

EFFECT OF TRIPLE ANTIBIOTIC PASTE, CALCIUM HYDROXIDE, LEDERMIX® ON VIABILITY OF PULP MESENCHYMAL STEM CELLS

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ABSTRACT

Objective: The goal of regenerative endodontic therapy is biological healing of the pulp tissue. It involves the disinfection of the canals with irrigants and medicaments. The medicaments that are currently used for this purpose are a triple antibiotic paste (TAP), calcium hydroxide (Ca(OH)₂), and Ledermix®, a paste containing demeclocycline and triamcinolone. Therefore, the purpose of this study was to evaluate the effects of TAP, Ca(OH)₂, and Ledermix® on the viability of dental pulp stem cells (DPSC).

Methods: Primary cultures of DPSC were obtained from immature third molars. Immunofluorescence assay using STRO-1 marker was performed to confirm the mesenchymal nature of the DPSC. The cells were exposed to TAP, Ca(OH)₂, and Ledermix® at concentrations of 0.1 and 1 mg/mL. Cell viability was analyzed using the MTT assay.

Results: Significant differences in viability were noted between the cells exposed to the medicaments and those in the control group (p<0.05).

Conclusions: All three medicaments decreased the viability of DPSC, with the Ledermix® paste demonstrating the highest toxic effect.

Keywords: Dental pulp stem cells, Triple antibiotic paste, Calcium hydroxide, Ledermix®, Cell viability.

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INTRODUCTION

The ultimate goal of endodontic treatment is the achievement of biological healing in the form of regeneration. Network regeneration is the process of healing by restoring the natural architecture and biological functions of damaged tissue, whereas repair is the restoration of tissue with replacement or scar tissue, without the return of function [1]. Regeneration restores the vitality of the tooth so that it can function like a healthy tooth. However, the conventional endodontic treatment procedures performed currently replace damaged tissue with biocompatible synthetic materials, but do not restore the biological functions to resemble that of normal tissues [2].

The development of science and network engineering technology is proving to be a breakthrough in the field of endodontics with the inclusion of stem cells, growth factors, and scaffolds, which are known as the triad of endodontic engineering. The first element of the tissue engineering triad is the stem cells. Studies on the isolation of dental stem cells have developed rapidly, especially after its isolation in pulp stem cells in 2000 [3]. Dental pulp stem cells (DPSC) have a high rate of proliferation even after many subcultures. In addition, DPSC have the ability to modify due to its high rate of differentiation. DPSC are present along with stem cells of apical papilla (SCAP) in the open apex; therefore, they continue to carry the morphogenetic information derived from SCAP. The characteristics and multiline differentiation potential of DPSC make them an important part of regenerative care [4].

Regenerative endodontic treatment has been a promising alternative treatment for the treatment of immature teeth with pulpal necrosis [5,6] and can replace conventional endodontic treatment methods when used in conjunction with tissue engineering [7-9]. This will shift the paradigm of endodontic treatment from conventional/reparative to regenerative [1,10].

Numerous studies on regenerative endodontics using successful revascularization procedures in immature teeth with pulp necrosis and

continued bleeding into the canal system due to over-instrumentation have been published so far [1,5]. The most important aspect of this clinical procedure is the chemical disinfection of the root canal system using irrigation materials and root canal medicaments for several weeks [5,6]. Root canal medicaments should ideally have antibacterial properties and provide a conducive environment so that stem cells can regenerate or heal [11,12]. Therefore, the concentration of the medicaments used in regenerative endodontic procedures should be effective against bacteria but not toxic to the stem cells [6].

METHODS

The study protocol had been approved by the Dental Research Ethics Committee, Faculty of Dentistry, Universitas Indonesia. Mesenchymal stem cells from the pulp of immature permanent third molars with open apices (primary culture) were used in this study. Inclusion criteria were as follows: Permanent third molar teeth indicated for extraction, from male or female patients aged <20 years, with radiographic evidence of immature teeth with open apices (>1.5 mm) during the development stage. The exclusion criteria were as follows: Permanent third molars with caries or pulp and periapical disease, cultures contaminated with fungi/bacteria, and cells that did not grow well during the first and subsequent passages.

Primary cells were isolated from newly extracted immature third molar teeth. Pulp tissue was removed, chopped into 2 mm×2 mm×1 mm fragments and incubated in a Petri dish with 0.25% trypsin for 5 min. The cells were added into 6-well plates containing complete Dulbecco's modified Eagle's medium and incubated at 37°C and 5% CO₂ until confluent.

For the immunofluorescence test, cells were plated in chamber slides at a density of 5000 cells and incubated at 37°C and 5% CO₂ for 3 days. On day 3, the medium was removed, and the cells were fixed using acetone-methanol absolute solution for 2 min. After fixation, the cells were washed with PBS solution and dried. Primary antibodies

were dissolved in PBS at concentrations of 100%, 50%, and 25%, and 100 μ L was added into each chamber. The cells were incubated with the antibodies at 37°C for 60 min; following which, they were washed again with PBS solution. Secondary antibody fluorescein isothiocyanate was added, and the cells were incubated at 37°C for 60 min. After washing with PBS, the cells were dyed with Evans Blue, rinsed with aquabidest, and dried. Subsequently, the cell specimens were observed under a fluorescence microscope.

The cells were divided into two groups, control and treatment cells exposed to the test materials triple antibiotic paste (TAP), Ledermix®, a paste containing demeclocycline and triamcinolone, and pure calcium hydroxide powder ($\text{Ca}(\text{OH})_2$), at concentrations of 0.1 and 1 mg/mL.

TAP was prepared by mixing 500 mg of metronidazole (Indofarma, Indonesia), 200 mg of ciprofloxacin (Bernofarm, Indonesia), and 100 mg of doxycycline (OGB Dextra, Indonesia) at a 1:1:1 ratio (3Mix) and added to a mixture of macrogol (M) and propylene glycol (P; ratio, 1:1), which acted as the solvent (MP). The antibiotic mixture and the solvent were stirred evenly until a putty consistency was achieved. A $(\text{OH})_2$ paste was prepared by mixing $\text{Ca}(\text{OH})_2$ powder (Merck, Germany) with aquadest to form a sandy paste-like consistency. TAP, Ledermix® (Riemser Pharma GmbH, Germany), and $\text{Ca}(\text{OH})_2$ pastes were diluted in the medium to concentrations of 0.1 and 1 mg/mL.

For the MTT test, the cells were incubated in 96-well plates for 24 h. The cell culture medium was discarded, and 100 μ L of test material was added per well. The cells were incubated at 37°C and 5% CO_2 for 48 h. MTT compound (100 μ L) was added, and the cells were incubated at 37°C and 5% CO_2 for 4 h. The cell supernatant was removed, and the formazan crystals formed were dissolved in 70% ethanol. Optical density (OD) readings were obtained using a microplate reader at a wavelength of 595 nm.

The data obtained were analyzed by one-way analysis of variance and *post hoc* Bonferroni's tests with a 95% confidence interval to compare the OD values between the control group and the treatment group.

RESULTS

An immunofluorescence test was performed using the STRO-1 antibody to confirm the mesenchymal nature of the primary culture cells from the dental pulp. Microscopic picture of the test results is shown in Fig. 1.

The figure above shows a red cell image with no primary antibody attached to the cell antigen. Thus, secondary antibodies cannot react with the primary antibody and produce a green luminescence. On the other hand, green luminescence indicates that the cell is positive against STRO-1 and is a mesenchymal stem cell.

Microscopic images of the cells in the control and treatment groups after incubation for 2 days on a 96-well plate are shown in Figs. 2-5.

Fig. 2 shows the microscopic images of the control cell cultures that were not exposed to bioactive compounds. Numerous, evenly dispersed fibroblasts presenting as small spindle-shaped cells with a thin cytoplasmic extension were noted. Microscopic images of cells in the TAP group revealed shortened cells that were in fewer numbers when compared with the control group (Fig. 3). The morphology of cells in the Ledermix® treatment group was also altered, and the numbers were reduced (Fig. 4), whereas in the $\text{Ca}(\text{OH})_2$ treatment group the morphology of the cells was similar to that in the control group with a sufficiently high number of cells (Fig. 5).

The OD was measured in the MTT assay, and the values were expressed as a percentage of the control group. The mean OD values and percentages of cell viability are shown in Table 1, whereas Table 2 illustrates the significant differences in cell viability between the treatment and control groups.

The highest value of cell viability among the cell groups exposed to the 0.1 mg/mL of the test material was noted with $\text{Ca}(\text{OH})_2$ (45.5% \pm 5.8%), and the lowest was noted with Ledermix® (15.1% \pm 3.4%). TAP was the most effective in reducing the viability of cells exposed to 1 mg/mL concentration of the test material, whereas Ledermix® appeared to be the least effective (Table 1).

The cell viability in all treatment groups was significantly lower than that of the control group at concentrations of both 0.1 and 1 mg/mL ($p < 0.05$).

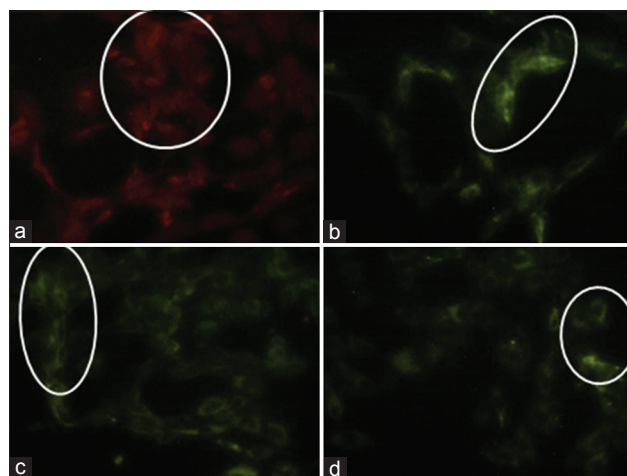


Fig. 1: Microscopic images of the immunofluorescence test results. (a) Control group, primary antibody STRO-1 was not added; (b) Group I, primary antibody added without dilution; (c) Group II, primary antibody added with $\times 2$ dilution; and (d) Group III, primary antibody added with $\times 4$ dilution

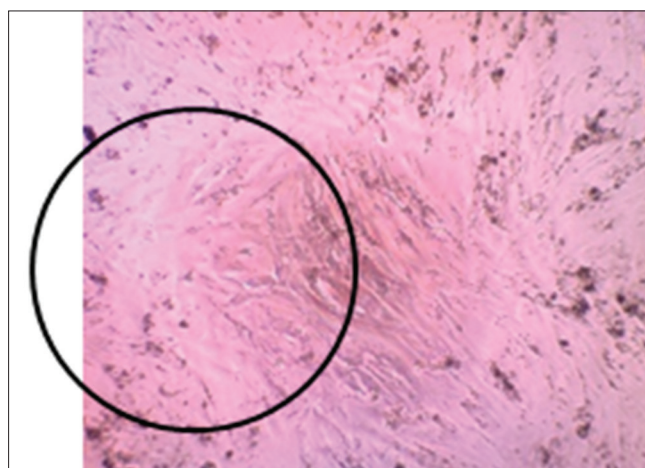


Fig. 2: Microscopic image of control cells in medium after incubation for 2 days ($\times 32$ magnification)

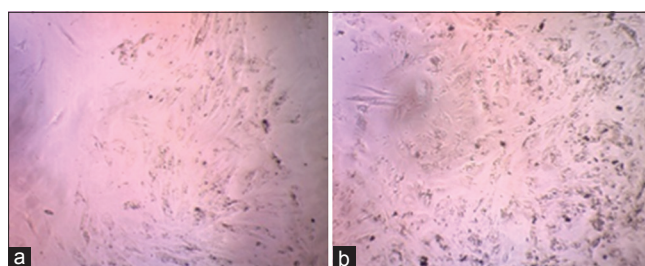


Fig. 3: Microscopic images of the triple antibiotic paste (TAP) treatment group cells after incubation for 2 days ($\times 32$ magnification). Treatment group exposed to TAP at a concentration of 0.1 mg/mL (a) and 1 mg/mL (b)

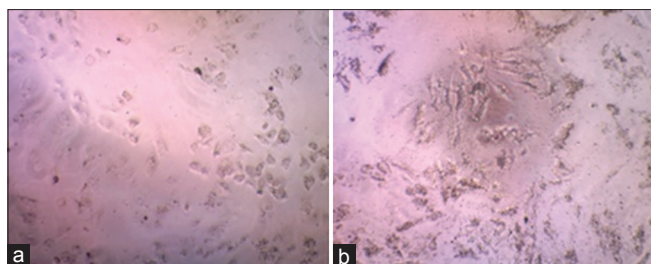


Fig. 4: Microscopic images of the Ledermix® treatment group cells after incubation for 2 days (×32 magnification). Treatment groups exposed to Ledermix® at a concentration of 0.1 mg/mL (a) and 1 mg/mL (b)

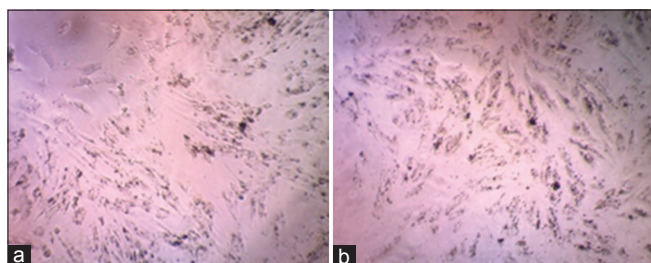


Fig. 5: Microscopic images of calcium hydroxide (Ca(OH)₂) treatment group cells after incubation for 2 days (×32 magnification). Treatment group exposed to Ca(OH)₂ at a concentration of 0.1 mg/mL (a) and 1 mg/mL (b)

Table 3 presents the differences in cell viability among the three treatment groups exposed to the test material at concentrations of 0.1 and 1 mg/ml. Statistically significant differences were not observed in the TAP treatment group, whereas both Ledermix® and Ca(OH)₂ treatment groups showed statistically significant differences between the two concentrations of the test material used in this study (p<0.05).

Table 4 shows that the viability of cells in the TAP group was significantly higher than that of the Ledermix® group when exposed to the test material at concentrations of 0.1 mg/mL (42.8% vs. 15.1%) and 1 mg/mL (37.2% vs. 7.4%), respectively (p<0.05). Similarly, the cell viability ratio of the Ca(OH)₂ treatment group at all concentrations was significantly higher than that of the Ledermix® group (p<0.05), but lower than that of the TAP treatment group (statistical significance notwithstanding).

DISCUSSION

The purpose of endodontic regeneration is the achievement of biological recovery, which reproduces the natural architecture and original biological functions of the tissue. A crucial stage in this treatment is the chemical disinfection of the root canal system by irrigation and placing of medicaments in the root canal for several weeks to kill bacteria and germs that are still present deep within the dentinal tubules. Very few studies on endodontic regeneration have been conducted in Indonesia; hence, this study aimed to evaluate the effects of root canal medicaments commonly used in root canal treatment on the viability of mesenchymal stem cells.

In this study, the primary culture of mesenchyme DPSC was used on the basis of the study by Pérez et al. (2003), who reported that the results of primary cells used to test an endodontic material would be much closer to the actual condition when compared with secondary cells [13]. SCAP is commonly used in regenerative studies because it has a higher proliferative capacity when compared with DPSC [14]. Nonetheless, we used DPSC in the current study because they carry morphogenetic information derived from SCAP. DPSC is an integral part of SCAP and is separated by an apical cell-rich zone at the odontogenesis

Table 1: Mean optical density values and percentages of cell viability between the control and treatment groups

Group	Mean value of OD±SD	Cell viability±SD (%)
Control	0.325±0.025	100±7.7
Concentration 0.1 mg/ml		
TAP	0.139±0.016	42.8±4.9
Ledermix®	0.049±0.011	15.1±3.4
Ca(OH) ₂	0.148±0.019	45.5±5.8
Concentration 1 mg/ml		
TAP	0.121±0.003	37.2±0.9
Ledermix®	0.024±0.006	7.4±1.8
Ca(OH) ₂	0.097±0.014	29.8±4.3

OD: Optical density, TAP: Triple antibiotic paste, SD: Standard deviation, Ca(OH)₂: Calcium hydroxide

Table 2: Significance differences in cell viability between the treatment and control groups at concentrations of 0.1 mg/mL and 1 mg/mL (p<0.05)

Concentration of test material	Control versus TAP	Control versus Ledermix®	Control versus Ca(OH) ₂
0.1 mg/ml	0.001*	0.000*	0.001*
1 mg/ml	0.015*	0.000*	0.001*

*p<0.05. Ca(OH)₂: Calcium hydroxide, TAP: Triple antibiotic paste

Table 3: Differences in cell viability among the three treatment groups exposed to the test material at concentrations of 0.1 and 1 mg/mL (p<0.05)

Treatment group	p value 0.1 mg/ml versus 1 mg/ml
TAP	1.000
Ledermix®	0.001*
Ca(OH) ₂	0.001*

*p<0.05. Ca(OH)₂: Calcium hydroxide, TAP: Triple antibiotic paste

Table 4: Differences in cell viability among the TAP, Ledermix®, and Ca(OH)₂ treatment groups based on the concentration of the test materials (p<0.05)

Group	TAP versus Ledermix®	TAP versus Ca(OH) ₂	Ca(OH) ₂ versus Ledermix®
0.1 mg/ml	0.002*	1.000	0.000*
1 mg/ml	0.000*	1.000	0.004*

*p<0.05. Ca(OH)₂: Calcium hydroxide, TAP: Triple antibiotic paste

stage, especially in immature teeth with incompletely closed root apices [15]. Chuensombat et al. (2013) reported that the survival of DPSC in infected root canals indicates their ability to regenerate [16]. Moreover, the volume of DPSC is 10 times higher than that of primary cells from SCAP, whereas the levels of contamination are lower because it is more coronally located, thus minimizing the risk of contact with the external environment at the time of collection.

There is heterogeneity in the mesenchymal stem cell population, with cells expressing different molecular markers. DPSC is an adult mesenchymal stem cell that can be characterized *in vitro* by STRO-1 [14,17,18], a monoclonal antibody that marks the subpopulation of mesenchymal stem cells as a colony-forming unit fibroblast because the morphology of mesenchymal stem cells resembles that of fibroblasts *in vitro* [19,20]. The selection of STRO-1 as a DPSC molecular marker in this study is based on previous studies, which demonstrated the consistent specificity of STRO-1 as a mesenchymal stem marker [18,21]. In the present study, STRO-1 was detected on DPSC using the indirect immunofluorescence test method.

Immunofluorescence is a histochemical staining technique that uses antigen-antibody reactions, where antibodies are characterized by a fluorescent dye and the antigen-antibody complex is visualized using a fluorescence microscope. The indirect immunofluorescence method was used because the signal amplification of secondary antibodies that bind to each primary antibody results in higher sensitivity [22-24].

There are several methods to test the cytotoxicity of a bioactive substance/compound. The cell viability parameter used in this study was the MTT test because it has a high sensitivity in evaluating the cytotoxicity of the test material; moreover, the steps used to conduct this procedure are relatively fast and easy to re-perform if necessary. This method is used to measure the metabolic activity of cell growth after exposure to the test material. The basic principle is to measure cellular activity based on the ability of succinate dehydrogenase in the mitochondria of the cell to reduce the MTT salts. This enzyme reacts with MTT and forms purple formazan crystals. The amount of formazan crystals formed is proportional to the activity of the living cells because these crystals are impermeable to cell membranes [25,26].

Cell viability in the treatment group was lower than that in the control group (Tables 1 and 2). Thus, the first hypothesis that TAP and Ledermix® can decrease the viability of DPSC was accepted, whereas the second hypothesis that Ca(OH)₂ can increase the viability of DPSC was rejected.

The viability of cells in the TAP group at concentrations of 0.1 and 1 mg/mL was significantly lower when compared with that in the control group. These results are consistent with those reported by Chuensombat *et al.* (2013), where TAP had a toxic effect on pulp and apical papilla cells at a concentration of 25 µg/mL. This is due to the low pH of the TAP (4–4.6), caused by the release of hydrogen ions from the hydrochloride group in minocycline hydrochloride and ciprofloxacin hydrochloride, thus causing unfavorable acidic conditions in the cell culture. The low pH also maintains TAP solubility enabling it to enter the cell, thereby increasing its cytotoxicity. It is known that metronidazole has no toxic properties due to its neutral pH [16]. Therefore, alternative antibiotics with neutral pH and potent antibacterial properties, instead of minocycline and ciprofloxacin, should be selected.

As seen in Table 1, the cell viability values in the Ledermix® treatment group were very low (<30%) and may be included in the high toxicity category based on the Dahl *et al.* criteria [27]. The decrease in cell viability with a concentration of 1 mg/mL Ledermix® was significantly higher than that with a concentration of 0.1 mg/mL. This finding is supported by Taylor *et al.* (1989) who reported that Ledermix® can kill mouse fibroblast cells at very low concentrations of 0.001 mg/mL [28]. The cytotoxic properties of Ledermix®, especially the corticosteroid component, are exerted by inhibiting phagocytosis and DNA/proteins synthesis during the cell mitosis process. Thus, cell replication is inhibited, and the healing process is impaired [28,29]. This was evidenced by Oliveira *et al.* (2009) who used mouse pulp cells exposed to antibiotic-corticosteroids (Otosporin®) for 72 h to demonstrate delays in tissue healing after treatment [30]. However, Taylor *et al.* (1989) stated that the toxic effects can be inhibited by mixing with Pulpdent® (Ca(OH)₂) [28]. These findings indicate that the use of Ledermix® as a medicament in regenerative endodontics should be avoided as it may interfere with the cell regeneration process. Alternatively, they may be used at very low concentrations or in conjunction with Ca(OH)₂.

The viability values of cells exposed to 0.1 or 1 mg/mL Ca(OH)₂ was also decreased when compared with the control group. This may be attributed to the high pH of the Ca(OH)₂ (2.5–12.8), which can cause cell/tissue necrosis immediately after contact, thus destroying cells that have the potential to regenerate [31]. Giro *et al.* (2010) examined the effects of Ca(OH)₂ on pulp cells during the direct pulp capping procedure and observed a layer of coagulation necrosis under the capping agent in all treatment groups after 7 days; however, pulp cells were still present within the necrotic tissue with very few infiltrates

of inflammatory cells [32]. Subsequently, after 30 days, the specimens showed normal elongation of the monolayer cells associated with hard tissue deposition near the wound area, thus implying that Ca(OH)₂ can be used over a long period of time. The formation of mineralized tissue after contact with Ca(OH)₂ can be seen on the 7th–10th day after application of the compound. In the present study, incubation after exposure of the test material was only for 2 days. The duration of this exposure was based on the number of cells planted (as many as 5000 cells) in the 96 well plates to achieve confluence within 48 h.

The test material used in this study was dissolved in the culture medium until the concentrations of 0.1 and 1 mg/mL were obtained. These concentration values were selected on the basis of a previous study by Ruparel *et al.* (2012), which states that TAP concentrations above 1 mg/mL have a lethal effect [6]. The concentrations of the test material were effective in reducing cell viability, especially in the Ledermix® and Ca(OH)₂ treatment groups, in the current study. However, in the TAP treatment group, the decrease in cell viability was not statistically significant. This contradicts with the results of the aforementioned study, where concentrations of 1 mg/mL TAP had a toxic effect, whereas, at a concentration of 0.1 mg/mL, no detectable toxic effect was noted; on the other hand, Ca(OH)₂ at high concentrations (100 mg/mL) increased cell proliferation [6]. These differences in results may be due to the different methods used, wherein a cell insert was used by Ruparel *et al.* so that the material did not come in direct contact with the cell, whereas in the present study, we used the direct contact method. The use of direct contact simulates the clinical setting where the root canal medication used in current endodontic treatment needs to be in direct contact to be more effective.

The root canal medicaments that were least effective in decreasing cell viability were TAP and Ca(OH)₂. Statistical significance notwithstanding, the cell viability value in the TAP group (concentration, 0.1 mg/ml) was lower than that of Ca(OH)₂ at a concentration of 0.1 mg/mL; on the other hand, the cell viability value in the TAP group (concentration, 1 mg/mL) was higher than that of Ca(OH)₂ at a concentration of 1 mg/mL. These findings indicate that TAP and Ca(OH)₂ have the same effects on the viability of DPSC cells.

The three root canal medicaments, TAP, Ledermix®, and Ca(OH)₂, were found to decrease the viability of dental pulp mesenchymal stem cells. The results of this *in vitro* study, which was purely a cellular study, cannot be compared directly with those of other *in vivo* studies. However, it is important to focus on the concentration of and duration of exposure to the medicinal ingredients before selection for use in regenerative care; the medicaments must be effective against bacteria and not cytotoxic for the stem cells. In addition, further *in vivo* studies in animals and humans are required to re-evaluate the cytotoxicity and biocompatibility of these medicaments.

CONCLUSIONS

From this study, it can be concluded that the root canal medicaments TAP, Ledermix®, and Ca(OH)₂ can decrease the viability of stem cells obtained from the pulp mesenchyme. TAP and Ca(OH)₂ are the least effective in reducing cell viability, whereas Ledermix® has the highest effect in decreasing the viability of the stem cells of the pulp.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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