plate containing 1mL of OM, the hDPSCs at a density of 5 × 10^3 cells/well were seeded and incubated for 24 hrs. After that, the cells were exposed to 10 µL of pulp capping material extract and incubated for 7 days in a 5% CO_2 incubator at 37°C.

After 7 days incubation period, the cells were lysed at 4°C for 2 hrs. Next, 100 µL of p-nitrophenyl phosphate (p-NPP) was added to 50 µL of the cellular lysate in a 96-well plate and incubated for 30 min. Then, a spectrophotometer (ELx 800; Bio-Tek Instruments Inc., Winooski, VT, USA) was used to measure the absorbance at 405 nm.

Each group was evaluated in triplicate with three independent experiments.

B. Calcium Deposition Identification:

The ARS assay was used to assess the mineralized nodules formation among the hDPSCs after being treated with the different MTA extracts. First, hDPSCs at a density of 5×10^3 cells/well were cultured with 200 µL of OM in 6-well plates and incubated for 24 hrs for adherence of cells. After that, 2 µL of each pulp capping material extract was added to cells and cultured for 14 days.

After 14 days of mineralization, the hDPSCs were washed three times with PBS after the removal of OM. Then, fixation of cells was done with formaldehyde 4% (Merck Millipore, USA) for 15 min at room temperature. For calcium deposition identification, 200 µL of 2% w/v Alizarin red stain (Sigma Aldrich, Saint Louis, USA) at pH 4.2 was added to the cells. Incubation for 30 min was done at room temperature with shaking. Next, the hDPSCs were then examined under an inverted microscope (Labomed Trinocular inverted phase contrast microscope model TCM400 microscope, USA) and images were captured by camera (Atlas 16MP Cmos USB Camera with PixelPro 3.0 software).

Alizarin red staining photometric analysis: hDPSCs at a density of 5×10^3 cells/well were seeded in 150 µL of OM in a 96-well plate and incubated for 24 hrs. Then, 15 µL of each material extract was added to cells and cultured for 14 days. Next, 50 µL of 10% acetic acid was added to the cells after OM removal, and incubation was done for 10 min at 85°C, followed by transfer to ice for 5 min. Centrifugation was done for 10 min at 4°C, and then 35 µL of the supernatant was transferred to a new Eppendorf tube where 10% ammonium hydroxide (Alfa Aesar, Tewksbury, MA, USA) was added. After this, alizarin red stain was added to 40 µL of the suspension in a 96-well plate. The absorbance of solubilized calcium-bound ARS was measured at 405 nm using a microplate reader (TECAN, Männedorf, Switzerland). All groups were evaluated in triplicates with three independent experiments.

SPSS Statistics software (Version 27.0 - IBM Corp, Armonk, NY, USA) was used to conduct the statistical analyses. The Shapiro-Wilk test was adopted to assess the continuous variables for normality. Since the normal distribution of the data was verified, one-way ANOVA test followed by Tukey's post-hoc test was used for multiple comparisons among the experimental groups at each time point. Statistical significance was considered at *p-value* < 0.05. All data was expressed at the mean ± standard deviation.

Results

Isolated hDPSCs Characterization:

The hDPSCs were characterized after isolation and culture using surface marker expression investigated by flow cytometric analysis. Noticeably, these cells showed negative expression of CD45 (3.8%), a hematopoietic stem cell marker, and on the other hand, they demonstrated bright expression of CD90 (97.7%) and CD105 (97.3%), the mesenchymal stem cell markers (Fig.1A). The results confirm the pure mesenchymal cells isolation (CD90+/CD105+); and the negative expression of CD45 proves the pure mesenchymal cells of non-hematopoietic origin.

The isolated cells from the pulp tissues had round shape, short microvilli, and bean shaped nuclei during baseline assessment. On Day 21, cells showed the typical morphology of well-differentiated cells with large, flattened spindle-shaped cells with hyperchromatic nuclei (Fig. 1B).

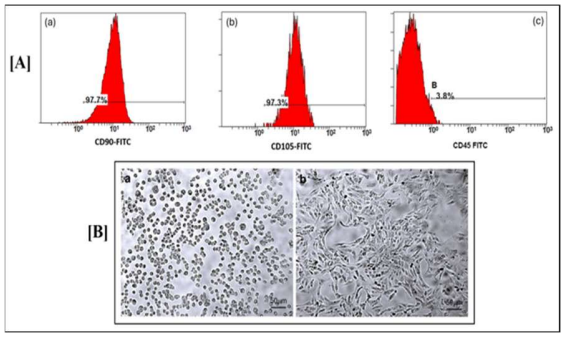

Fig. 1: hDPSCs' Characterization. [A] Flow cytometric analysis: Representative dot plot graphs showing: (a) CD90 bright expression (97.7%), (b) CD 105 bright expression (97.3%), and (c) CD45 negative expression (3.8%). [B] Representative images showing: (a) Cell morphology at baseline, and (b) Cell morphology on day 21. Magnification power is 10×. Scale bar indicates 50 µm. Labomed inverted microscope and Labomed Camera software were used for capturing images. FITC: fluorescein isothiocyanate, hDPSCs: human dental pulp stem cells.

The Effect of Different MTA Mixtures on hDPSCs Viability and Proliferation:

After cell culture with different MTA pulp capping material extracts, the highest mean percentage of cell viability was recorded in the MTA/Distilled group (154.24 ± 45.76), followed by the MTA/i-PRF group (136.18 ± 30.29) and the MTA/Voltaren group (118.59 ± 26.22), respectively, with significant difference among them (*p-value* < 0.001). On the other hand, the mean percentage cell viability of the MTA/Epifenac group was (112.30 ± 29.47), which was insignificantly lower than that of the MTA/Voltaren group with *p-value* = 0.758. All the experimental groups showed statistically significant high mean percentage cell viability when compared to the positive control group (100 ± 7.21) with *p-value* < 0.001, except for the MTA/Epifenac group that revealed no significant difference (*p-value* = 0.165) (Fig. 2).

In addition, the time factor affected the percentage of cell viability in response to the different MTA mixtures used. To illustrate, on Day 3 of culture, the highest mean percentage of viable cells was observed (151.40 ± 33.18), in comparison to Day 1 (116.88 ± 35.33) and Day 7 (104.51 ± 13.61) of culture with a statistically significant difference in the percentage of viability during different time intervals (*p-value* < 0.05).

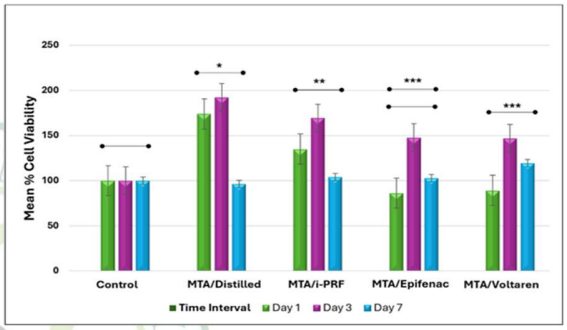

Fig. 2: Bar chart showing the mean % hDPSCs viability evaluated by MTT assay in response to different MTA pulp capping material mixtures at different time intervals. The error bars correspond to mean ± standard deviation. Different asterisks indicate statistically significant differences among groups and when compared with control (*p-value* < *0.05*).

The hDPSCs Odontogenic Differentiation (Mineralization) Potential:

A. ALP Activity:

The ALP enzyme activity of the hDPSCs was investigated on Day 7 of culture. The highest activity was significantly recorded in the MTA/Voltaren group (0.860 ± 0.049), followed by the MTA/i-PRF group (0.628 ± 0.092) and the MTA/Epifenac group (0.366 ± 0.024), respectively, when compared to the negative control group (0.123 ± 0.012), with statistically significant difference between them (*p-value* < 0.05). The MTA/Epifenac group showed a non-statistically significantly higher ALP activity when compared to the MTA/Distilled group (0.304 ± 0.010), with *p-value* = 0.811. However, both groups expressed statistically significant higher ALP activity when compared to the negative control group (*p-value* < 0.05) (Fig. 3[A]).

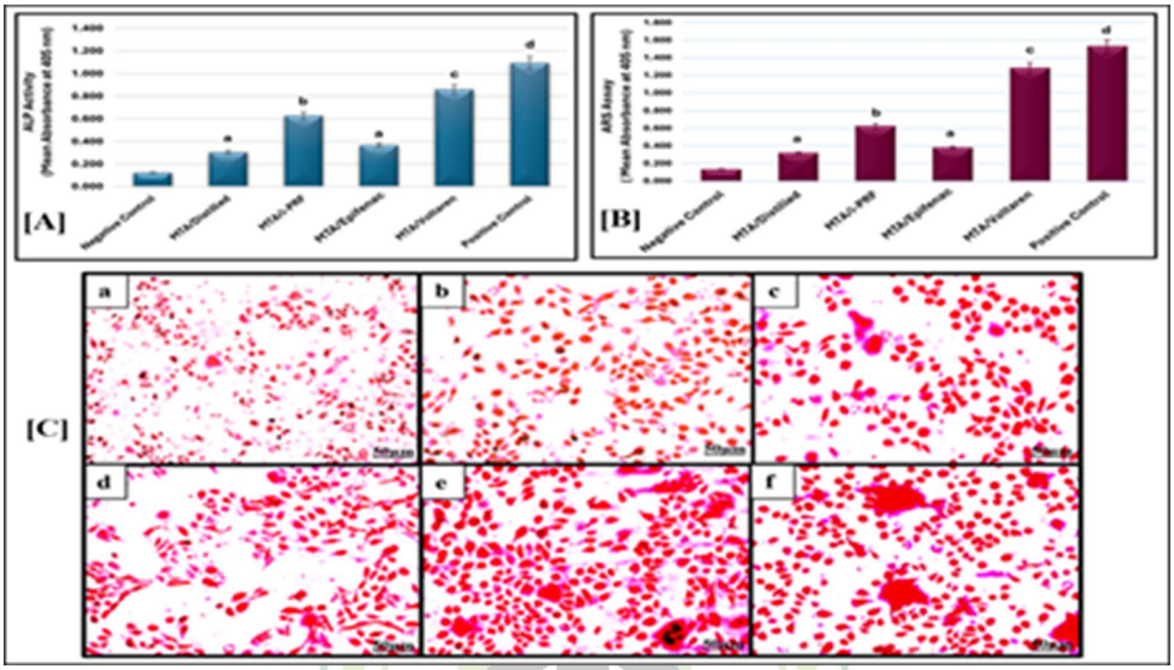

Fig. 3: Alkaline phosphatase enzyme activity and alizarin red stain assay. [A] and [B] Bar charts showing the effect of different MTA pulp capping material mixtures on ALP activity and ARS assay respectively. Different letters indicate statistically significant differences among groups and when compared to the controls (p-value < 0.05). [C] Representative images of transmitted light microscopy at 20× magnification showing calcium nodules formation in hDPSCs on Day 14 after alizarin red staining. (a): Negative control, (b): MTA/Distilled, (c): MTA/i-PRF, (d): MTA/Epifenac, (e): MTA/Voltaren, (f): Positive control. Scale bar indicates 50 µm.

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B. Alizarin Red-based Assay:

The mineralization potential of hDPSCs treated with different MTA pulp capping materials mixtures and the amount of calcium deposited in differentiated cells were evaluated on Day 14 of culture using ARS assay (Fig. 3[B],[C]). Quantified analysis of alizarin red exhibited the highest mean absorbance values and calcium deposition in the MTA/Voltaren group (1.278 ± 0.029, Fig. 3[C]e), followed by the MTA/i-PRF group (0.621 ± 0.032, Fig. 3[C]c), and then the MTA/Epifenac group (0.376 ± 0.032, Fig. 3[C]d), with statistically significant differences between them and with the control groups (*p-value* < 0.001). In contrast, the MTA/Distilled group (0.316 ± 0.028, Fig. 3[C]b) showed a non-statistically significant lower mean absorbance value when compared to the MTA/Epifenac group; however, both still recorded statistically significant higher values than that of the negative control group (0.130 ± 0.020, Fig. 3[C]a).

Discussion

The new MTA mixtures used in our study were proposed in a trial to augment the properties of MTA as a DPC agent to help in modulating the initial inflammation that occurs during the pulp capping procedure either from the preceded bacterial invasion or

from the direct application of MTA on pulp tissues. Three different liquids were used in this study together with the routinely used distilled water to mix MTA powder. These liquids were a platelet derivative in the form of liquid PRF and two different concentrations of commercially available DCS drugs: high-concentration Voltaren and low-concentration Epifenac. Previous studies supported the fact that liquid PRF and DCS have the ability to control the inflammatory pulpal reaction.[8,13]

Liquid PRF used in our research was prepared by centrifugation of whole venous blood at speed of 700 rpm for 3 min.[14] Centrifugation is generally carried out to separate blood cells according to their density into layers. Standard speed described for PRF centrifugation was high speed from 2700 to 3000 rpm for 10-12 min.[15] Recently, lower speed and less time for centrifugation were proved to result in a PRF clot with higher platelets and leukocytes concentration and increased amount of growth factors' release.[17] Further trials to reduce speed and time even more, resulted in the separation of blood cells layers before clot formation leading to liquid PRF formation.[14] Liquid PRF remains in the injectable form for 20-25 min before starting to clot. So, it can be used in this liquid form with all the PRF superior regenerative properties being a 100% autologous biomaterial with a high concentration of defensive leukocytes.[8]

In the present study, hDPSCs isolated from wisdom teeth showed bright expression to CD90/CD105 surface markers but negative expression to CD45. Our findings agree with those reported by the International Society of Cellular Therapy (2006) declaring that pure mesenchymal cells express CD90 - CD105 but lack CD45 expression.[18] Furthermore, this research aimed to assess the four different MTA mixtures as DPC materials and compare their effects, when cultured with hDPSCs, on cell viability, proliferation, and odontoblastic differentiation (mineralization) potentials. Such assessments may give us, as clinicians, an idea about the cellular regenerative activity of hDPSCs in conjunction with the proposed mixtures and their ability of dentin bridge formation during vital pulp therapy.

Essential information was provided by our results regarding the biocompatibility of different MTA mixtures as potential DPC materials where all mixtures expressed good biocompatibility with hDPSCs and none of them affected cell viability when compared to the positive control group. This favorable biological performance was relatively low on Day 1 of culture. Then, it significantly increased on Day 3 and declined again on Day 7. Still, this change in performance at different time intervals did not influence the decision to use the tested mixtures in further mineralization assessment as all of them expressed high mean percentage viability above 100%.

The MTA/Distilled group exhibited significantly the highest mean percentage of cell viability and proliferation when compared to all the other groups. Our finding is partly in accordance with Araújo et al. who found that MTA maintained the viability of stem cells derived from human exfoliated deciduous teeth and allowed for continuous cellular proliferation.[19] Similarly, Costa et al. declared that MTA increased the umbilical vein endothelial cells growth and stimulated the human mesenchymal stem cells proliferation.[20] On the contrary, other studies reported that MTA might adversely affect cellular viability and proliferation.[21,22] This variability in findings may be explained by the different experimental conditions among the studies, which might have influenced cellular response, including cell types, method and duration of the assessment, usage of MTA during setting or after being set, and whether the MTA was used in direct contact with cells or was in an extract form.

The biocompatibility of i-PRF and DCS on hDPSCs when mixed with MTA has been affirmed in our work. When MTA was mixed with i-PRF, it showed a higher mean percentage of cell viability and proliferation when compared to positive control; however, it was still lower than that of the MTA/Distilled group. Several studies highlighted the ability of liquid PRF to maintain cell viability and promote cellular proliferation when compared to control.[8,14,23] Additionally, PRF was proved in previous research to be as biocompatible as MTA, or even more, when used as a pulp capping agent and was in direct contact with cells.[24,25] Furthermore, when liquid PRF was cultured within the cells after stimulating an inflammatory environment, PRF improved the regenerative capability of hDPSCs while attenuating the inflammatory reaction.[8] This is mainly due to the high leukocyte content of liquid PRF that is responsible for fighting against bacterial invasion and controlling inflammation, promoting wound healing.[8] The enhanced cell viability and proliferation associated with the MTA/i-PRF mixture may be attributed to the fact that liquid PRF directly releases different essential growth factors, such as transforming growth factor-β, platelet-derived growth factor, and vascular endothelial growth factor, which are strongly associated with cellular growth and proliferation as well as the processes of pro-inflammation and angiogenesis.[26] Thus, it promotes cellular regeneration during tissue healing after vital pulp therapy when mixed with MTA.

Both DCS concentrations in MTA mixtures expressed an increased mean percentage of cell viability and proliferation when compared to positive control; however, it was still less than that of the MTA/Distilled and MTA/i-PRF groups. The MTA/Epifenac group showed an insignificantly higher percentage than that of the control; however, it remained comparable to that of the MTA/Voltaren group. Many previous publications demonstrated the effective antimicrobial activity and biofilm inhibition ability of DCS either when used alone or associated with Biodentine and calcium hydroxide paste.[11,12,27]

Obviously, the current research is the first to assess the cellular viability and biocompatibility of MTA mixtures with different DCS concentrations. The association of calcium hydroxide paste with DCS was proved to promote cell viability in the pre-osteoblast cell line and to induce a less intense inflammatory action, when compared to calcium hydroxide paste alone.[13] Likewise, a tricalcium silicate experimental cement having DCS in its components employed similar cellular proliferation as MTA, and this biological response was dependent on the concentration of DCS used.[28] Moreover, Refaat et al. showed that DCS enhanced hDPSCs proliferation when different concentrations were used.[29] In contrast, a recent study by Adamičková et al. suggested that DCS usage might have a suppressing effect on hDPSCs proliferation and viability.[30] These contradictory findings are mainly related to the concentrations and doses of DCS used in the different studies conducted. The positive biological behavior of cells in our study may be attributed to the DCS concentration used and the relatively low dose applied for mixing MTA.

In our in vitro study, the mineralization ability of different MTA mixtures on hDPSCs was examined based on the ALP activity and ARS assays. ALP enzyme activity is an early indicator for any signs of cellular mineralization. Elevated ALP activity is usually associated with an increased ability for dentin bridge formation, which is crucial for the success of the DPC procedure. Furthermore, extracellular calcium deposition within pulp stem cells was quantitatively assessed using the ARS

assay to gain a thorough understanding of the material properties.

Compared to both MTA/Distilled and negative control groups, MTA mixed with Voltaren showed significantly superior mineralization potential and calcium nodules formation, followed by MTA mixed with i-PRF, when tested on hDPSCs. The MTA/Epifenac group, when compared to the MTA/Voltaren and MTA/i-PRF groups, showed significantly reduced cellular mineralization; however, it was relatively comparable to that of the MTA/Distilled group. This implies that these mixtures may positively enhance the formation of reparative dentin. MTA or its extracts was reported to have a positive impact on ALP enzyme activity and ARS assay.[31,32] This is mainly attributed to calcium and silicon ions released from MTA that promote the odontoblastic cellular differentiation through different pathways and subsequently mineralized tissue formation.[33]

The long-term systemic administration of DCS has been known to have direct inhibitory effect on animals' bone formation.[34] However, Grossner et al. stated that this negative impact on bone was subsidiary, and diclofenac enhanced mineral deposition in human mesenchymal stem cells.[35] Hadjicharalambous et al. showed that DCS negatively influenced pre-osteoblast cell viability with reduced ALP activity at Day 7; however, it showed increased calcium deposits formation with ARS assay after 21 days.[36] In contrast, DCS did not influence the bone regeneration process and healing in rat model.[37] This different cellular behavior depends mainly on DCS exposure time and concentration and on whether it is systemically administrated or locally applied. The actual mechanism through which DCS inhibits bone formation should be explored through further histological studies. The amount of Voltaren used in our study to mix MTA did not inhibit the mineralization process. This interesting effect of MTA/Voltaren mixture on mineralization potential and calcium nodules formation may be attributed to the propylene glycol content of the Voltaren liquid which increases calcium ion release, especially when incorporated with calcium-silicate based cement, as proved by previous studies.[28,38]

The positive impact of the MTA/i-PRF mixture on mineralization potential of hDPSCs was proved in our study. Wang et al. examined the influence of i-PRF on the biological behavior of osteoblasts and found that i-PRF significantly induced ALP activity and ARS assay at Days 7 and 14, respectively, when compared to control.[23] Chai et al. also reported that when i-PRF was cultured with hDPSCs, it stimulated greater ALP activity and ARS staining, maintaining a regenerative environment supportive of odontoblastic differentiation and hard tissue formation even in inflammatory conditions.[8] Additionally, Dou et al. proved that PRF can enhance mineralization, similar to MTA, when cultured with hDPSCs.[25] It could be hypothesized that i-PRF added benefit to MTA mainly because of the physiologically incorporated leukocytes in i-PRF as well as the fibrin proteins, which will coagulate thereafter, that greatly influence the odontoblastic differentiation and mineralization potentials.

Despite the promising findings of our study, the effect of these mixing liquids on the physicochemical properties of MTA should be further examined to suggest their potential application in clinical practice. Our in vitro study still has limitations since MTA was used in the set condition and not when freshly mixed. Another limitation is the examination of these mixtures in a controlled cellular environment with no inflammation, which does not reflect the actual clinical condition. Further ex vivo and in vivo research is needed regarding these mixing liquids and the newly suggested MTA

mixtures for long-term evaluation and to support the results we obtained.

Conclusion

In conclusion, under the experimental conditions of our research, MTA mixed with either Voltaren as DCS or i-PRF augmented the MTA cellular regenerative capability in terms of cellular viability and mineralization potential, and these mixtures may be potentially used as direct pulp capping material for vital pulp therapy. Further research is needed to validate this.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

Ethical approval for our research proposal was obtained from the Research Ethics Committee - Suez University, SUEZ Med-IRB with approval no. (23). A written informed consent was obtained from all donors to have their permission to use their extracted teeth in this research work for isolation of human dental pulp stem cells.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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