In Vitro Evaluation of the Effect of Oleanolic Acid as a Potential Root Canal Medicament on Viability and Proliferation of Dental Pulp Stem Cells

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Abstract

Objective In light of the potential drawbacks associated with certain intracanal medicaments, such as triple antibiotic paste (TAP) and calcium hydroxide ($Ca(OH_2)$), the introduction of herbal agents has ushered in a new era in the field of dentistry. Consequently, this study aimed to explore the impact of oleanolic acid (OA) on the viability and proliferation of dental pulp stem cells (DPSCs), comparing its effects to those of conventional intracanal medicaments, TAP and $Ca(OH_2)$.

Materials and Methods DPSCs were derived from the third molars of an adult donor. Flow cytometry was utilized to do a phenotypic study on DPSCs. The methyl-thiazol tetrazolium (MTT) test was used to evaluate cellular viability. The cells were subjected to various concentrations of TAP and $Ca(OH)_2$ (5, 2.5, 1, 0.5, and 0.25 mg/mL), in addition to OA (40, 20, 10, 5, and 2.5 µM). A cell proliferation experiment assessed the cell growth precisely at 3, 5, and 7 days.

Results DPSCs were characterized by flow cytometry. The mesenchymal markers (CD73, CD90, and CD105) had a positive expression. However, the hematological markers (CD14, CD34, and CD45) showed negligible expression. A notable reduction in cellular viability was seen in cells subjected to concentrations exceeding 0.5 mg/mL of TAP and $Ca(OH)_2$ compared to the cells that were not treated ($p < 0.05$). The cells treated with different concentrations of OA 2.5, 5, 10, and 20 µM did not exhibit any significant variance in cell viability compared to untreated cells ($p > 0.05$). Moreover, the concentrations of OA (20, 10, and 5 µM) showed high proliferation level compared to TAP and $Ca(OH_2)$ especially 5µM of OA after 7 days ($p < 0.05$).

Conclusion Our results revealed that OA exerted significant effect on the viability and proliferation of DPSCs compared to TAP and $Ca(OH_2)$.

Keywords
- oleanolic acid
- triple antibiotic paste
- calcium hydroxide
- dental pulp stem cells
- cell viability
- cell proliferation
- intracanal medicaments
- immature teeth

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Introduction

The occurrence of pulpal necrosis in juvenile permanent teeth is a significant clinical concern in endodontics. It does not expect this clinical case to have further root growth, leading to thin and delicate dentinal walls. Consequently, the tooth becomes more vulnerable to fractures.[1] Historically, the management of these teeth involved the use of intracanal calcium hydroxide ($Ca(OH)_2$) for apexification protocols, with the aim of stimulating the formation of a natural calcific barrier, subsequently followed by the root canal system's obturation.[2] Then, the methodology changed to incorporate the insertion of a synthetic apical barrier composed of mineral trioxide aggregate, reducing the number of dental appointments required and yielding equivalent positive results.[3,4]

Over the past decade, many published cases and case series have documented the implementation of a novel therapeutic approach known as revascularization or regenerative endodontics. This protocol has demonstrated the potential to facilitate ongoing root growth and elicit favorable outcomes regarding pulp vitality tests for specific immature teeth undergoing treatment.[5] Regenerative endodontic therapy often commences by chemical debridement of the root canal with minimum or no instrumentation. Then this is followed by disinfection step using an intracanal medicament, such as triple antibiotic paste (TAP) consisting of ciprofloxacin, metronidazole, and minocycline, or $Ca(OH)_2$ dressing.[2] TAP offers several advantages in regenerative endodontics, particularly in the treatment of immature permanent teeth with necrotic pulp.[6] TAP provides potent antimicrobial properties, reducing inflammation and disinfecting the root canal system, creating an optimal environment for tissue regeneration.[7] However, TAP has some disadvantages. Perhaps the most prominent drawback is its potential cytotoxicity, particularly concerning stem cells.[8] This cytotoxic effect may result in diminished cell viability and proliferation, thus compromising the regenerative potential of dental pulp.[9] The antibiotics in TAP, particularly minocycline, have a known side effect of tooth discoloration.[10] Moreover, the application of TAP can stimulate the generation of reactive oxygen species within the root canal, potentially causing oxidative stress and tissue damage.[11]

$CaOH_2$ is of common intracanal medicament. It has several benefits, including its capacity to promote healing and repair preiapical tissue,[12] to stop internal resorption, to neutralize low pH of acids,[13] and its capability to inhibit pathogen growth.[14] Its biocompatibility and capacity to stimulate the formation of tertiary dentin make $Ca(OH)_2$ a valuable asset in various dental procedures, including direct and indirect pulp capping, apexification, and overall pulp health maintenance.[15] Notably, it is cost-effective, easy to apply, and gradually resorbable, making it a practical choice in dental treatments.[16] Nevertheless, the high pH of $Ca(OH)_2$ may affect the stem cells viability, reducing their ability to regenerate.[17] Moreover, the quick decomposition of $Ca(OH)_2$ presents difficulties by preventing the formation of critical soft tissue needed for appropriate tooth differentiation in the canal space.[18,19]

The increasing popularity of herbal remedies in dentistry and medicine is driven by their biocompatibility, heightened antibacterial activity, and antioxidant and anti-inflammatory properties. Herbal medicine's rapid growth is attributed to its medicinal benefits, accessibility, and minimal side effects. With rising microbial antibiotic resistance, researchers are exploring new treatment options for oral disorders.[20] Various herbal medicaments like glycyrrhizin[21] and Rosmarinic acid (RA)[22] are being investigated as alternative intracanal treatments, creating an ideal microenvironment for regenerative endodontics due to their biocompatibility and antibacterial, anti-inflammatory, and antioxidant effects. Compounds like acetyl-11-keto-β-boswellic acid,[23] and Asiatic acid[24] also show promise in enhancing collagen synthesis and stimulating mesenchymal stem cell osteogenic differentiation, making them potential solutions to traditional materials' limitations like $Ca(OH)_2$ and TAP in promoting dentin-pulp complex regeneration.

Oleanolic acid (OA), a naturally occurring pentacyclic triterpenoid, is the aglycone for several saponins and holds significant bioactive properties within medicinal plants.[25] OA is a prevalent compound in several food items, particularly vegetable oils. It may be obtained by extracting from the leaves and roots of numerous plant species, including *Olea europaea, Viscum album*, and *Aralia chinensis*.[26,27] OA demonstrates a range of biological features, encompassing anti-inflammatory, antidiabetic, and hepatoprotective actions.[28,29] In recent years, a growing number of research has demonstrated that OA displays metabolic activity in several cellular processes, such as apoptosis,[30] differentiation, and cell cycle arrest.[31] Bian et al[32] showed that OA has osteoprotective effect. Another study concluded that OA is a promising bioactive agent for bone tissue regeneration.[33] It has inhibitory action against the majority of oral infections.[34]

In the current study, we assessed for the first time the impact of OA on viability and proliferation of proliferation of dental pulp stem cells (DPSCs) as potential intracanal medicament compared with TAP and $Ca(OH)_2$.

Materials and Methods

Ethical Consideration

The DPSCs were obtained from the third molars of an adult donor who had wisdom teeth extraction at the Faculty of Dentistry, Mansoura University. The research was conducted in accordance with the approved ethical protocol by Faculty of Dentistry local ethical committee, Mansoura University (ID: A11040820). The research procedures employed in this study adhered to the ethical guidelines of Declaration of Helsinki with an informed consent being provided by the participants in the study.

Drugs Preparation

OA (Cat.No. T2865, TargetMol, Wellesley Hills, MA, United States) was prepared with a stock solution 10mM in dimethyl sulfoxide (DMSO cat no. 2650, Sigma-Aldrich, United States). Metronidazole (250 mg, Sanofi Co, Egypt), minocycline chlorhydrate (100 mg), and ciprofloxacin chlorhydrate (250 mg,

European pharmaceuticals Co, Egypt) were combined to make TAP. The mixture was manipulated at 1 mg/mL to achieve a 1:1:1 ratio. $Ca(OH)_2$ was employed since it is readily commercially available.

Isolation of Dental Pulp Stem Cells

The dental pulp tissue was acquired using a portable pulp isolator equipment, which is a patented invention created by the research team affiliated with the Faculty of Dentistry at Mansoura University (WO2016162041A9). This procedure was conducted in a manner that adhered to aseptic conditions. The removal of the pulp tissue was conducted by a manual dental excavator. The pulp underwent three cycles of washing using phosphate-buffered saline (Cat. no. 10010023, Thermo Scientific, United States). The pulp tissue was cut into small pieces about 0.5 mm or smaller in diameter using 10-cm culture dishes. The pulp tissue was treated with enzymatic digestion (5 mL of collagenase type I) at 37°C for 15 minutes. The tissue sample was centrifuged, the supernatant was removed, and the pellet was diluted with Dulbecco's Modified Eagles Medium low glucose (DMEM Cat no. D5523, Sigma-Aldrich, United States). This medium contained 10% fetal bovine serum (Cat no. F9665; Sigma-Aldrich, United States) and 100 IU/mL penicillin/streptomycin (cat no. 15070063, Gibco, Thermo Scientific, Life Technologies Corp., United States). Then the sample was transferred to a tissue culture flask and incubated in a CO_2 incubator at 37°C and 5% CO_2.

Flow Cytometry Analysis

The use of flow cytometry was applied in order to investigate DPSCs and determine the presence of a distinct group of surface markers associated with mesenchymal stem cells. The used antibodies in this study were CD90- FITC, CD73-PE, CD105- PE, CD34-PE, CD14-FITC, and CD45-FITC, all of them were purchased from BD Biosciences, United States. One million cells were suspended in a buffer formulated to facilitate flow cytometry labeling. Following that, a volume of 100 µL of the cell suspension was exposed to an incubation procedure, including antibodies, which took place in a controlled environment devoid of light for 30 minutes at a temperature of 4°C. The expression profiles were examined using a CytoFLEX S flow cytometer produced by Beckman Coulter.

Cell Viability Assay

The cell viability experiment was conducted using the methylthiazoltetrazolium (MTT) test kit obtained from Abcam, following the manufacturer's instructions. A total of 1×104 cells were cultured in each well of a 96-well plate and incubated overnight at 37°C with 5% CO_2. The cells were exposed to different concentrations of TAP and $Ca(OH)_2$ (5, 2.5, 1, 0.5, and 0.25 mg/mL) and OA (40, 20, 10, 5, and 2.5 µM). The medium was removed from the cells after a period of three days, and afterward, the cells were subjected to treatment with MTT for a duration of 4 hours. Subsequently, the crystal violet formazan compound was solubilized using 100 µL per well of DMSO for 15 minutes. Formazan was quantified by employing a spectrophotometer set at a wavelength of 570 nm. The viability was detected by the following equation (OD Sample –OD blank / OD control – OD blank) $\times$ 100%.[35]

Cell Proliferation Assay

In a 24-well plate, 5×10^4, cells were seeded for 24 hours. Then, the cells were treated with drug concentrations that gave the highest cell viability. The cells were subjected to culture for three distinct time periods: 3, 5, and 7 days. The media was discarded, and the cells were split by 0.25% trypsin/ethylenediamenetetraacetic acid solution. The process of cell counting involved the combination of 10µL of cell suspension with 10µL of trypan blue solution (Cat no. 25-900-CI, CORNING, United States). Then, 10 µL of the sample was put on a hemocytometer and analyzed under a light microscope.

Statistical Analysis

The data were tested using the SPSS 20.0.1 software. After performing a normality test on the dataset, analysis of variance was studied. Following that, Tukey's tests were utilized for making comparisons. The level of significance was determined to be p-value less than 0.05.

Results

Characterization of Cells by Flow Cytometry

DPSCs underwent phenotypic characterization using flow cytometry. The performed research revealed that a significant proportion of cells had the expression of mesenchymal markers, namely CD73 (99.47%), CD90 (96.43%), and CD105 (96.37%). Conversely, limited or negligible expression of hematological markers CD14 (0.41%), CD34 (0.31%), and CD45 (0.22%) was observed (►**Fig. 1**).

Cell Viability Assay

The MTT test was used to examine the cytotoxic effects of TAP, $Ca(OH)_2$, and OA on DPSCs. The viability of cells treated with concentrations of $Ca(OH)_2$ and TAP (1, 2.5, and 5 mg/mL) showed a significant decrease in viability when compared to untreated cells ($p < 0.05$). However, there was no statistically significant change in viability observed between cells treated with concentrations of 0.5 and 0.25 mg/mL of TAP and $Ca(OH)_2$ and untreated cells ($p > 0.05$; ►**Fig. 2A, B**). Therefore, TAP and $Ca(OH)_2$ with a 0.5 mg/mL concentration were chosen for the following experiment.

Moreover, DPSCs were subjected to varying concentrations of OA. Compared to those not treated, the cells with concentrations of 2.5, 5, 10, and 20 µM did not exhibit any significant variance in cell viability compared to the intreated cells ($p > 0.05$). At the same time, the cells treated with a concentration of 40 µM showed a notable decrease in cell viability ($p < 0.05$; ►**Fig. 2C**).

Cell Proliferation

DPSCs were exposed to several experimental conditions, encompassing TAP, $Ca(OH)_2$, and OA treatments. The experiment included a group that remained untreated. The cells

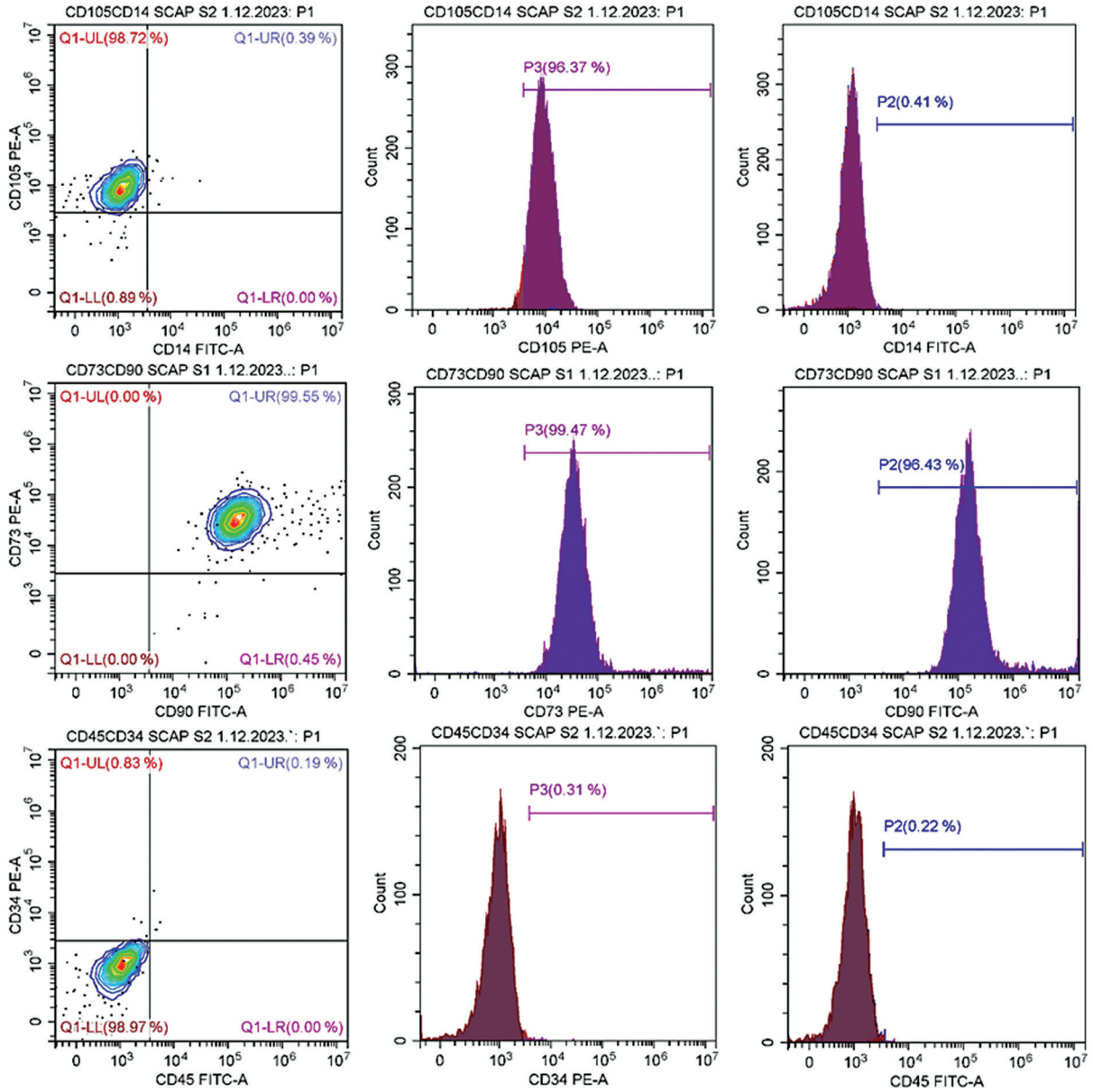

Fig. 1 Phenotypic analysis of mesenchymal stem cells markers expression in dental pulp stem cells.

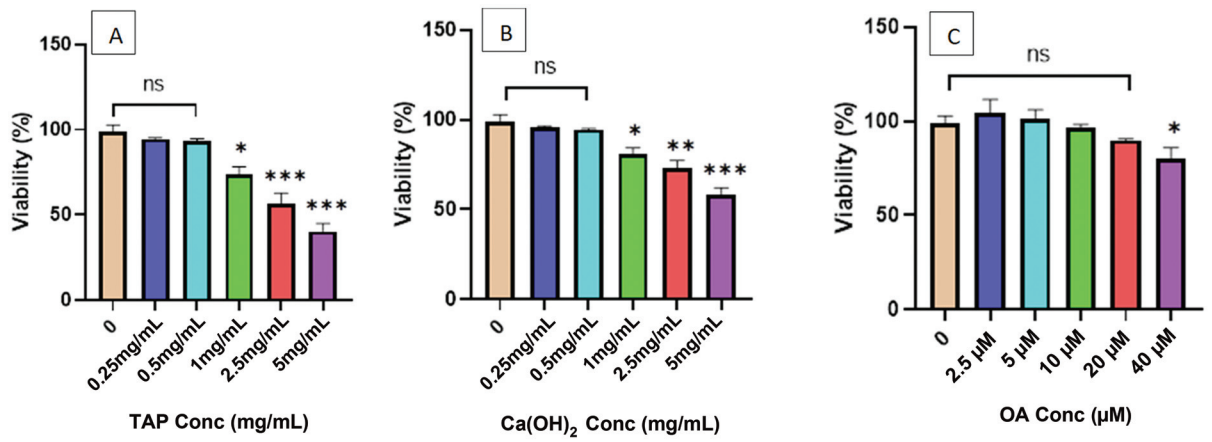

Fig. 2 Viability of dental pulp stem cells treated with different concentrations of (**A**) triple antibiotic paste (TAP), (**B**) calcium hydroxide ($Ca(OH)_2$), and (**C**) oleanolic acid (OA) for 3 days.

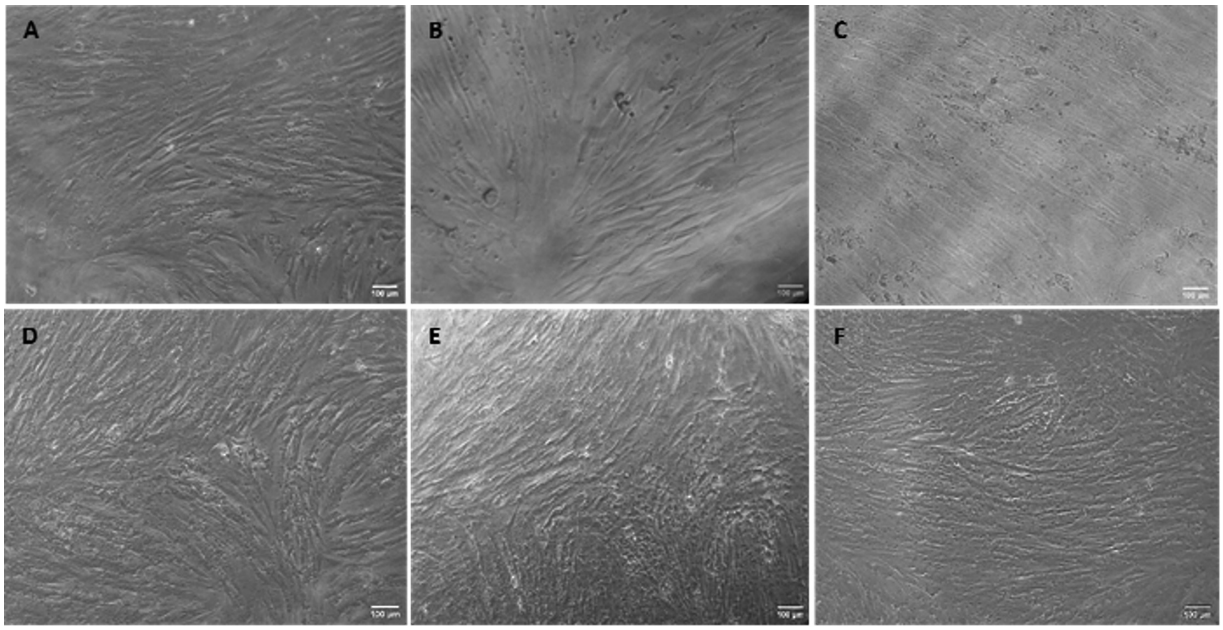

Fig. 3 Light inverted microscopy images of dental pulp stem cells showing; (**A**): control group, (**B**) triple antibiotic paste group, (**C**) calcium hydroxide group, (**D**) oleanolic acid 20µM, (**E**) oleanolic acid 10µM, and (**F**) oleanolic acid 5µM after 3 days, X: 100.

were incubated with TAP, $Ca(OH)_2$, and OA for 3, 5, and 7 days (►**Figs. 3–5**), and the cell density was evaluated (►**Supplementary Material Fig. S1**). Following 3 days of culturing, a noteworthy decline in cell density was seen when comparing the TAP treated cells to the untreated cells ($p < 0.05$). Conversely, no statistical difference was noticed between the two concentrations (10 and 5 µM) of OA and the untreated cells ($p > 0.05$). The concentrations 10 and 5 µM showed higher cell density than TAP.

After 5 days, a notable reduction in cellular density was seen when comparing the TAP concentration to the untreated cells ($p < 0.05$). The $Ca(OH)_2$ and the three used concentrations of OA exhibited no statistically significant change when compared to the untreated cells ($p > 0.05$). The concentration of 20 µM showed significant high cell density than TAP and $Ca(OH)_2$ ($p < 0.05$).

After7 days, the samples subjected to three distinct concentrations of OA (20, 10, and 5 µM) did not exhibit any notable variance in cell density when compared to the untreated cells ($p > 0.05$). However, the same OA-treated cells had a greater cell density in comparison to the TAP and $Ca(OH)_2$ samples ($p < 0.05$). This study's findings suggest that applying OA therapy at concentrations of 20 , 10, and 5 µM enhanced cell density after 7 days of culture.

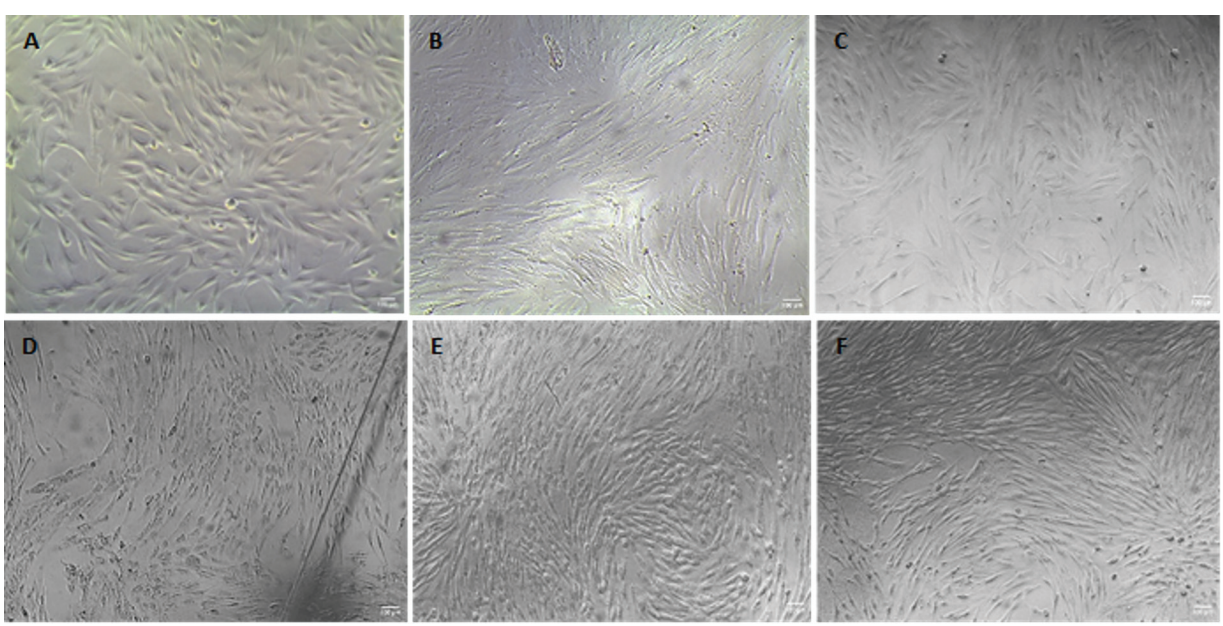

Fig. 4 Light inverted microscopy images of dental pulp stem cells showing; (**A**): control group, (**B**) triple antibiotic paste group, (**C**) calcium hydroxide group, (**D**) oleanolic acid 20µM, (**E**) oleanolic acid 10µM, and (**F**) oleanolic acid 5µM after 5 days, X: 100.

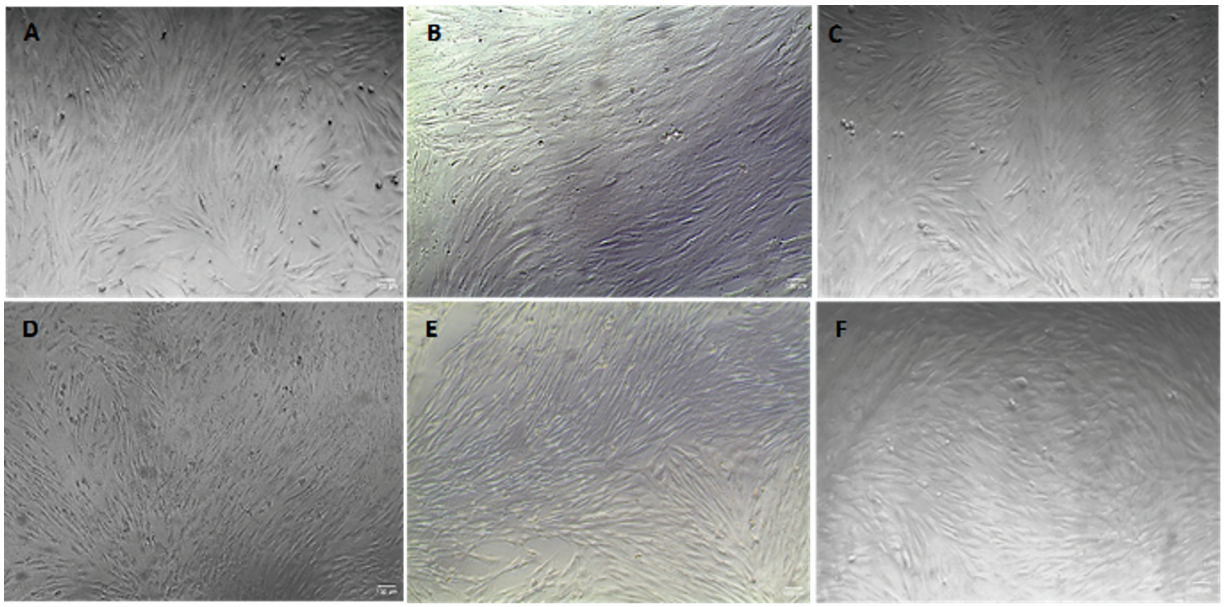

Fig. 5 Light inverted microscopy images of dental pulp stem cells showing; (**A**): control group, (**B**) triple antibiotic paste group, (**C**) calcium hydroxide group, (**D**) oleanolic acid 20μM, (**E**) oleanolic acid 10μM, and (**F**) oleanolic acid 5μM after 7days, X: 100.

Discussion

The efficacy of endodontic therapy is closely tied to the efficient elimination of bacterial colonization within the pulp cavity.[36] Hence, the incorporation of antibacterial agents, intracanal irrigants, and medicines plays a pivotal role in eliminating remaining microorganisms and attaining a desirable outcome in the context of root canal treatment.

$Ca(OH)_2$ is a frequently employed intracanal medicament. The substance exhibits various advantages, including its capacity to dissolve tissue[37] and its possession of antimicrobial capabilities.[38] However, previous studies have shown evidence that dental products, namely those that include $Ca(OH)_2$, exhibit fatal impacts on DPSCs.[39] Moreover, there is a potential risk of uncontrolled calcification induced by $Ca(OH)_2$, which may hinder the regeneration of functional pulp tissue.[40] Additionally, there is a possibility of overfilling or extruding $Ca(OH)_2$ into the periapical area, which can result in complications such as periapical inflammation and pain.[41]

TAP is another example of an intracanal medicament, composed of ciprofloxacin, metronidazole, and minocycline.[42] It is used for disinfecting the root canal system in root canal treatment and pulpal regeneration.[43,44]

Notably, *Enterococcus faecalis* bacteria are inherently resistant to the antibiotics in TAP, potentially leading to incomplete root canal disinfection.[45] Moreover, in certain instances, TAP may cause changes in the mechanical properties of dentin within the root canal, impacting the overall strength of the tooth.[46,47] Moreover, several experiments have yielded evidence that supports the ineffectiveness of $Ca(OH)_2$ in eliminating microbial cells when used against *Streptococcus sanguis* and *E. faecalis*.[48,49]

There has been a significant increase in the popularity of using medication derived from natural plants as a solution to overcome the restrictions associated with commercially available intracanal medicaments.[50] Grape seed extract has shown a role in bone remodeling within alveolar, jaw, and skeletal bone.[51] A *Moringa oleifera*-based dentifrice proving more effective in reducing gingival and plaque index scores.[52] Furthermore, in a controlled clinical trial, a gel containing 10% *Lippia sidoides* essential oil is found to be effective in controlling plaque and gingivitis, with results comparable to chlorhexidine.[53]

OA is a versatile antibacterial agent that has a wide range of activity against various microorganisms. It is extensively utilized in the medical field for the management of inflammatory and infectious conditions.[54,55] The findings of the research indicate that OA has the ability to impede the infiltration of inflammatory cells, diminish the cellular response to oxidative stress, and hinder cell apoptosis.[56] Furthermore, Kim et al documented that OA exhibits antibacterial properties, as evidenced by its ability to effectively eradicate *L. monocytogenes, E. faecium, and E. faecalis* through the disruption of the bacterial cell membrane.[57] Hence, this study aimed to assess the impact of OA on the viability and proliferation of DPSCs in order to determine its potential as a substitute pericanal medicament in comparison to commercially available options.

This study employed DPSCs generated from dental tissue, which may enhance the potential utility of the test. This section provides a detailed explanation of the methodology applied to conduct flow cytometric analysis on DPSCs in order to evaluate the expression of surface antigens and determine the presence of the mesenchymal phenotype in these cells. The biomarkers used for the analysis of DPSCs typically encompass CD29, CD34, CD73, CD90, CD105, and CD146, all of which are recognized as being expressed in mesenchymal stem cells. Furthermore, the examination of CD34 and CD45, both of which are expressed in hematopoietic stem cells, is also being explored.[58] The findings of our study demonstrated the presence of CD73, CD90, and CD105 with positive expression. In contrast, the DPSCs

exhibited a lack of expression for hematological markers CD14, CD34, and CD45.

The viability of DPSCs was assessed by employing the MTT assay to determine the impact of commonly utilized intracanal medicaments, specifically TAP and $Ca(OH)_2$, on their survival. Additionally, the effect of OA was also evaluated. The MTT assay is commonly employed as a colorimetric technique for evaluating cell viability and cytotoxicity of different drugs at varying concentrations.[59] Our data showed that using TAP and $Ca(OH)_2$ with concentrations above 0.5 mg/mL reduced the cell viability after 3 days, while concentrations below 0.5mg/mL did not show an cytotoxic effects. This discovery aligns with the recommendations established by the American Association of Endodontists (2016). The recommended guidelines propose the utilization of TAP at a dosage not exceeding 1 mg/mL (with a range of 0.1–1 mg/mL) in the context of regenerative endodontic therapy. This approach aims to mitigate any potential adverse effects on stem cells derived from the apical papilla.[59]

Also, Alsalleeh et al demonstrated a minor cytotoxic effect of $Ca(OH)_2$ when present at a concentration of 0.5 mg/mL, suggesting that the high pH of Ca(OH)2 at high concentration is cytotoxic and decrease the cells viability.[60] Similarly, Bhandi et al found that the cytotoxic effect of $Ca(OH)_2$ depends on both its concentration and the length of time it is exposed to cells. $Ca(OH)_2$ concentrations as high as 0.25 mg/mL were tolerated by DPSCs after 6 hours. However, only a tenth of that concentration had a detrimental effect on DPSCs after 24 and 48 hours of exposure.[61]

On the other hand, varied concentration of OA ranging from 2.5 to 40µM was tested on DPSCs for 3 days. MTT results manifested that OA concentration from 2.5 to 20µM was safe on cells, while OA with 40 µM showed decrease in cell viability. Our findings exhibit similarities to the study conducted by Shu et al, in which they reported a considerable induction of cell death in mesenchymal stem cells following a 12-hour treatment of OA using three different doses. Specifically, a dose of 30 µM resulted in significant cell death, but lower doses of 10 and 20 µM did not lead to any noticeable cell death in the cultures.[33] In contrast, the investigation of the vitality of neural stem cells treated with OA was conducted using the CCK-8 test. The test revealed a marginal rise in absorbance on the second day following culture; however, this increase did not reach statistical significance. On the third day following treatment, there was a notable rise in absorbance, indicating an increase in cell viability. Notably, the groups treated with 20 and 40 µM OA exhibited considerably higher cell viability compared to the group treated with 10 µM OA. These findings suggest a dose-dependent relationship between OA therapy and the observed increase in cell viability.[62]

Additionally, the effect of TAP and $Ca(OH)_2$ on DPSCs proliferation was assessed after 3, 5, and 7 days and compared with OA. Cells were treated with 0.5 mg/mL of TAP and $Ca(OH)_2$ and 5, 10, and 20µM of OA. Data revealed a higher proliferation rate of OA compared to TAP at the different three times, while after 7 days, the three used concentration of OA showed higher proliferation rate compared to both TAP and $Ca(OH)_2$. The findings of this study suggest that the use of OA is a safer alternative compared to commercially available intracanal medicaments, even when evaluated over a period of 7 days. Furthermore, the absence of difference in cell viability between cells treated with OA and untreated cells may be attributed to the differentiation capacity of OA, as previously indicated in the study conducted by Zhou et al. In their research, it was demonstrated that OA induces cell cycle arrest and activates the JNK pathway in both normal and malignant cells, while also suppressing the mTOR pathway.[63] The aforementioned signaling pathways are of significant importance in the process of mesenchymal stem cell differentiation.

Conclusion

Based upon the study findings, it seems that OA does not exhibit any cytotoxic effects on DPSCs. However, additional research is required to well understand the underlying mechanism by which OA could promote DPSCs differentiation pathways.

Clinical Significance

OA could have the capability for application in revascularization process of necrotic immature teeth.

Conflict of Interest

None declared.

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