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ORIGINAL ARTICLE

# *In vitro* cytotoxic effects of different intracanal medicaments on the co-culture of dental mesenchymal stem cells using the MTT assay


## ABSTRACT

***Aim:*** *This* in vitro *study aimed to assess the cytotoxic effects of different intracanal medicaments on the co-culture of dental mesenchymal stem cells.*
***Materials and Methods:*** *The Periodontal Ligament Stem Cells (PDLSCs) and the stem cells of the apical papilla (SCAPs) were isolated from a human teeth. The cells were passaged and underwent mono-culture and co-culture. The cells were then exposed to different concentrations of calcium hydroxide/chlorhexidine, calcium hydroxide/distilled water, double antibiotic paste/ chlorhexidine, and double antibiotic paste/distilled water. The cytotoxicity and cell viability was evaluated and data were analyzed by independent t-test, ANOVA, and Tukey's test (alpha=0.05).*
***Results:*** *The viability of PDLSCs in co-culture was higher than that in mono-culture following exposure to different concentrations of medicaments. However, when distilled water was used as the vehicle for intracanal medicaments, the viability of SCAPs in mono-culture was higher than that in co-culture. In use of DAP/CHX, the viability of SCAP in co-culture was higher than that in mono-culture (P=0.000). This difference was not significant in exposure of SCAP to DAP/CHX (P>0.05).*
***Conclusions:*** *Generally, the viability of PDLSCs in co-culture was higher than that in mono-culture, and distilled water was a better vehicle than CHX for intracanal medicaments.*



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## Introduction

Root canal disinfection by use of irrigating solutions and intracanal medicaments is a fundamental step in endodontic regeneration (1). Thus, intracanal medicaments play an important role in elimination of microorganisms (2), neutralization of endotoxins, preventing the entry of salivary microorganisms into the canal, reduction of pain and periradicular inflammation, and induction of mineralized tissue regeneration. Therefore, the byproducts of the medicaments should be biocompatible because they are in close contact with the periapical tissue (3). Otherwise, these products can trigger the release of many inflammatory factors such as histamines, kinins, and neuropeptides that can induce tissue destruction and slow down the healing process (4).

Several *in vitro* and in vivo studies have tried to optimize root canal disinfection in endodontic regeneration (5, 6). The optimization techniques have mainly focused on the survival and function of stem cells in the periapical region (7). Several chemical agents such as calcium hydroxide (CH), chlorhexidine (CHX), and antibiotics have been suggested to preserve and induce the dental mesenchymal stem cells (8-11). These products are all known for their optimal bacteriostatic and bactericidal effects; however, considering their potential side effects, some concerns exist regarding their cytotoxicity for host cells, especially dental mesenchymal stem cells (12-14).

CH can induce moderate angioblastic proliferation and formation of a fibrous capsule and moderate amounts of collagen fibers over time (15). Nonetheless, CH has lower cytotoxicity than other commonly used endodontic medicaments (16).

Evidence shows that CHX gluconate induces inflammatory reactions and tissue necrosis (17), delays the granulation tissue formation and tissue healing, inhibits the mitochondrial activity, and causes endoplasmic reticulum stress and cell death (18), brown extrinsic tooth and tongue staining, taste disturbance, dryness of mouth and burning sensation: these side effects limit its acceptability to users and its and long term use (19). Trevino et al. (9) evaluated the effect of 2% CHX on stem cells of the apical papilla (SCAP) by immunomagnetic separation, and showed that a combination of CHX/EDTA, and sodium hypochlorite/CHX/iodine potassium iodide/EDTA decreased the viability of the cells to 0%.

CHX is an effective agent against the microbial biofilms. Its main advantage over sodium hypochlorite is its lower cytotoxicity and no odor or bad taste. Although CHX has both bactericidal and bacteriostatic properties, it does not have tissue-dissolving capacity compared with other irrigating solutions (20). Another study used the methyl thiazolyl tetrazolium (MTT) assay and reported that CHX and saline had minimum cytotoxicity for SCAP (21). Kim et al. (22) demonstrated that double antibiotic paste (DAP) without EDTA significantly decreased the adhesion of dental pulp stem cells (DPSCs) to dentin, which can be due to the residual cytotoxicity of DAP. Alternatively, it may be due to changes in surface topography of dentin, which may interfere with the adhesion of DPSCs (22). Another study reported that 1 to 100 mg/mL DAP had direct adverse effects on SCAP and dental pulp fibroblasts (23, 24).

A noteworthy issue is that all the aforementioned studies were conducted on single-culture media; while, stem cells in the periapical region of immature teeth with an apical lesion are not independent of other cells under in vivo conditions. However, inter-cellular interactions in co-culture can be generalized to the in vivo conditions. In indirect co-culture, the cells are independently cultured in cell culture inserts on porous membranes with 0.4 µm pore size and $2x10^6$ pores/cm to allow cell proliferation. This technique has higher reproducibility and reliability *in vitro* (25). Since the cytotoxic effects of intracanal medicaments used in endodontic regeneration have not been evaluated in co-culture under *in vitro* conditions, this study aimed to assess the cytotoxic effects of different intracanal

medicaments on periodontal ligament stem cells (PDLSCs) and SCAP in co-culture and mono-culture using the MTT assay.

## Materials and Methods

The PDLSCs and SCAP were isolated from the apical region of an immature impacted mandibular third molar of a 19-year-old patient after obtaining his written informed consent. The study was approved by the ethics committee of Zahedan University of Medical Sciences (IR.ZAUMS.REC.1398.150). The extracted tooth was immediately rinsed with sterile phosphate buffered saline (PBS; BRL, Grand Island, NY, USA) and stored in sterile saline. Using a scalpel, the periodontal ligament residues and the apical papilla were removed, and the tissues were diced and immersed in 5 mg/mL collagenase (Sigma Aldrich, France) at 37 °C for 1 h. The SCAP and the PDLSCs were isolated by enzymatic digestion. The cells were seeded on 25 $cm^2$ cell culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented by 15% fetal bovine serum and 1% penicillin/streptomycin, and incubated at 37 °C with 92.4% humidity and 5% $CO_2$. After a couple of days, adhesion of cells to the bottom of the flask was observed under an inverted microscope. After reaching 80% confluence, the cells were passaged. The contents of the flask were divided between three flasks. Third passage (or higher) cells were used for the experiment. The flasks were emptied from the culture medium and rinsed with 2-3 mL of FBS. Next, 700 λ to 1 mL of trypsin-EDTA was added to each flask. After 5 min, the adhered elongated cells transformed to round suspended cells. The cells were transferred into separate falcon tubes, and DMEM was added to neutralize trypsin. Next, 5 mL of DMEM was added to the flasks for the next passages.
Using a Neubauer chamber, the number of cells in each 1 mL of the suspension in falcon tubes was calculated. Next, PDLSCs were added to the 6 wells of a 24-well plate. SCAPs were added to another 6 wells. In the remaining 12 wells that had insert (SPL Life Science, Gyeonggi-do, South Korea), PDLSCs were placed at the bottom and SCAPs were added into the inserts. Each well contained 24,000 cells in 1 mL of culture medium. In the insert plates, 12,500 cells were at the bottom and 12,500 cells were placed in the insert along with 1 mL of the culture medium. After 24 h, the cells completely adhered to the wells and then the medicaments were added to the culture medium.

*Preparation of specimens:*
DAP composed of ciprofloxacin (Sigma Aldrich, France) and metronidazole (Sigma Aldrich, France) with 1:1 ratio was dissolved in distilled water. Also, combinations of DAP with 2% CHX (Cerkamed, Pawłowski, Poland) were prepared in 0.125, 0.250, and 1 mg/mL concentrations. CH powder (Sigma Aldrich, France) was also dissolved in distilled water and 2% CHX, and prepared in the abovementioned concentrations similar to DAP. According to ISO 1993, the medicaments were added to DMEM and incubated at 37 °C and 5% $CO_2$ for 48 h. They were then filtered through a 0.2 µm filter. The stem cells were treated with different concentrations of the medicaments. After 3 days, the plate was removed from the incubator and the inserts were placed in a different plate. The contents of the wells were emptied and replaced with 1 mL of the culture medium containing 10% MTT (5 mg/mL of MTT salt was dissolved in PBS and filtered using a 0.2 µm filter. It was then diluted 10 times by serum-containing culture medium). The plates were incubated for 4 h and then 1 mL of dimethyl sulfoxide (DMSO) was added to each well (the co-culture plates received 0.5 mL of DMSO since the number of cells at the bottom of the plate and in the insert was half). After several times of pipetting of each well to dissolve the formazan crystals, the optical density values were read by a spectrophotometer at 540 nm wavelength, and recorded.

Cytotoxicity was determined by estimating the percentage of viable cells, and the measurements were repeated in triplicate for each concentration. Single-cultured and co-cultured cells in DMEM without any medicament served as the control group.

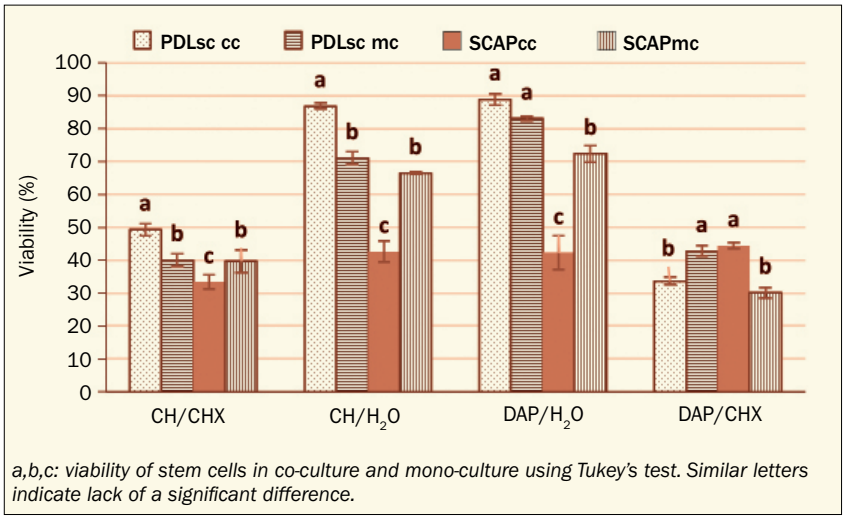
*Statistical analysis*

Statistical analysis of the data was performed by SPSS version 25. The mean and standard deviation of the percentage of viable PDLSCs and SCAP after exposure to different concentrations of medicaments were calculated and reported separately for the mono-culture and co-culture. Binary comparisons (stem cell types or culture types) were performed by independent t-test; while, multiple comparisons (different concentrations or different types of medicaments) were performed by one-way ANOVA. In case of significant results, pairwise comparisons were performed using the Tukey's post-hoc test. Level of significance was set at 0.05.

## Results

The results showed 100% viability of both SCAP and PDLSCs in the control group in both co-culture and mono-culture. Diagrams in figures 1-3 show the viability of SCAP and PDLSCs treated with different concentrations of medicaments in co-culture and mono-culture forms.

**Figure 1**
Comparison of stem cell viability in co-culture (cc) and mono-culture (mc) following exposure to 0.125 mg/mL concentration of medicaments (CH calcium hydroxide, CHX chlorhexidine, DAP Double Antibiotic Paste).



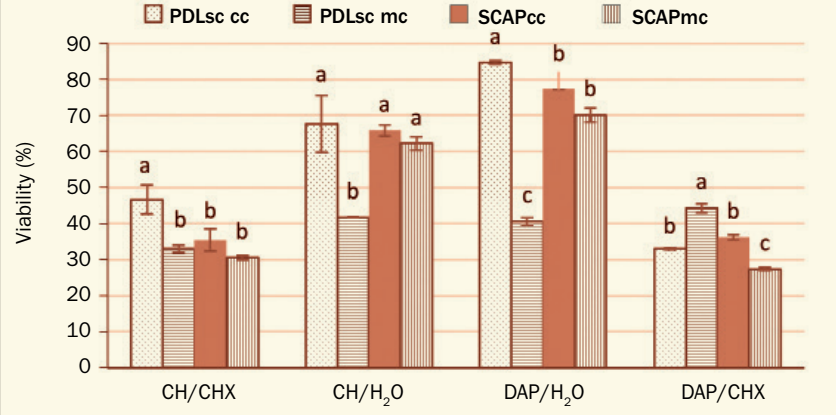

*a,b,c: viability of stem cells in co-culture and mono-culture using Tukey's test. Similar letters indicate lack of a significant difference.*

*Co-culture*

Increasing the concentration of CH/CHX in co-culture of PDLSCs significantly decreased their viability (P=0.047). However, this increase had no significant effect on the viability of SCAP (P=0.597). Also, in all concentrations, the viability of SCAP was significantly lower than that of PDLSCs (P=0.000). The current results showed that in co-culture, increasing the concentration of CH/distilled water had no significant effect on the viability of SCAP or PDLSCs (P>0.05). However, the viability of PDLSCs was higher than that of SCAP in all three concentrations (P=0.000). The current results showed that increasing the concentration of DAP/distilled water and DAP/CHX significantly decreased the viability of PDLSCs (P=0.004, P=0.003, respectively). However, this increase in concentration had no significant effect on the viability of SCAP (P>0.05). Both medicaments in all three concentrations significantly decreased the viability of SCAP compared with PDLSCs (P=0.000).

*Mono-culture*

In mono-culture, increasing the concentration of CH/CHX significantly decreased the viability of PDLSCs and SCAP (P=0.008 and P=0.003, respectively). Increasing the concentration of CH/distilled water significantly decreased the viability of PDLSCs only (P=0.016), and had no significant effect on the viability of SCAP (P>0.05). Also, the current results showed that increasing the concentration of DAP/distilled water significantly decreased the viability of both PDLSCs and SCAP (P=0.037 and P=0.021, respectively). However, a significant difference was noted in the viability of PDLSCs and SCAP only in 0.250 and 0.125 mg/mL concentrations (P=0.000 and P=0.01, respectively). This difference was not significant in 1 mg/mL concentration (P>0.05). Also, the current results showed that increasing the concentration of DAP/CHX significantly decreased the viability of PDLSCs (P=0.000) while it had no significant effect on the viability of SCAP (P>0.05). A significant difference was noted in the viability of




*a,b,c: viability of stem cells in co-culture and mono-culture using Tukey's test. Similar letters indicate lack of a significant difference.*

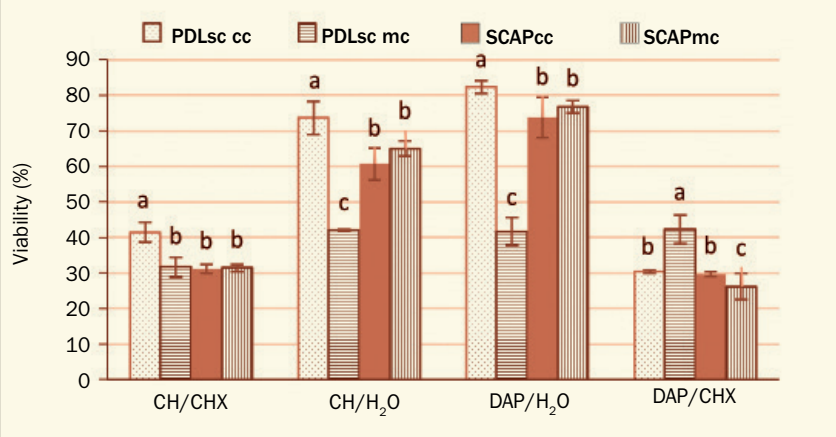
**Figure 2**
Comparison of stem cell viability in co-culture(cc) and mono-culture(mc) following exposure to 0.250 mg/mL concentration of medicaments (CH calcium hydroxide, CHX chlorhexidine, DAP Double Antibiotic Paste).

PDLSCs and SCAP only in 0.250 and 0.125 mg/mL concentrations (P=0.000) while this difference was not significant in 1 mg/mL concentration (P>0.05).

*Comparison of co-culture and mono-culture*

The viability of PDLSCs in different tested concentrations of medicaments was higher in co-culture than mono-culture. However, this difference in some concentrations was not significant (P>0.05). SCAP exposed to CH/distilled water and DAP/distilled water showed higher viability in mono-culture. However, this difference was not significant in exposure to CH/CHX (P>0.05). In exposure to DAP/CHX, cell viability in co-culture was higher than that in mono-culture (P=0.000) (Figures 1-3).

**Figure 3**
Comparison of stem cell viability in co-culture(cc) and mono-culture(mc) following exposure to 1 mg/mL concentration of medicaments (CH calcium hydroxide, CHX chlorhexidine, DAP Double Antibiotic Paste).




*a,b,c: viability of stem cells in co-cultureand mono-cultureusing Tukey's test. Similar letters indicate lack of a significant difference.*

## Discussion

Tissue engineering is a multidisciplinary science aiming to generate new tissue and regulation of the immune response (26) or immunomodulatory activity (27, 28) in order to restore the function of organs and injured tissues due to a disease condition or trauma (29). In tissue engineering, attention must be paid to three components namely the scaffold, signaling factors, and cells. All the available *in vitro* studies on endodontic regeneration have used mono-culture for this purpose. In other words, the heterogeneity of the cells in the periapical region has not been considered. Thus, co-culture was used in this study.

A successful endodontic treatment depends on optimal efficacy of mechanical and chemical debridement of the root canal system. Since mechanical instrumentation of infected canals in immature teeth is contraindicated due to immature root development and fragility of dentinal walls, chemical debridement is the main disinfection technique in endodontic regeneration (30).

In designing studies on cytotoxicity of materials, biocompatibility of chemical agents used as intracanal medicament is particularly important due to their direct contact with the periapical tissue, since they can induce cell cytotoxicity and cell death. On the other hand, cell type is another important factor to consider (31, 32). In this respect, dental mesenchymal stem cells, especially SCAP are specifically important. It should be noted that a cellular heterogeneity exists in the apical papilla due to the presence of cells other than the SCAP (33, 34). This heterogeneity is also seen among the stem cells and the markers they express (34). SCAPs are among these cells, which have a mesenchymal origin. Under *in vitro* conditions, they can differentiate into different cell lines and show very good clonal expansion capacity (35). SCAPs are of particular interest in endodontic regeneration due to their anatomical location at the site of root development (i.e. right next to the root end). Also, they are beneficial in cell delivery strategies (36). They can produce relatively higher and more homogenous den-

**Table 1**

**Comparison of viability of SCAP and PDLSCs in presence of different concentrations of medicaments in co-culture and mono-culture**

| Culture | Stem cells | Medicament | 0.125 mg/mL | | 0.250 mg/mL | | 1.00 mg/mL | | p value |
|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | Std. deviation | Mean | Std. deviation | Mean | Std. deviation | |
| Co-Culture | PDLsc | CH/CHX | 49.297$^{aC}$ | 1.827 | 46.653$^{aC}$ | 4.057 | 41.367$^{cC}$ | 2.804 | 0.047 |
| | | CH/$H_2O$ | 86.843$^{B}$ | 0.94 | 67.577$^{B}$ | 7.889 | 73.663$^{B}$ | 4.604 | 0.158 |
| | | DAP/H2O | 88.880$^{aA}$ | 1.677 | 84.667$^{bA}$ | 0.443 | 82.233$^{cA}$ | 1.859 | 0.004 |
| | | DAP/CHX | 33.677$^{aD}$ | 1.137 | 33.030$^{aD}$ | 0.191 | 30.370$^{bD}$ | 0.426 | 0.003 |
| | | **p value*** | 0.000 | | 0.001 | | 0.000 | | |
| | SCAP | CH/CHX | 33.350$^{B}$ | 2.245 | 32.987$^{C}$ | 0.975 | 31.593$^{B}$ | 2.789 | 0.597 |
| | | CH/$H_2O$ | 42.597$^{A}$ | 3.19 | 41.707$^{B}$ | 0.152 | 41.983$^{A}$ | 0.255 | 0.838 |
| | | DAP/$H_2O$ | 42.260$^{A}$ | 5.173 | 40.633$^{B}$ | 1.101 | 41.660$^{A}$ | 3.877 | 0.871 |
| | | DAP/CHX | 44.423$^{A}$ | 1.003 | 44.333$^{A}$ | 1.196 | 42.247$^{A}$ | 4.084 | 0.527 |
| | | **p value*** | 0.013 | | 0.000 | | 0.008 | | |
| Mono-Culture | PDLsc | CH/CHX | 40.047$^{aC}$ | 1.915 | 35.477$^{bC}$ | 3.116 | 31.117$^{cC}$ | 1.143 | 0.008 |
| | | CH/H2O | 70.933$^{aB}$ | 1.938 | 65.807$^{bB}$ | 1.496 | 60.720$^{bB}$ | 4.508 | 0.016 |
| | | DAP/H2O | 83.067$^{aA}$ | 0.726 | 77.263$^{bA}$ | 0.750 | 73.790$^{bA}$ | 5.627 | 0.037 |
| | | DAP/CHX | 42.787$^{aC}$ | 1.651 | 36.210$^{bC}$ | 0.706 | 29.677$^{cC}$ | 0.59 | 0.000 |
| | | **p value*** | 0.000 | | 0.000 | | 0.000 | | |
| | SCAP | CH/CHX | 39.677$^{aC}$ | 3.394 | 30.560$^{bC}$ | 0.577 | 31.380$^{bC}$ | 0.994 | 0.003 |
| | | CH/$H_2O$ | 66.390$^{B}$ | 0.34 | 62.173$^{B}$ | 1.877 | 65.043$^{B}$ | 2.131 | 0.051 |
| | | DAP/H2O | 72.317$^{bA}$ | 2.558 | 70.123$^{bA}$ | 1.887 | 76.783$^{aA}$ | 1.744 | 0.021 |
| | | DAP/CHX | 30.067$^{D}$ | 1.501 | 27.400$^{C}$ | 0.395 | 26.100$^{C}$ | 3.747 | 0.188 |
| | | **p value*** | 0.000 | | 0.000 | | 0.000 | | |

*P value: One-way ANOVA for the comparison of different concentrations.*
*P value*: One-way ANOVA for the comparison of different intracanal medicaments.*
*a,b,c: comparison of concentrations regarding cytotoxicity by Tukey's post-hoc test. Similar letters indicate lack of a significant difference.*
*A,B,C: comparison of medicaments regarding cytotoxicity by Tukey's epost-hoc test. Similar letters indicate lack of a significant difference.*

tin-like tissue (35). Also, they have higher proliferation rate, dentin regeneration capacity and cell motility than DPSCs (33). PDLSCs are a type of somatic stem cells that have the potential to differentiate into various cell types. Moreover, they have strong self-renewal capacity. Thus, they are considered a promising population of stem cells for periodontal regenerative treatments. Moreover, PDLSCs are proliferated easier and faster and are more efficacious than other somatic stem cells (37). Thus, two different types of stem cells namely SCAP and PDLSCs in co-culture and mono-culture forms were used in this study, which further adds to the uniqueness of this study (Tables 1 and 2).

Our results indicated that in co-culture, the concentration of CH/CHX had no significant effect on the viability of the two types of stem cells. However, at all three concentrations, SCAP showed significantly lower viability than PDLSCs. It has been demonstrated that addition of CHX to CH significantly increases the cytotoxicity of CH (38). Our findings confirmed this statement.

Our results indicated that in mono-culture, the viability of SCAP and PDLSCs treated with different concentrations of CH/CHX was not significantly different. In other

**Table 2**

**Comparison of cell viability in co-culture and mono-culture following exposure to different intracanal medicaments**

| Medicament | Stem cells | 0.125 mg/mL | | 0.250 mg/mL | | 1.00 mg/mL | |
|---|---|---|---|---|---|---|---|
| | | Mean | Std. deviation | Mean | Std. deviation | Mean | Std. deviation |
| CH/CHX | PDLSCs cc | 49.297 | 1.827 | 46.653 | 4.057 | 41.367 | 2.804 |
| | PDLSCs mc | 40.047 | 1.915 | 35.477 | 3.116 | 31.117 | 1.143 |
| | **p value*** | 0.007 | | 0.004 | | 0.002 | |
| | SCAP cc | 33.350 | 2.245 | 32.987 | 0.975 | 31.593 | 2.789 |
| | SCAP mc | 39.677 | 3.394 | 30.560 | 0.577 | 31.380 | 0.994 |
| | **p value*** | 0.051 | | 0.680 | | 0.999 | |
| CH/H2O | PDLSCs cc | 86.843 | 0.940 | 67.577 | 7.889 | 73.663 | 4.604 |
| | PDLSCs mc | 70.933 | 1.938 | 65.807 | 1.496 | 60.720 | 4.508 |
| | **p value*** | 0.000 | | 0.995 | | 0.007 | |
| | SCAP cc | 42.597 | 3.190 | 41.707 | 0.152 | 41.983 | 0.255 |
| | SCAP mc | 66.390 | 0.340 | 62.173 | 1.877 | 65.043 | 2.131 |
| | **p value*** | 0.000 | | 0.091 | | 0.000 | |
| DAP/$H_2O$ | PDLSCs cc | 88.880 | 1.677 | 84.667 | 0.443 | 82.233 | 1.859 |
| | PDLSCs mc | 83.067 | 0.726 | 77.263 | 0.750 | 73.790 | 5.627 |
| | **p value*** | 0.165 | | 0.000 | | 0.084 | |
| | SCAP cc | 42.260 | 5.173 | 40.633 | 1.101 | 41.660 | 3.877 |
| | SCAP mc | 72.317 | 2.558 | 70.123 | 1.887 | 76.783 | 1.744 |
| | **p value*** | 0.000 | | 0.000 | | 0.000 | |
| DAP/CHX | PDLSCs cc | 33.677 | 1.137 | 33.030 | 0.191 | 30.370 | 0.426 |
| | PDLSCs mc | 42.787 | 1.651 | 36.210 | 0.706 | 29.677 | 0.590 |
| | **p value*** | 0.000 | | 0.003 | | 0.990 | |
| | SCAP cc | 44.423 | 1.003 | 44.333 | 1.196 | 42.247 | 4.084 |
| | SCAP mc | 30.067 | 1.501 | 27.400 | 0.395 | 26.100 | 3.747 |
| | **p value*** | 0.000 | | 0.000 | | 0.000 | |

*P value*: independent t-test for the comparison of the two culture types*

words, both cell types showed similar response to the cytotoxic effects of these medicaments. A previous study showed that CH and CH reinforced with CHX in 0.016 to 0.00025 percent concentrations inhibited the proliferation of L929 fibroblasts. CH reinforced with CHX and CH powder in 0.016 percent concentration showed 75% and 45% cytotoxicity at 72 h, respectively (39).

This study showed that in mono-culture, the viability of PDLSCs treated with CH/distilled water significantly decreased by an increase in concentration. The difference in this respect was not significant between the two cell types. But, in co-culture, increasing the concentration had no significant effect on cell viability. However, PDLSCs showed significantly higher viability than SCAP in all concentrations. Increased proliferation of both DPSCs and PDLSCs in co-culture has been previously shown. Also, the expression of mRNA of dentin sialophosphoprotein increases in co-culture of PDLSCs and DPSCs. Immunohistochemical analysis showed overexpression of dentin sialoprotein in both cell types in co-culture compared with mono-culture. Prolonging the culture period further increased the expression of dentin

sialoprotein. The expression of osteopontin also increased in both cell types in co-culture (40).

Evidence shows that CH has cytotoxic effects on 3T3 fibroblasts after 48 h; this effect was cytostatic and reversible such that after 7 days of incubation, cell proliferation returned to normal (41). Another study demonstrated that after 3 days of incubation, all tested materials were cytotoxic for DPSCs compared with the control group. Mineral trioxide aggregate with 77% cell viability had minimum cytotoxicity while CH with 26% and Biodentine with 16% cell viability had the lowest cell viability compared with the control group. Also, microscopic assessments showed that a reduction in the number of cells and morphological changes in treated cells occurred with all tested medicaments, particularly CH compared with the control group (42). Moreover, in contrast to the results of this study, it was reported that CH/distilled water had the significantly lowest cytotoxicity for the mono-culture of SCAP (38). Although the exact mechanism of cytotoxicity of these medicaments has yet to be fully understood, primary release of calcium ions, ionic activity and release of toxic components, and changes in pH of the tested material can all affect cell behavior (43).

Furthermore, our results indicated that SCAP treated with DAP/CHX in mono-culture had the significantly lowest cell viability compared with the control group. A previous study showed that in WST-1 assays, all antibiotic dilutions except for 0.125 mg/mL concentration significantly decreased cell viability while in the lactate dehydrogenase (LDH) assays, minimum tested concentration of DAP (0.5, 0.25, 0.125 mg/mL) and minimum concentration of triple antibiotic paste (0.25, and 0.125 mg/mL) were not cytotoxic for DPSCs (44). Also, it was shown that addition of CHX to modified triple antibiotic paste significantly decreased the viability of SCAP (38). This difference in the results can be attributed to the methods employed for assessment of cytotoxicity and type of stem cells. Our results revealed that in co-culture, increasing the concentration of DAP/distilled water significantly decreased the viability of PDLSCs. However, in all concentrations, SCAP showed significantly lower viability than PDLSCs. But, in mono-culture, increasing the concentration of DAP/distilled water significantly decreased the viability of both cell types. In 0.250 and 0.125 mg/mL concentrations, the viability of PDLSCs was significantly higher than that of SCAPs. Sabrah et al. (44) evaluated the effect of DAP in 0.125, 0.25, 0.5, 1 and 10 mg/mL concentrations using the LDH and cell viability assays and concluded that in all antibiotic concentrations except for 0.125 mg/mL, the viability of DPSCs significantly decreased. Also, LDH assays revealed that low (0.5, 0.25 and 0.125 mg/mL) concentrations of DAP were non-toxic for DPSCs (44). Another study showed that DAP in 1, 10 and 100 mg/mL concentrations had cytotoxic effects on the viability of SCAP and significantly decreased their viability (23).

Our results showed that the viability of PDLSCs in co-culture was higher than that in mono-culture; although this difference was not significant following exposure to DAP/distilled water. In contrast, the viability of SCAP in mono-culture was higher than that in co-culture, except for exposure to DAP/CHX, where cell viability was significantly higher in co-culture. The indirect co-culture technique was employed in this study. Indirect co-culture techniques are systems in which, two or more distinct cell types are cultured in the same environment.However, the cell culture environments are physically apart. This physical separation is often performed by using a Transwell or a well insert/Boyden chamber. Indirect co-cultures are as simple as monolayer cultures and are easily controlled. Despite separation of cells, they allow inter-cellular interactions to continue. Thus, they are often used to study some particular aspects such as cellular interaction mechanisms and cell behavior. It seems that SCAP in co-culture may protect the PDLSCs by signaling via the soluble factors. However, this theory is in need of further investigations. Indirect co-culture allows the cells to reside in environments with the same architecture as the natural

target tissue specific for each cell type and at the same time have efficient communication with other cells. Signaling in indirect co-culture between the cells occurs through paracrine signaling by use of soluble factors while physical separation does not allow the cells to have direct interactions (45). Also, paracrine signaling is an important factor for behavioral regulation of stem cells and terminally differentiated cells in the co-culture (46).

## Conclusions

Despite the limitations of this *in vitro* study, the results showed that addition of CHX to CH and DAP significantly decreases the viability of PDLSCs and SCAP in both co-culture and mono-culture. Therefore, the use of low concentrations of intracanal medicament is highly recommended.

## Clinical Relevance

Inftracanal medicaments and the viability of dental mesenchymal stem cells play a important role in endodontic regeneration, so in invitro studies, the use of co-culture media mimics more invivo conditions.

## Conflict of Interest

None.

## Acknowledgements

None.

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