Vol 10 No 1 (2022) DOI 10.5195/d3000.2022.146

Mode of cell death of co-cultured dental mesenchymal stem cells following exposure to endodontic irrigating solutions

Eshagh Ali Saberi, Narges Farhad Mollashahi, Mahboobe Ahmadi, Arezoo Pirhaji

Department of Endodontics, Faculty of Dentistry, Oral and Dental Diseases Research Center, Zahedan University of Medical Sciences, Zahedan, Iran.

Abstract

Aims: This in vitro study aimed to determine the mode of cell death of co-cultured dental mesenchymal stem cells following exposure to different irrigating solutions.

Methods and Material: Mesenchymal stem cells of the apical papilla (SCAPs) and periodontal ligament stem cells (PDLSCs) were isolated from the periapical region of a third molar of an 18-year-old patient and cultured. Adequate number of cells in each well, 6-well plate inserts were placed over the wells. The cells were then randomly exposed to 17% ethylenediaminetetraacetic acid (EDTA), 1.5% sodium hypochlorite (NaOCL), 2% chlorhexidine (CHX) and saline. After 1, 5, and 10 minutes cells were trypsinized and underwent flow cytometry. Apoptotic and necrotic cells were quantified. Data were analyzed using the One-way ANOVA and Tukey's post-hoc test.

Results: With increasing exposure time, cell viability is decrease and the mode of cells death was necrosis in CHX and NaOCL groups and necrosis plus late apoptosis in 1 and 10 minutes in EDTA group.

Conclusion: Maximum cell death occurred following exposure to EDTA while minimum cell death occurred following exposure to CHX. Necrosis was the dominant mode of cell death in all groups.

Keywords: Co-culture; Apoptosis; Necrosis; Irrigants; Cells; Cultured.

Citation: Ali Saberi E, et al. (2022) Mode of cell death of co-cultured dental mesenchymal stem cells following exposure to endodontic irrigating solutions Dentistry 3000. 1:a001 doi:10.5195/d3000.2022.146
Received: February 5, 2021
Accepted: August 10, 2021
Published: March 21, 2022

Email: saberiendo@yahoo.com

INTRODUCTION

The mode of cell death is often divided into two groups of apoptosis (programmed cell death) and necrosis (accidental cell death) for the purpose of simplification. However, a more advanced classification defines 11 types of cell death such as apoptosis, necrosis, autophagy, oncosis, pyroptosis, etc. Numerous biochemical and morphological differences exist between apoptosis and necrosis. Apoptosis is a coordinated biochemical process that leads to cell death and is classified into three types of apoptosis, apoptosis-like and necrosis-like cell death [1]. In this process, the cell, itself, is responsible for its death; thus, it is also referred to as cell suicide. The characteristics of an apoptotic cell include condensed chromatin in cell nucleus, DNA fragmentation, transfer of phosphatidylserine from the inner half to the outer half of the cell membrane, membrane ballooning and production of apoptotic bodies. In necrosis, all internal cellular organelles, especially the mitochondria, are swollen, and disintegration of cell membrane and cell lysis occur. Cell death by necrosis is associated with extensive tissue destruction. Apoptosis can be evaluated by a number of techniques such as evaluation of cytotoxicity and morphological changes, DNA laddering, flow cytometry and the TUNEL test [2].

The basic principles of flow-cytometry are based on the fact that in initial phases of apoptosis, phosphatidylserine that is naturally located in the inner half of the membrane is transferred to the outer half of membrane due to the impaired activity of ATP-dependent

Vol 10 No 1 (2022) DOI 10.5195/d3000.2022.146

translocase enzyme or activation of other enzymatic systems such as scramblase. The transfer of phosphatidylserine to the outer half of the cell membrane is a natural signal for detection of apoptotic cells by the macrophages and adjacent cells [1,2].

Evidence shows that annexin V, which is an anticoagulation protein with 35 KD molecular weight, has high affinity for binding to phosphatidylserine in presence of calcium. Fluorescein isothiocyanate (FITC)-conjugated annexin V is used for quantification of apoptotic cells by flow cytometry [3]. The membrane integrity is lost and the cell membrane becomes highly permeable in necrotic cells. Thus, the propidium iodide fluorescent intercalating agent can pass through the membrane and bind to DNA. In apoptotic cells, the membrane integrity is preserved. Thus, the propidium iodide cannot enter the cells. On the other hand, phosphatidylserine has been transferred to the outer half of the cell membrane in these cells and annexin binds to it. Thus, by simultaneous staining of cells with these two compounds, apoptotic and necrotic cells can be differentiated [4].

Regeneration is a biological process to replace structures such as dentin, root and dentin-pulp complex. Disinfection of the root canal system is an important step in the process of regeneration. Ideal disinfecting solutions should have excellent antimicrobial properties and minimal cytotoxicity [5]. Evidence shows that irrigating solutions have cytotoxic effects on stem cells and gingival fibroblasts. mollashahi et al. evaluated the cytotoxic effects of commonly used endodontic irrigating solutions on stem cells of the apical papilla (SCAPs). They found that chlorhexidine (CHX) had the highest cytotoxicity followed by NaOCL, QMIX, EDTA, MTAD and saline [6]. Barnhart et al. demonstrated the cytotoxic effects of irrigating solutions on gingival fibroblasts [7]. Most studies on cytotoxicity have used single culture of cells while stem cells are not independent of other cells in vivo. Cell-to-cell interactions in the culture media in vitro are highly complex and play an important role in the scaffold in tissue regeneration [8].

To the best of the authors' knowledge, no previous study has evaluated the mode of cell death following exposure to different endodontic irrigating solutions. Thus, this study aimed to assess the mode of cell death of co-cultured stem cells of the apical papilla(SCAPs) and periodontal ligament stem cells (PDLSCs) following exposure to commonly used endodontic irrigating solutions.

MATERIALS AND METHODS

This study was approved by the ethics committee of Zahedan University of Medical Sciences (IR.ZAUMS.REC.1397.280). The stem cells of the apical papilla and periodontal ligament stem cells were isolated from the periapical region of a third molar tooth of an 18-year-old donor. Two-thirds of the root had been formed and the cells were isolated using enzymatic digestion. Written informed consent was obtained from the donor. Immediately after tooth extraction, the tooth was rinsed with phosphate buffered saline (PBS; Gibco BRL, Grand Island, NY, USA) and stored in a sterile solution. The tooth was then decoronated at the cementoenamel junction and its PDL and apical papilla were removed. The tissues were diced into small pieces and immersed in 5 mg/mL of dispase II (Invitrogen, Karlsruhe, Germany) at 37°C for one hour. Single-cell culture was obtained by filtering the solutions using a 70 nm cell filter (BD Biosciences, Heidelberg, Germany). The cells were transferred to cell culture flask with Dulbecco's modified Eagle's medium containing 15% fetal bovine serum and 1% penicillin/streptomycin and incubated in presence of 95% O2/5% CO2. The solution was refreshed every 2 days and after reaching 80% cell confluence at the bottom of the flask, the cells were passaged and the flask contents were distributed among three flasks. Third passage cells were used for the next experiments (figure 1).

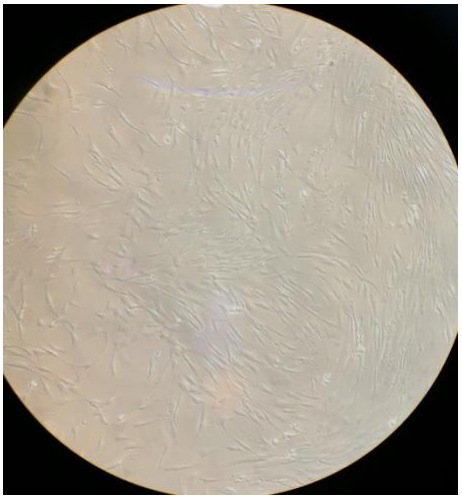
Characterization of isolated cells

In order to Characterization of isolated cells, expression of CD105, CD45, CD146, CD73, STRO-1, and CD90 surface markers measured by

Vol 10 No 1 (2022) DOI 10.5195/d3000.2022.146

flow-cytometry. For this purpose, after counting the cells, they were fixed in 4% paraformaldehyde at 4°C for 30 min and permeabilized using 0.2% triton-X100 for 30 min. The fixed cells were then rinsed with PBS, and incubated in primary antibodies (Table 1) diluted in 10 mg/mL BSA for overnight. Finally, following incubation with secondary antibodies (Table 1) at 37°C for 2 h, the expression analysis were performed by a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed by WinMDI 2.9 software.

Figure 1. Inverted microscopic photographs of the morphology of mesenchymal stem cells



Assessment of cell death

The insert plates (0.4 µm; SPL Life Science, Gyeonggi-do, South Korea) were used for treatment of the cells. After culturing the cells to reach adequate cell count in each well of a 6-well plate, the inserts were placed over the wells. stem cells of the apical papilla were placed at the top layer while periodontal ligament stem cells were placed at the bottom of the plates (80,000 cells per well) [9]. The wells were then randomly divided into 4 groups for treatment of cells with 2% CHX (Clorhexidina S, Dentscare LTDA, Joinville, SC, Brazil), 17% EDTA (MD-cleanser, Meta Biomed, Chungju, Korea), 1.5 % NaOCL (Bojneh, Tehran, Iran) and saline. The cells were subjected to flow cytometry (Becton Dickinson) after 1, 5 and 10 minutes of exposure to the irrigating solutions. After treatment of the cells, the overlaying medium was removed and after trypsinization and removal of trypsinized cells, the cell suspension was transferred into a tube for 15 minutes and was then centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and the cells were washed with PBS. Next, 500 µL of the bonding buffer present in the kit and 100 µL of annexin V + FITC were added and the mixture was incubated at room temperature for 15 minutes. The additional dye was washed with PBS, and 5 µL of propidium iodide fluorescent intercalating agent was also added to the cell suspension and around 10,000 cells underwent flow cytometry. Annexin V + FITC creates green fluorescence and is used for quantification of apoptotic cells. The propidium iodide with red fluorescence is used for quantification of necrotic cells and their differentiation from apoptotic cells. The FL1 filter was used for annexin and the FL3 filter was used for propidium iodide. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA) software (latest version). The mean and standard deviation of the number of apoptotic and necrotic cells in the groups at different time points were calculated and reported. Pairwise comparisons at different time points were carried out using the One-way ANOVA and Tukey's post-hoc test. Type one error (alpha) was considered to be 0.05 and each test was repeated in triplicate.

RESULTS

The expression of specific mesenchymal stem cell markers, including CD146, STRO-1, CD105, CD90, and CD73 as well as a hematopoietic stem cell marker (CD45) at the passage 4 revealed a substantial expression of all MSC-related markers. Figure 2 exhibited a representative histogram for each protein approved that over 95% of the cells expressed mesenchymal cell markers (CD73, CD90, CD105) and around 90% of the cells expressed dental stem cell markers (CD146, STRO-1). However, the cells did not express CD45 as a prominent hematopoietic stem cell marker (0.45%).

The present results revealed that over 95% of SCAPs and PDLSCs in the control and saline groups were viable in the co-culture, and no significant difference was noted in their viability. The passage of time had no significant effect on their viability either.

Vol 10 No 1 (2022) DOI 10.5195/d3000.2022.146

Table 1. Antibodies used for identification of mesenchymal cells

Name	Host	Company	Cat-No
Anti h- CD105	Mouse	Millipore	MABT117
Anti h –CD45	Mouse	Millipore	05-1413
Anti h –CD146	Mouse	Chemichon	MAB16985
Anti h– CD73	Mouse	Chemichon(Millipore)	MABD122
Antih –STRO-1	Mouse	Millipore	MAB4315
Anti h – CD90	Mouse	Millipore	CBL415
Anti h-Mouse IgM-FITC	Goat	MP Biomedical	672311
Antih-Mouse IgG-PE	goat	sigma	P9670

Figure 2. Expression of mesenchymal stem cell markers. Representative histogram of the expression of CD105, CD90, CD73, CD146, STRO-1 and CD45. Green histogram indicates cell fluorescence with control antibody (isotype control) and purple histogram indicates cell fluorescence with the respective antibody.

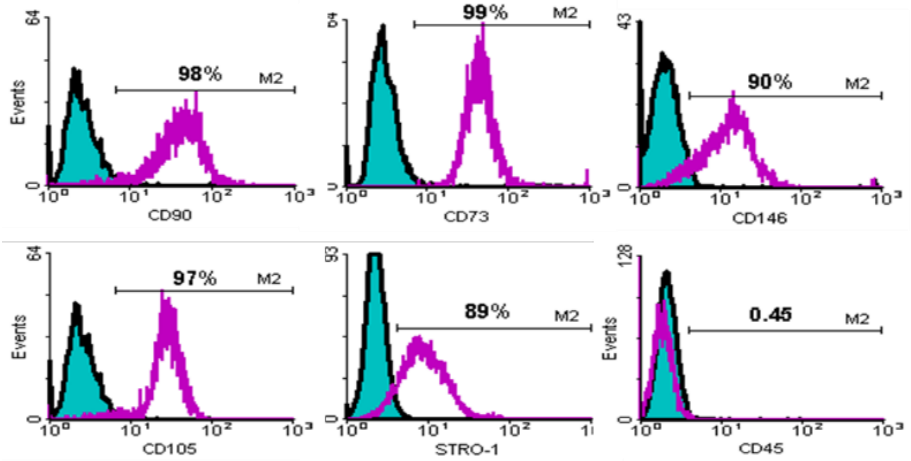


Table 2 and 3 and figure 3 and 4 shows the viability and mode of cell death of stem cells treated with different irrigating solutions over time.

The results showed that increasing the exposure time significantly decreased the viability of stem cells treated with CHX, sodium hypochlorite and EDTA. The majority of the cells underwent necrosis, except in the CHX group in which, PDLSCs underwent early apoptosis at 1 and 10 min.

Vol 10 No 1 (2022) DOI 10.5195/d3000.2022.146

By an increase in exposure time, the necrotizing effect of sodium hypochlorite and EDTA increased on stem cells; although no significant difference was noted between sodium hypochlorite and EDTA at 10 min. In general, the cytotoxicity of the irrigating solutions was as follows:

Control group=saline<2% CHX<1.5% NaOCL<17% EDTA

Table 2. Cell viability,early apoptosis, late apoptosis and necrosis in co-cultured PDLSCs at different times of treatment with endodontic irrigating solutions.

Exposure time (minute)	Group	total number of cells	Viable cells	Early Apoptosis	Late Apoptosis	Necrosis
			Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
1	Control	80000	78632^{a}±1.00	432^{b}±59.2	80^{b}±56	848^{c}±256
	Saline	80000	79504^{a}±176	144^{b}±120	32^{b}±24	320^{c}±120
	CHX	80000	76360^{a}±106	1864^{a}±1296	960^{b}±280	816^{c}±512
	NaOCL	80000	53248^{b}±2128	2280^{a}±200	3584^{a}±320	20992^{b}±2448
	EDTA	80000	45112^{b}±1632	184^{b}±96	168^{b}±8	34488^{a}±1728
	P value		0.00	0.00	0.00	0.00
5	Control	80000	78632^{a}±1008	432^{b}±592	80^{b}±56	848^{b}±1056
	Saline	80000	78888^{a}±568	288^{b}±216	48^{b}±8	784^{b}±728
	CHX	80000	70448^{b}±3944	3784^{a}±4896	824^{a}±696	4704^{b}±3488
	NaOCL	80000	45848^{c}±1832	48^{b}±16	1568^{a}±48	31888^{a}±1736
	EDTA	80000	45928^{c}±2408	1736^{a}±40	64^{b}±16	32192^{a}±2360
	P value		0.00	0.00	0.00	0.00
10	Control	80000	78632^{a}±1008	432^{b}±592	80^{b}±56	848^{b}±1056
	Saline	80000	77896^{a}±72	56^{b}±16	48^{b}±0.00	2000^{b}±48
	CHX	80000	63984^{b}±3360	9800^{a}±7936	1992^{b}±2280	4216^{b}±5176
	NaOCL	80000	38704^{c}±1512	536^{b}±472	2192^{b}±104	38480^{a}±1184
	EDTA	80000	18288^{c}±2080	216^{b}±24	14288^{a}±520	47512^{a}±1208
	P value		0.00	0.043	0.00	0.00

SD: Standard deviation; P value: One-way ANOVA.for the comparison of irrigating solutions; a,b,c: comparison of irrigating solutions regarding mode of cell death by Tukey's post-hoc test. Similar letters indicate lack of a significant difference.

Table 3. Cell viability,early apoptosis, late apoptosis and necrosis in co-cultured SCAPs at different times of treatment with endodontic irrigating solutions.

Vol 10 No 1 (2022) DOI 10.5195/d3000.2022.146

Exposure time(minute)	Group	total number of cells	Viable cells	Early Apoptosis	Late Apoptosis	Necrosis
			Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
1	Control	80000	79392^{a}±208	240^{b}±72	48^{c}±40	152^{c}±184
	Saline	80000	78800^{a}±840	232^{b}±64	80^{c}±64	816^{c}±864
	CHX	80000	65832^{b}±1640	760^{b}±64	1376^{c}±416	11640^{b}±1440
	NaOCL	80000	57976^{b}±1080	6008^{a}±432	6616^{b}±200	9096^{b}±696
	EDTA	80000	31832^{c}±1592	1032^{b}±328	22528^{a}±464	24400^{a}±1208
	P value		0.00	0.00	0.00	0.00
5	Control	80000	79392^{a}±208	240^{b}±72	48^{c}±40	152^{d}±184
	Saline	80000	77016^{a}±728	344^{b}±336	192^{c}±272	2520^{d}±768
	CHX	80000	60080^{b}±600	544^{b}±40	4024^{b}±40	15112^{c}±416
	NaOCL	80000	48992^{b}±680	3456^{a}±464	1368^{c}±64	25528^{b}±496
	EDTA	80000	28472^{c}±872	3608^{a}±40	6880^{a}±288	40664^{a}±608
	P value		0.00	0.00	0.00	0.00
10	Control	80000	79392^{a}±208	240^{b}±72	48^{b}±40	152^{c}±184
	Saline	80000	76216^{a}±920	192^{b}±56	72^{b}±56	3240^{c}±760
	CHX	80000	53512^{b}±3176	5808^{a}±4728	4360^{b}±360	16104^{b}±1600
	NaOCL	80000	36720^{c}±1944	136^{b}±8	800^{b}±8	42760^{a}±2136
	EDTA	80000	16008^{d}±1208	904^{b}±56	28888^{a}±464	33904^{a}±792
	P value		0.00	0.00	0.00	0.00

SD: Standard deviation; P value: One-way ANOVA.for the comparison of irrigating solutions; a,b,c: comparison of irrigating solutions regarding mode of cell death by Tukey's post-hoc test. Similar letters indicate lack of a significant difference.

Figure 3. Flow cytometry graph of annexin/pi for EDTA treated PDLSCs: (A) control group, (B) after 1 minute, (C) after 5 minutes, (D) after 10 minutes. In this graph, the upper left (annexin -/ pi +) indicates the necrotic cells, the lower left (annexin -/ pi -) indicates the viable cells, the upper right (annexin +/ pi +) indicates the late apoptotic cells, and the lower right (annexin +/ pi -) indicates the early apoptotic cells.

Vol 10 No 1 (2022) DOI 10.5195/d3000.2022.146

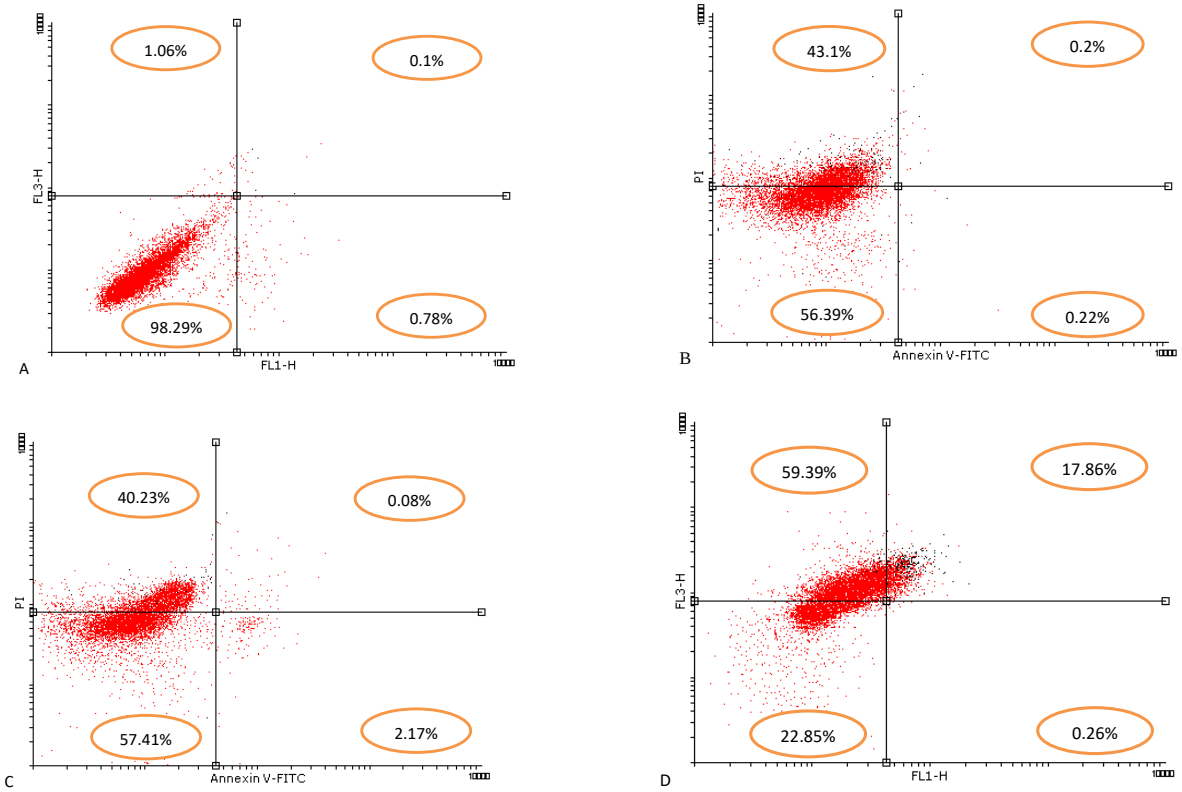
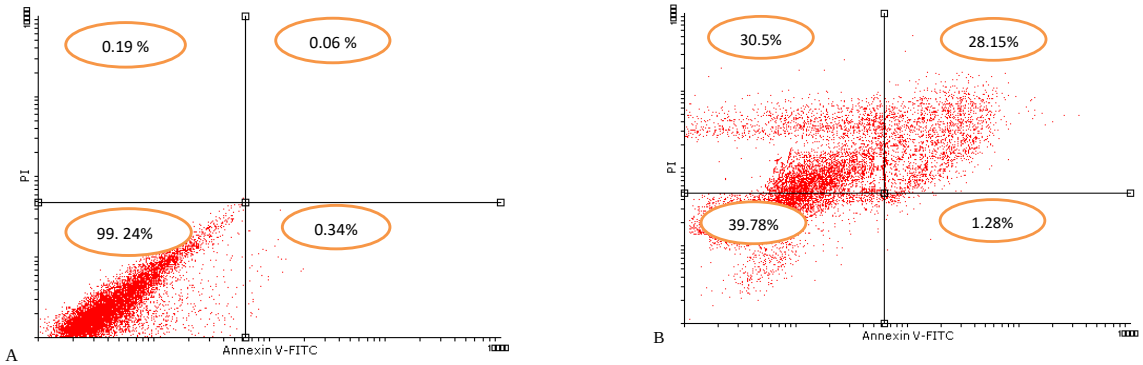
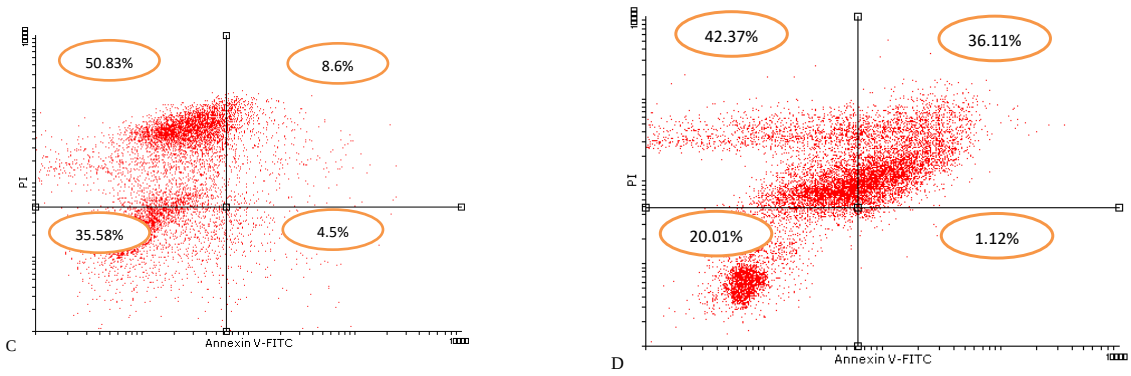


Figure 4. Flow cytometry graph of annexin/pi for EDTA treated SCAPs: (A) control group, (B) after 1 minute, (C) after 5 minutes, (D) after 10 minutes. In this graph, the upper left (annexin -/ pi +) indicates the necrotic cells, the lower left (annexin -/ pi -) indicates the viable cells, the upper right (annexin +/ pi +) indicates the late apoptotic cells, and the lower right (annexin +/ pi -) indicates the early apoptotic cells.



Vol 10 No 1 (2022) DOI 10.5195/d3000.2022.146



DISCUSSION

This study compared the mode of cell death of co-cultured cells following exposure to four different endodontic irrigating solutions namely 2% CHX, 17% EDTA, 1.5% NaOCL and saline after different exposure times. The number of viable cells decreased in all groups following an increase in exposure time. Necrosis was the dominant mode of cell death in groups treated with CHX, NaOCL and EDTA. Many studies have evaluated the cytotoxicity of different endodontic irrigating solutions against single culture of cells [3,6,10-12]. In this study, we evaluated the mode of cell death of co-cultured cells following their exposure to different irrigating solutions. Evaluation of co-culture of cells was the main difference between our methodology and that of previous studies. Use of a co-culture of cells helps in assessment of the role of cell interactions and their mutual effects on each other. Also, it is a strong tool in tissue engineering and plays a role in regeneration of tissues and organs. It is also effective in guiding and protecting the process of tissue regeneration due to interactions of different cells. The interaction of cells plays an important role in regeneration of tissues and organs, and affects cell mitosis, differentiation and physiology [13,14].

The cytotoxic effects of NaOCL on L929 fibroblasts [15], human dental pulp stem cells [16] and SCAPs [17] have been previously confirmed. Our results revealed that necrosis was the dominant mode of cell death following exposure to 1.5% NaOCL such that after 10 minutes of exposure, almost half of the cells died of necrosis. Alkahtani et al. showed that 5% NaOCL induced necrosis of mesenchymal stem cells of the bone marrow. Light microscopic assessment revealed that in addition to a reduction in number of viable cells after 2 hours of treatment with NaOCL, the remaining cells were smaller and rounder. The cells had lost their inter-cellular attachments and their cell wall had been disintegrated. These are typical characteristics of necrotic cells. On the other hand, flow cytometric assessment of cells exposed to NaOCL indicated positive response to both propidium iodide and annexin dyes [11]. Their results were in agreement with our findings. Longo et al. indicated that 2% NaOCL caused apoptosis of human KB cells, which was in contrast to our findings. Apoptosis is attributed to generation of free radicals. The chloroamination reactions cause generation of free radicals such as hydroxyl ions, that can cause an alkaline environment and accelerate biological stresses in different parts of the cells. Thus, the difference in cell response depends on the concentration of hypochlorite and can be related to higher production of free radicals by cells exposed to 2% NaOCL. Free radicals damage the cell DNA and can cause chromosomal injury [18].

The current results revealed that necrosis was the dominant mode of cell death following exposure to different endodontic irrigating solutions. Necrosis is the passive form of cell death that occurs as the result of cell membrane rupture and release of cell components into the extracellular matrix [19]. However, apoptosis is the active form of cell death characterized by cell shrinkage, condensation of nuclear chromatin and fragmentation of the nucleus [20]. Apoptotic cells, in contrast to necrotic cells, are quickly phagocytosed in vivo without causing inflammatory reactions. For this reason, cell death by apoptosis and necrosis are biologically different. After cell necrosis, severe inflammatory reactions occur, causing severe tissue damage [21].

The present results revealed minimum cell death in SCAPs treated

Vol 10 No 1 (2022) DOI 10.5195/d3000.2022.146

with CHX in the co-culture at 10 min, compared with sodium hypochlorite and EDTA. The mode of cell death was mainly necrosis in them, followed by early apoptosis; whereas, PDLSCs treated with CHX mainly underwent early apoptosis at 1 and 10 min. Hernandez et al. demonstrated that combination of 0.12% CHX and ProRoot MTA induced apoptosis in macrophages and fibroblasts while combination of ProRoot MTA and distilled water could not induce apoptosis. They assessed the cell cycle and concluded that CHX increased the ratio of cells in G1 phase and decreased the ratio of cells in S phase. S phase is the DNA transcription phase and cell mitosis occurs in this phase. In fact, combination of CHX and ProRoot MTA had anti-proliferative effects on the cells [22]. Newton et al. showed that CHX ruptures the external membrane of the mitochondria and releases apoptosis-inducing proteins such as cytochrome C [23]. Faria et al. evaluated apoptosis and necrosis following the use of CHX on L929 fibroblasts using flow cytometry after 24 hours. They used 0.000125% to 0.016% concentrations of CHX and the results showed that concentrations higher than 0.004% of CHX caused cell death by necrosis while lower concentrations induced apoptosis. They suggested increased expression of heat-shock protein as an indicator of cellular stress [15]. Their results were in agreement with our findings since in our study, the dominant mode of cell death following exposure to 2% CHX was necrosis. li et al, demonstrated that CHX had cytotoxic effects on RAW264.7 macrophages in a dose-dependent and time-dependent manner. Type of cell death shifted from apoptosis to necrosis by an increase in concentration of CHX, and dose-dependent DNA damage occurred [24]. Giannelli et al. reported that CHX induced apoptosis and necrosis of osteoblasts, endothelial cells and fibroblasts. The viable cell count depends on the dosage and duration of exposure to CHX. Apoptosis and necrosis are both characterized by impaired mitochondrial function, increased intracellular calcium level and oxidative stress [25].

Our results indicated death of around 80% of the cells 10 minutes after exposure to EDTA; necrosis occurred in 2/3 and late apoptosis occurred in 1/3 of the cells. Following an increase in concentration of intracellular calcium due to the activity of Ca ATPase pump, or increased entry of calcium into the nucleus and mitochondrial organelles, caspases are activated, inducing apoptosis [26]. Many studies have suggested the correlation of apoptosis with cationic ions. Sakabe et al. showed that EDTA induced apoptosis of neuro-2A cells and fragmentation of DNA [27]. Ostby demonstrated that exposure of periapical tissues to 17% EDTA did not cause any cell or tissue damage. Moreover, they did not report any cell necrosis following exposure of dental pulp cells to EDTA [28], which was in contrast to our findings. Amaral et al. showed that 17% EDTA had direct effects on macrophages and caused some changes in their cell membrane via chelator ions such as magnesium and calcium, which can enhance apoptosis [29].

In brief, the cytotoxicity of CHX, NaOCL and EDTA significantly increased by increased exposure time.

In co-culture, the mode of stem cells death treated with sodium hypochlorite and EDTA is necrosis, while chlorhexidine at 1 and 10 Minutes often causes the PDLSCs death through early apoptosis. Despite the limitations of this in vitro study, it appears that co-culture better simulates the clinical setting than single culture.

REFERENCES

1. Imaging of apoptosis. Tait JF. J Nucl Med. 2008;49:1573-6.
2. Cytotoxicity effect of Cladribine on the MCF-7 human breast cancer cell line. Hashemi M, Karami-Tehrani F, Ghavami S. Iran Biomed J. 2004;8(1):7-12.
3. Evaluation of chlorhexidine toxicity injected in the paw of mice and added to cultured l929 fibroblasts. Faria G, Celes MR, De Rossi A, Silva LAB, Silva JS, Rossi MA. J Endod. 2007;33:715-22.
4. Adenosine and deoxyadenosine induces apoptosis in oestrogen receptor-positive and-negative human breast cancer cells via the intrinsic pathway. Cell Prolif. 2005;38:269-85.
5. Evaluation of cytotoxicity of MTAD using the MTT-tetrazolium method. Zhang W, Torabinejad M, Li Y. J Endod. 2003;29:654-7.
6. Evaluation of cytotoxic effects of various endodontic irrigation solutions on the survival of stem cell of human apical papilla. Mollashahi NF, Saberi E, Karkehabadi H. Iran Endod J. 2016;11:293.
7. An in vitro evaluation of the cytotoxicity of various endodontic irrigants on human gingival fibroblasts. Barnhart BD, Chuang A, Dalle Lucca JJ, Roberts S, Liewehr F, Joyce AP. J Endod. 200531:613-5..
8. Cell culture models of higher complexity in tissue engineering and regenerative medicine. Kirkpatrick CJ,

Vol 10 No 1 (2022) DOI 10.5195/d3000.2022.146

Fuchs S, Hermanns MI, Peters K, Unger RE.Biomaterials. 2007;28:5193-8.

9. A review of the three-dimensional cell culture technique: Approaches, advantages and applications. Zhang W, Zhuang A, Gu P, Zhou H, Fan X. Curr Stem Cell Res Ther. 2016;11:370-80.

10. Cytotoxicity of endodontic irrigants on human periodontal ligament cells. Karkehabadi H, Yousefifakhr H, Zadsirjan S. Iran Endod J. 2018;13:390.

11. Cytotoxicity of QMix™ endodontic irrigating solution on human bone marrow mesenchymal stem cells. AlKahtani A, Alkahtany SM, Mahmood A, Elsafadi MA, Aldahmash AM, Anil S. BMC oral health. 2014;14:27

12. Effects of chlorhexidine on stem cells from exfoliated deciduous teeth. Tu Y-Y, Yang C-Y, Chen R-S, Chen M-H. Taiwan Yi Xue Hui Za Zhi. 2015;114:17-22.

13. Controlling cell interactions by micropatterning in co-cultures: Hepatocytes and 3T3 fibroblasts. Bhatia SN, Yarmush ML, Toner M. J Biomed Mater Res A. 1997;34:189-99.

14. Osteogenic differentiation is selectively promoted by morphogenetic signals from chondrocytes and synergized by a nutrient rich growth environment. Gerstenfeld L, Barnes G, Shea C, Einhorn T. Connect Tissue Res. 2003;44:85-91.

15. Chlorhexidine-induced apoptosis or necrosis in L929 fibroblasts: A role for endoplasmic reticulum stress. Faria G, Cardoso CR, Larson RE, Silva JS, Rossi MA. Toxicol Appl Pharmacol. 2009;234:256-65.

16. Effect of sodium hypochlorite on human pulp cells: an in vitro study. Essner MD, Javed A, Eleazer PD. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2011;112:662-6.

17. Effect of irrigants on the survival of human stem cells of the apical papilla in a platelet-rich plasma scaffold in human root tips. Trevino EG, Patwardhan AN, Henry MA, Perry G, Dybdal-Hargreaves N, Hargreaves KM, et al. J Endod. 2011;37:1109-15.

18. Cytotoxicity and genotoxicity of endodontic irrigants on human cells. Longo JPF, Valois CA, Tapajs ßCC, Santos MdFMA, de Azevedo RB. Arch Oral Res. 2010;6.135-40

19. Adhesive resin induces apoptosis and cell-cycle arrest of pulp cells. Mantellini M, Botero T, Yaman P, Dennison J, Hanks C, Nr J. J Dent Res. 2003;82:592-6.

20. The pathways of cell death: oncosis, apoptosis, and necrosis. Trump BE, Berezesky IK, Chang SH, Phelps PC. Toxicol Pathol. 1997;25:82-8.

21. The coinitiator DMABEE induces death by apoptosis and necrosis in human monoblastoid cells. Cimpan MR, Matre R, Skaug N, Lie SA, Lygre H. Clin Oral Investig. 2005;9:168-72.

22. Effect of ProRoot® MTA mixed with chlorhexidine on apoptosis and cell cycle of fibroblasts and macrophages in vitro. Hernandez E, Botero T, Mantellini M, McDonald N, Nr J. Int Endod J. 2005;38:137-43.

23. New data on biological effects of chlorhexidine: Fe2+ induced lipid peroxidation and mitochondrial permeability transition. Newton APN, Cadena SMS, Rocha MEM, Carnieri EGS, De Oliveira MBM. Toxicol Lett. 2004;151:407-16.

24. Cytotoxicity and genotoxicity of chlorhexidine on macrophages in vitro. Li YC, Kuan YH, Lee SS, Huang FM, Chang YC. Environ Toxicol. 2014;29:452-8.

25. Effect of chlorhexidine digluconate on different cell types: a molecular and ultrastructural investigation. Giannelli M, Chellini F, Margheri M, Tonelli P, Tani A. Toxicol In Vitro. 2008;22:308-17.

26. Apoptosis in development. Meier P, Finch A, Evan G. Nature. 2000;407:796.

27. Induction of apoptosis in Neuro-2A cells by Zn2+ chelating. Sakabe I, Paul S, Dansithong W, Shinozawa T. Cell Struct Funct. 1998;23:95-9.

28. Chelating in root canal therapy. Ethylene-diamine tetraacetic acid for cleansing and widening of root canals. Ostby N. Odontol Tidskr. 1957;65:3-11.

29. Cytotoxicity analysis of EDTA and citric acid applied on murine resident macrophages culture. Amaral K, Rogero M, Fock R, Borelli P, Gavini G. Int Endod J. 2007;40:338-43.