

Original Article

Cytotoxicity and induced apoptosis of a new bioceramic cement containing simvastatin on stem cells from human exfoliated deciduous teeth

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ABSTRACT

Background: This study aims to compare cytotoxicity and induced apoptosis of a new bioceramic cement containing different concentrations of simvastatin on stem cells from human exfoliated deciduous teeth (SHED).

Materials and Methods: This research was an *in vitro* study. To evaluate the cytotoxicity and induced apoptosis of the bioceramic cement containing different concentrations of simvastatin, the SHED were exposed to the cement during 1, 3, and 7 days. Pure bioceramic cement and pure simvastatin with concentrations of 1, 0.1, and 0.01 μM were also tested to evaluate the possible synergic effect. Mineral trioxide aggregate (MTA) as the gold standard of pulp dressing materials was compared. MTT assay and Annexin V assay were used to evaluate cytotoxicity and induced apoptosis, respectively. The data were analyzed using ANOVA and Tukey *post hoc* tests at the significance level of 0.05.

Results: During 7 days, MTA, bioceramic cement, simvastatin 0.1 and 0.01 μM , and bioceramic cement containing 0.1 and 0.01 μM simvastatin increased ($P < 0.05$) and simvastatin with concentration of 1 μM decreased the cell viability ($P < 0.05$). Except for MTA and bioceramic cement containing 0.1 and 0.01 μM simvastatin, all other compounds induced apoptosis within 7 days ($P < 0.05$).

Conclusion: After 7 days, the viability of the SHED in the presence of a new bioceramic cement containing 0.1 and 0.01 μM simvastatin was not compromised. Moreover, this cement showed superior results than MTA and provided an environment for cell proliferation. This finding appears to be due to the pharmacological effects of low concentrations of simvastatin.

Key Words: Apoptosis, mineral trioxide aggregate cement, simvastatin, toxicity

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INTRODUCTION

Dental caries is the most common chronic infectious disease of childhood. Despite significant advances in preventive dentistry, many children still suffer from the disease and its consequences including

pain, infection, chewing and eating disorders, space loss, psychological problems, and missing school hours.^[1] In many cases, the disease progression causes reversible pulp inflammation, which requires advanced

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treatments, including indirect pulp treatment, direct pulp treatment, and pulpotomy. In more severe cases with irreversible pulpitis or pulp necrosis, nonvital pulp treatment might be indicated.^[2]

Pulpotomy is the most common pulp treatment method in primary dentition with the ultimate goal of maintaining the health and integrity of the oral and dental structures.^[3,4] This treatment is indicated when the pulp is exposed by caries or mechanically exposed during caries removal. Clinical and radiographic criteria must confirm that inflammation is limited to the coronal pulp.^[5] Technically, the coronal pulp is completely removed, and after gaining homeostasis, the remaining root pulp is treated with different approaches and dressing materials.^[4]

Pharmacologic and nonpharmacologic techniques of pulpotomy are categorized as devitalizing (e.g., formocresol, electrosurgery, laser), preserving (e.g., ferric sulfate, sodium hypochlorite), and regenerating (e.g., calcium hydroxide, calcium silicate-based cements) based on their effect on the remaining radicular pulp.^[6,7]

Ideally, the materials used for pulpotomy should have antibacterial activity and easy manipulation, provide a tight seal, be affordable and not interfere with physiological root resorption.^[6] Moreover, the therapeutic agent should not induce an inflammatory process. It is well known that all materials may irritate the pulp tissue and cause some degree of inflammation. The inflammatory response can activate odontoclast progenitor cells, differentiate them to odontoclasts, and promote the process of internal root resorption.^[8,9]

A material with all these properties has not yet been manufactured, and studies in this field have been continued since the 1900s with the introduction of Buckley formocresol.

Although calcium silicate-based cements (e.g., mineral trioxide aggregate [MTA] and CEM Cement) have revealed promising results in several clinical studies,^[10] they have shortcomings for regular application in pediatric dentistry. Tooth discoloration, long setting time, and high treatment costs for patients in developing countries are among those.^[11]

To overcome these disadvantages, a series of studies are designed to evaluate the biological properties of a new bioceramic cement containing simvastatin on stem cells from human exfoliated deciduous teeth (SHED).

The new hydroxyapatite-based bioceramic cement containing tricalcium silicate, silicon hydroxyapatite, and strontium hydroxyapatite has been prepared and introduced in 2019 at the Dental Materials Research center, Mashhad University of Medical Sciences. Studies on this new material have been very limited.^[12]

In recent years, there has been a tendency toward the use of simvastatin in dentistry.^[13,14] Suppressing the expression of pro-inflammatory mediators and increasing the expression of odontoblastic/osteoblastic markers such as dentin sialophosphoprotein, alkaline phosphatase, osteopontin, osteocalcin, and bone morphogenic protein 2 and deposition of mineralized matrix by low concentrations of simvastatin (0.1–1 μ M) has been reported in studies.^[13,15]

As the first step, we aimed to determine if adding simvastatin to the bioceramic cement will affect its biological properties on SHED. Hence, the aim of this *in vitro* study was to compare the cytotoxicity and induced apoptosis of a new bioceramic cement containing different concentrations of simvastatin on SHED by MTT and Annexin V assays, respectively.

MATERIALS AND METHODS

This *in vitro* study was conducted in August 2020 in the Dental Research Center of Mashhad University of Medical Sciences with the ethics committee registration code IR.MUMS.DENTISTRY.REC.1399.005.

Preparation of bioceramic cement

Bioceramic cement (Mashhad University of Medical Sciences, Iran) containing 50% by weight of tricalcium silicate/dicalcium silicate, 25% by weight of strontium-doped hydroxyapatite, and 25% by weight of silicon-doped hydroxyapatite has been prepared.

Silicon-hydroxyapatite was prepared as described through a sol-gel method in an aqueous-alcoholic medium, assuming the substitution of silicate ions instead of phosphate. For this purpose, 0.02 mol of tetraethyl orthosilicate (TEOS) in 100 cc of water/ethanol solution was first placed on a magnetic stirrer to complete the hydrolysis. Then, 0.28 mol of sodium dihydrogen phosphate salt was dissolved in 100 cc of deionized distilled water and added to the container containing TEOS. The pH of the solution was adjusted to 10 using normal sodium hydroxide. As a source of calcium ions, 0.5 mol of calcium

chloride in 200 cc of water was used. So that the final molar ratio is established:

$$[\text{Ca} + 2]/[\text{P} + \text{Si}] = 1.67$$

Calcium chloride solution was gradually added to the solution containing phosphate and silica over 1 h. The pH of the reaction vessel was fixed at 10 using 1N sodium hydroxide. The product was placed on a stirrer at 80°C for 12 h. After 12 h, the liquid phase was separated using a centrifuge at 4000 rpm, and the resulting solid was dried at ambient temperature and sintered at 800°C for 10 h with a temperature gradient of 10°C/min.

The synthesis of strontium-hydroxyapatite was performed by the sol-gel method in an aqueous medium, assuming the substitution of strontium ions instead of calcium. For this purpose, 0.05 mol of strontium chloride and 0.45 mol of calcium chloride were dissolved in 200cc of deionized distilled water. Then, 0.3 mol of sodium dihydrogen phosphate salt was dissolved in 200cc of deionized distilled water. The pH of the solution was adjusted to 10 using 1N sodium hydroxide. Hence that the final molar ratio is established:

$$[\text{Ca} + 2 + \text{Sr} + 2]/[\text{Pi}] = 1.67$$

Strontium chloride/calcium chloride solution was gradually added to the phosphate solution over 1 h using a decanter. Using the pH meter, the pH of the reaction vessel was fixed at 10 using 1N sodium hydroxide. The resulting material was placed on a stirrer at 25°C for 72 h. After 72 h, the liquid phase was separated using a centrifuge at 4000 rpm, and the resulting solid was dried at ambient temperature and sintered at 800°C for 10 h with a temperature gradient of 10°C/min.

Calcium silicate was prepared using a sol-gel method in an aqueous-alcoholic medium. First, 0.5 mol TEOS was mixed in 200 cc of deionized-distilled water and nitric acid (as a catalyst) to complete hydrolysis. Then, 1.5 mol of calcium nitrate was added and stirred at 80°C until gel formation. The resulting gel was dried in an oven at 120°C, and the white powder was placed at 1200°C for 10 h. After heat treatment, the mass of the resulting ceramic was ground by a mortar and ball-milling operation for 24 h in a container containing acetone and glass balls with a diameter of 3 mm. After drying, the resulting powder was sieved with a sieve size of 37 microns.

The synthesized ceramic was characterized by X-ray diffraction (XRD), (X' Pert PW 3040/60, Philips,

The Netherlands) at $2\theta = 20-80$. The morphology of ceramic particles was studied using scanning electron microscopy.

To add simvastatin to bioceramic cement, first concentrations of 0.01, 0.1, and 1 μM simvastatin (Sigma-Aldrich, Germany) were prepared in the liquid phase. According to the molecular weight of simvastatin (418.56 g/mol), 100 mg of this substance was dissolved in 50 ml of distilled water and uniformly mixed to form a suspension with a concentration of 1000 μM . By diluting this solution, 1, 0.1, and 0.01 μM solutions were obtained. Each of the concentrations was mixed with the bioceramic cement with 1:1 weight ratios to obtain a paste consistency. To prepare pure bioceramic cement, cement powder and distilled water were mixed with a 1:1 weight ratio.

Each compound was poured into tablet-shaped plastic molds and placed in an incubator (LEEC, England) with 100% humidity and 37°C for completion of the setting reaction. Then, the tablets were transferred to the cell culture laboratory for cellular experiments. The tablets were placed in the culture medium for 48 h to exchange particles.

Culture medium preparation

To prepare the culture medium, an appropriate amount of D-MEM (Modified Eagles Medium Dulbeccos) filtered medium was used. The acidity of the culture medium was adjusted by hydrochloric acid and NaOH in the range of 7.4. Then a combination of 10% fetal calf serum and 1% antibiotics including 1000 U/ml penicillin and 10 mg/ml streptomycin in a proportion of 1:10 was added. The solution was stored in a sterile container in the refrigerator until use.

Cell preparation

The cells were cultured and passaged to reach a sufficient number. The SHED were cultured in the logarithmic phase of proliferation in the culture medium. These cells were incubated in 5% CO_2 at 37°C. The culture medium was changed every 2 days if its color changed until a monolayer of cells covered the flask. Cell passage was performed when the cell confluency reached 70%–80%.

For cell passage, the outdated culture medium was removed, and 2 mm of trypsin enzyme was poured on the cells. Incubation was performed for 5 min at 37°C. Then, 2 ml of culture medium containing 10% FBS (Gibco, USA) was added to

the plate to stop the lethal activity of trypsin. The cells isolated from the bottom of the plate were transferred to a 15 ml sterile tube and centrifuged for 5 min at 1900 rpm. After washing the cells with PBS (Phosphate Buffered Saline), a few milliliters of fresh culture medium were added to the cell sediment at the bottom of the centrifuge tube and vortexed (Velp, Iran). Then, 2 ml of the resulting suspension (at a concentration of 10^4 cells/ml) was poured into a flask and stored in a CO₂ incubator. SHED at passages 3–5 were used.

Cell viability by MTT assay

MTT is a standard laboratory test to determine the cytotoxicity of various substances. This test is based on mitochondrial activity. Linear changes in mitochondrial activity may be associated with an increase or decrease in the number of living cells. In this test, cells break the yellow tetrazolium ring by mitochondrial dehydrogenase, producing NADH and NADPH, leading to the formation of a purple precipitate of formazan. The precipitate is then dissolved in isopropanol or dimethyl sulfoxide. Cells that are not alive lack such activity and do not cause discoloration. Thus, the intensity of the purple color indicates the number of viable cells or, in other words, cell proliferation. The color intensity at 570 nm is measured by the ELISA plate reader and is directly related to the number of cells with metabolic activity.^[16]

In this study, MTT was performed following the ISO/EN-109935^[14] on the SHED as target cells. MTT solution (Sigma-Aldrich, Germany) was prepared in PBS with a concentration of 5 mg/ml and was filtered and sterilized with a 0.2 µm filter. The cells were implanted in 24-well plates. Prepared culture medium containing particles of cements (bioceramic cement containing simvastatin in 3 concentrations of 0.01 µM, 0.1 µM, and 1 µM (CEM/SIM 0.01, CEM/SIM 0.1, CEM/SIM 1), pure bioceramic cement (CEM), simvastatin with concentrations of 0.01 µM, 0.1 µM and 1 µM (SIM 0.01, SIM 0.1, SIM 1) and MTA (Angelus, Brazil)) were added to the relevant wells. After the exposure at 1, 3, and 7 days, the culture medium was drained, and the cells were washed with PBS. Wells were filled with 250 µl of culture medium in addition to 25 µl of MTT solution. The cells were incubated at 37°C and 5% CO₂ for 3-4 h. The culture medium was drained, and 160 µl of DMSO (dimethyl sulfoxide) (Merck, Germany) was added to dissolve the formazan

crystals. The cells were transferred to 96-well plates. The quantity of formazan was determined by ELISA (Enzyme-Linked Immunosorbent Assay) (Biotek, USA) at 540 nm. Pure culture medium and a toxic material were considered as negative control and positive control groups, respectively. The percentage of the viable cells was calculated using the following formula:

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of negative control} - \text{Absorbance of blank}} \times 100$$

Induced apoptosis by Annexin V

Annexin V is an available test to quantify the extent of apoptosis and cellular necrosis affected by the stimulus. The surface of healthy cells is made up of lipids that are asymmetrically present on the inner and outer layers of the cell membrane. One of these lipids, called phosphatidylserine, is usually confined to the inner layer of the membrane and is located only in the vicinity of the cytoplasm. During apoptosis, this lipid asymmetry changes, and phosphatidylserine will be located in the outer layer of the membrane, as well. Annexin V is a calcium-binding protein that binds to this lipid, and its fluorescent form can be used to detect phosphatidylserine in the outer layer of the membrane of apoptotic cells. In addition, Annexin V can stain necrotic cells. In this case, the cells lack the membrane integrity that gives this protein access to all areas.^[17] Flow cytometry (BD Biosciences, Singapore) is used to detect dislocated phosphatidylserine and fragmented DNA that are bound to Annexin and Propidium Iodide, respectively.

The “FITC Annexin V Apoptosis Detection Kit with PI” (Biolegend, 640914, USA) was used to evaluate the extent of induced apoptosis, genotoxicity, and DNA damage. The cells were incubated in 48-well plates with 10^4 cells cultured in each well for 24 h at 37°C and 5% CO₂. Then, the cells were treated with CEM/SIM 0.01, CEM/SIM 0.1 and CEM/SIM 1, pure bioceramic cement, SIM 0.01, SIM 0.1, and SIM 1 as well as MTA at 1, 3, and 7 days. The cells were separated from the bottom of the plate by trypsin-EDTA (Gibco, USA) and washed twice with PBS. After centrifuge, the cells were collected in a 15 ml Falcon tube with a density of 10^6 cells/ml. The centrifuged cells were then resuspended in the binding buffer by the sampler. Before flowcytometry,

5 μ l of Annexin V and 10 μ l of Propidium iodide were added to each tube. The tubes were incubated at room temperature and away from ambient light for 30 min. Finally, the samples were read by flow cytometry. The rate of apoptosis and necrosis was evaluated based on cell migration and absorption of propidium iodide dye and analyzed by FlowJo software (BD Biosciences, Canada).

Statistical analysis

Cell viability and apoptosis, evaluated by MTT assay and Annexin V, are presented as the mean percentage \pm standard deviation. Due to biological experiments and triple replications of each of target concentration, the sample size is not considered in such studies.

The results of the MTT assay and Annexin V biological tests were analyzed by analysis of variance (ANOVA). As the statistical significance for analyzed variables was determined, the Tukey-*post-hoc* test was performed. Analysis was conducted by GraphPad Prism software, version 9 (GraphPad, USA) at the significance level of 0.05 ($P < 0.05$).

RESULTS

Electron microscopy images showed that a dense hexagonal crystal structure with sub-micron dimensions resembling calcium silicate was formed in the calcium silicate sample. In two examples of apatite ceramics, hexagonal crystals similar to the structure of apatite are evident. In the silicon hydroxyapatite sample, the crystals are elongated, and in the strontium hydroxyapatite sample, plate-shaped crystals with larger dimensions are observed [Figure 1].

Analysis of the XRD pattern confirmed the formation of the apatite structure in the presence of silicon and strontium ions and the replacement of the strontium and silicon ions in the hydroxyapatite structure. The XRD model for silicate composition also showed that the reaction product was a mixture of dicalcium silicate and tricalcium silicate [Figure 2]. Figures 1d and d show calcium hydroxide in the form of portlandite after the cement set.

Cell viability by MTT assay

The findings of the MTT assay showed that during 7 days, the percentage of viable cells increased in a culture medium containing bioceramic cement. Among simvastatin concentrations, SIM 0.01 caused

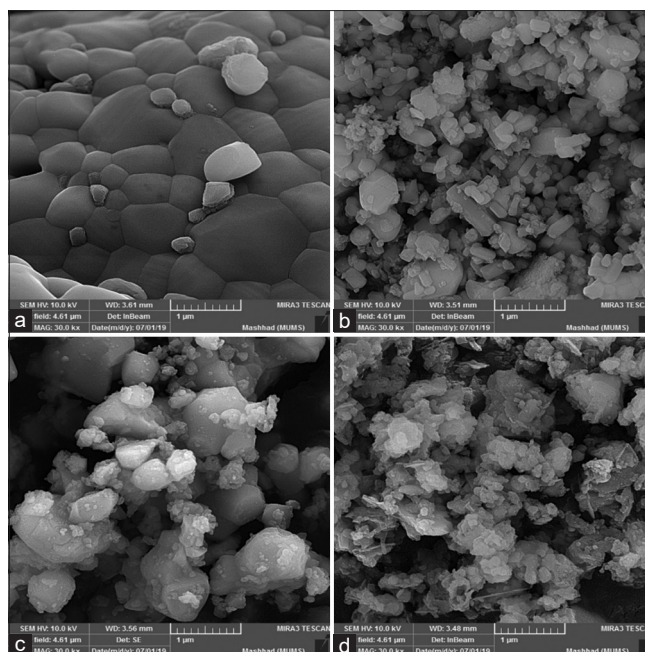


Figure 1: scanning electron microscope view. (a) Calcium silicate, (b) Silicon-doped hydroxyapatite, (c) strontium-doped hydroxyapatite, (d) set-cement

the maximum percentage of viable cells over 7 days (101%). Only in this concentration, the percentage of viable cells increased over time. Furthermore, along with the decreased concentration of simvastatin, the percentage of viable cells has increased. On day 7, this difference is statistically significant for all three concentrations compared to each other. It is also evident that over 7 days, CEM/SIM 0.01 caused the maximum percentage of viable cells in the culture medium with bioceramic cement containing simvastatin (108%). It is noteworthy that unlike CEM/SIM 1, CEM/SIM 0.01 and CEM/SIM 0.1 increase the percentage of viable cells over time. For comparison with the gold standard, it could be noted that after 7 days, the percentage of viable cells in the culture medium containing bioceramic cement, SIM 0.01, CEM/SIM 0.01, and CEM/SIM 0.1 was higher compared to MTA. This superiority is statistically significant for the bioceramic cement with concentration of 0.01 ($P < 0.0001$), CEM/SIM 0.1 ($P = 0.004$) and CEM/SIM 0.01 ($P < 0.0001$).

Adding simvastatin to the bioceramic cement resulted in different measures compared to each of them alone. It could be noted that after 7 days, CEM/SIM 0.1 caused a higher percentage of viable cells than the bioceramic cement ($P < 0.9$) and SIM 0.1 ($P < 0.0001$). Furthermore, after seven days, CEM/SIM 0.01 caused in higher percentage of viable

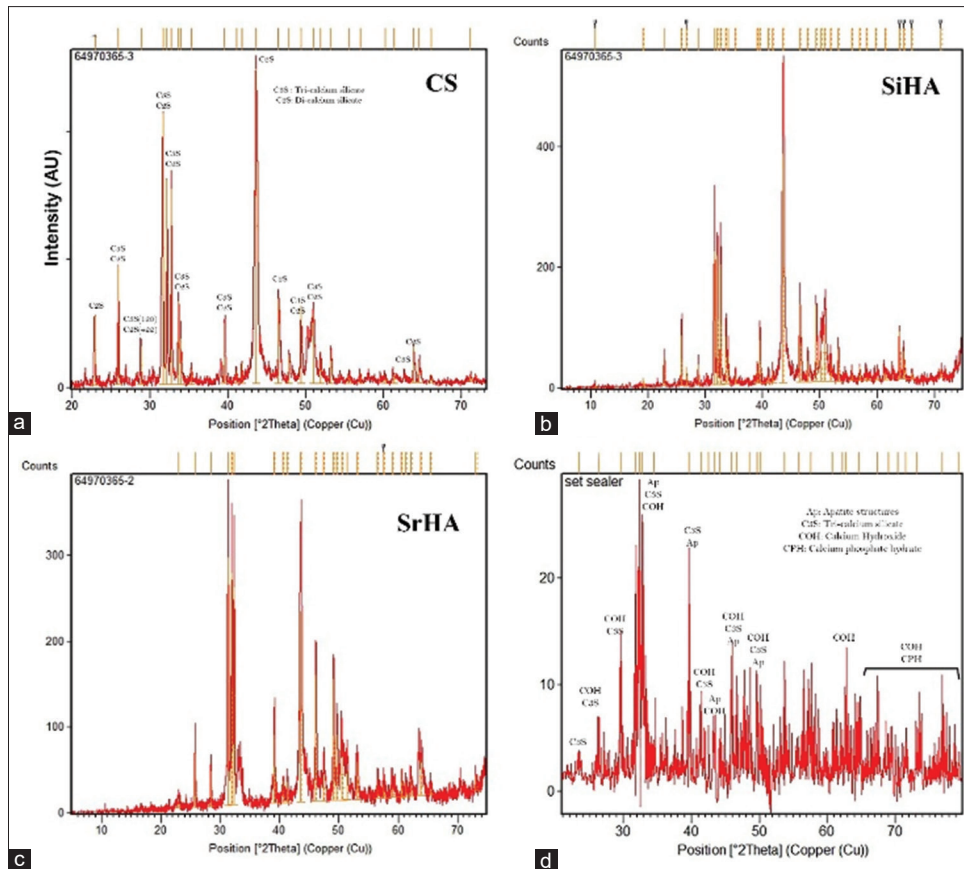


Figure 2: X-ray diffraction pattern. (a) Calcium silicate, (b) Silicon-doped hydroxyapatite, (c) strontium-doped hydroxyapatite, (d) set-cement

cells than the bioceramic cement ($P = 0.2$) and SIM 0.01 ($P < 0.0001$).

Table 1 and Figure 3 present the descriptive statistics and comparison graph of MTT assay, respectively. Results of the pairwise multiple comparisons obtained from Tukey-*post-hoc* test are presented in Table 2.

Cell apoptosis by Annexin V

The results of Annexin V show that the percentage of apoptotic cells in the culture medium containing bioceramic cement increased during 7 days. SIM 0.01 caused the minimum percentage of apoptotic cells among different concentrations of this substance (14%), and in all three concentrations, the percentage of apoptotic cells increased over time. Furthermore, along with decreased concentrations, the percentage of apoptotic cells decreased. This decrease on day 7 was statistically significant for all three concentrations compared to each other ($P < 0.0001$). By adding simvastatin to the bioceramic cement, CEM/SIM 0.01 caused the minimum percentage of apoptotic after 7 days (9%). It is noteworthy that the percentage of apoptotic cells in the culture

Table 1: Descriptive statistics of MTT assay

Compound	Mean (%)±SD		
	1 day	3 days	7 days
CEM	94.933±0.379	100.3±0.7	104.033±0.95
SIM 1	85.933±0.404	76.1±0.361	69.867±0.513
SIM 0.1	91.253±0.809	81.2±0.852	88.23±0.856
SIM 0.01	95.067±0.306	98.967±0.252	101±0.6
CEM/SIM 1	92.7±0.721	89.767±2.977	88.7±1.311
CEM/SIM 0.1	94.933±0.379	101.3±2.152	105.833±1.041
CEM/SIM 0.01	96.2±0.541	103.32±1.29	108.23±0.995
MTA	83.276±2.075	92.89±3.955	99.935±5.33
Control +	1.087±0.218	1.003±0.2	1.09±0.215
Control -	100±0	123±2	142±2.246

CEM: Bioceramic cement with full concentration, SIM 1: Simvastatin with concentration of 1 μ M, SIM 0.1: Simvastatin with concentration of 0.1 μ M, SIM 0.01: Simvastatin with concentration of 0.01 μ M, CEM/SIM 1: Bioceramic cement containing simvastatin 1 μ M, CEM/SIM 0.1: Bioceramic cement containing simvastatin 0.1 μ M, CEM/SIM 0.01: Bioceramic cement containing simvastatin 0.01 μ M, MTA: Mineral trioxide aggregate, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide

medium with bioceramic cement containing all three concentrations of simvastatin increased over time. For comparison with the gold standard, it could be noted that the percentage of apoptotic cells in the culture medium containing MTA increased over 7 days, but

Table 2. Pairwise multiple comparisons of MTT assay

Reference group	Comparison group	Statistical significance after (P)			
		1 day	3 days	7 days	
CEM	CEM	-	0.01 ^{a,*}	0.4 ^b	
	SIM1	>0.0001	<0.0001*	<0.0001*	
	SIM 0.01	>0.9	>0.9	0.8	
	SIM 0.1	0.5	<0.0001*	<0.0001*	
	CEM/SIM 0.1	>0.9	>0.9	>0.9	
	CEM/SIM 1	>0.9	<0.0001*	<0.0001*	
	CEM/SIM 0.01	>0.9	0.8	0.2	
	MTA	<0.0001*	<0.0001*	0.2	
	Control +	<0.0001*	<0.0001*	<0.0001*	
	Control -	0.03*	<0.0001*	<0.0001*	
SIM 1	SIM 1	-	<0.0001 ^{a,*}	<0.001 ^{b,*}	
	SIM 0.01	<0.0001*	<0.0001*	<0.0001*	
	SIM 0.1	0.02*	0.03*	<0.0001*	
	CEM/SIM 0.1	<0.0001*	<0.0001*	<0.0001*	
	CEM/SIM 1	0.003*	<0.0001*	<0.0001*	
	CEM/SIM 0.01	<0.0001*	<0.0001*	<0.0001*	
	MTA	>0.9	<0.0001*	<0.0001*	
	Control +	<0.0001*	<0.0001*	<0.0001*	
	Control -	<0.0001*	<0.0001*	<0.0001*	
	SIM 0.1	SIM 0.1	-	<0.0001 ^{a,*}	0.0001 ^{b,*}
SIM 0.01		0.4	<0.0001*	<0.0001*	
CEM/SIM 1		>0.9	<0.0001*	>0.9	
CEM/SIM 0.1		0.5	<0.0001*	<0.0001*	
CEM/SIM 0.01		>0.05	<0.0001*	<0.0001*	
MTA		<0.0001*	<0.0001*	<0.0001*	
Control +		<0.0001*	<0.0001*	<0.0001*	
Control -		<0.0001*	<0.0001*	<0.0001*	
SIM 0.01		SIM 0.01	-	0.3 ^a	>0.9 ^b
		CEM/SIM 1	>0.9	<0.0001*	<0.0001*
	CEM/SIM 0.1	>0.9	>0.9	0.06	
	CEM/SIM 0.01	>0.9	0.1	<0.0001*	
	MTA	<0.0001*	0.002*	>0.9	
	Control +	<0.0001*	<0.0001*	<0.0001*	
	Control -	>0.05	<0.0001*	<0.0001*	
	CEM/SIM 1	CEM/SIM 1	-	0.8 ^a	>0.9 ^b
		CEM/SIM 0.1	>0.9	<0.0001*	<0.0001*
		CEM/SIM 0.01	0.6	<0.0001*	<0.0001*
MTA		<0.0001*	0.8	<0.0001*	
Control +		<0.0001*	<0.0001*	<0.0001*	
Control -		<0.0001*	<0.0001*	<0.0001*	
CEM/SIM 0.1		CEM/SIM 0.1	-	0.001 ^{a,*}	0.0002 ^{b,*}
		CEM/SIM 0.01	>0.9	>0.9	>0.9
		MTA	<0.0001*	<0.0001*	0.004*
		Control +	<0.0001*	<0.0001*	<0.0001*
	Control -	0.03*	<0.0001*	<0.0001*	
	CEM/SIM 0.01	CEM/SIM 0.01	-	0.0001 ^{a,*}	<0.05 ^b
		MTA	<0.0001*	<0.0001*	<0.0001*
		Control +	<0.0001*	<0.0001*	<0.0001*
		Control -	>0.9	<0.0001*	<0.0001*

Table 2. Contd...

Reference group	Comparison group	Statistical significance after (P)		
		1 day	3 days	7 days
MTA	MTA	-	<0.0001 ^{a,*}	0.0001 ^{b,*}
	Control +	<0.0001*	<0.0001*	<0.0001*
	Control -	<0.0001*	<0.0001*	<0.0001*

^aComparison between day 3 and 1, ^bComparison between day 7 and 3, *Statistically significant, Green: Numerical superiority of the reference group, Red: Numerical superiority of the comparison group. CEM: Bioceramic cement with full concentration, SIM 1: Simvastatin with concentration of 1 µM, SIM 0.1: Simvastatin with concentration of 0.1 µM, SIM 0.01: Simvastatin with concentration of 0.01 µM, CEM/SIM 1: Bioceramic cement containing simvastatin 1 µM, CEM/SIM 0.1: Bioceramic cement containing simvastatin 0.1 µM, CEM/SIM 0.01: Bioceramic cement containing simvastatin 0.01 µM, MTA: Mineral trioxide aggregate, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide

the measures were lower than all other substances, and this difference was statistically significant for almost all comparisons (except for CEM/SIM 0.01).

As shown for cell viability, adding simvastatin to the bioceramic cement resulted in different measures compared to each of them alone. After 7 days, the percentage of apoptotic cells in the culture medium containing CEM/SIM 1, CEM/SIM 0.1, and CEM/SIM 0.01 were lower than the culture medium containing each of the materials alone (e.g., bioceramic cement or simvastatin 1 µM) ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, and $P = 0.03$, respectively).

Table 3 and Figure 4 present the descriptive statistics and comparison graph of Annexin V, respectively. Results of the Pairwise multiple comparisons obtained from Tukey-Post hoc test is presented in Table 4.

DISCUSSION

Evaluating biological effects is one of the most important aspects of dental material studies. Such substances can be clinically accepted if they do not show cytotoxicity in contact with oral tissues and do not cause irreversible inflammatory reactions by creating necrotic areas.^[18] Cytotoxicity and induced apoptosis tests are commonly used to evaluate the biocompatibility of dental materials.^[19,20] In the present study, MTT assay and Annexin v were used to evaluate the cytotoxicity and induced apoptosis, respectively. Among all types of viability assays, MTT is a safe, convenient, and low-cost colorimetric technique with many applications in investigations and clinic.^[21] Annexin V is commercially available labeled with either fluorescent markers for direct detection or indirect labeling. This test has the advantages of high

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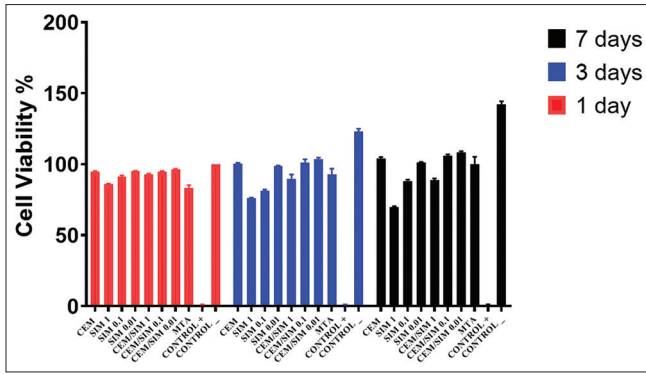


Figure 3: MTT assay comparison graph. CEM: Bioceramic cement with full concentration; SIM 1: Simvastatin with concentration of 1 μM ; SIM 0.1: Simvastatin with concentration of 0.1 μM ; SIM 0.01: Simvastatin with concentration of 0.01 μM ; CEM/SIM 1: Bioceramic cement containing simvastatin 1 μM ; CEM/SIM 0.1: Bioceramic cement containing simvastatin 0.1 μM ; CEM/SIM 0.01: Bioceramic cement containing simvastatin 0.01 μM

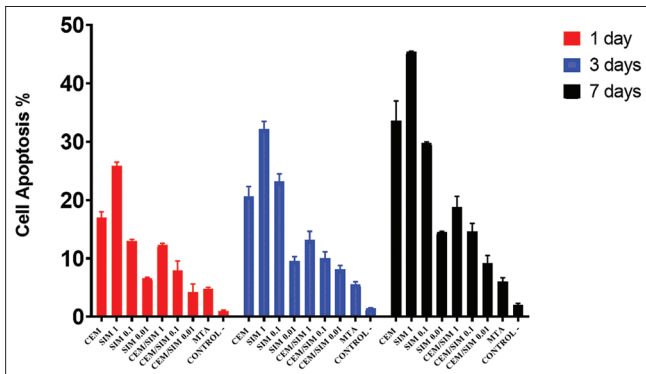


Figure 4: Annexin V comparison graph. CEM: Bioceramic cement with full concentration; SIM 1: Simvastatin with concentration of 1 μM ; SIM 0.1: Simvastatin with concentration of 0.1 μM ; SIM 0.01: Simvastatin with concentration of 0.01 μM ; CEM/SIM 1: Bioceramic cement containing simvastatin 1 μM ; CEM/SIM 0.1: Bioceramic cement containing simvastatin 0.1 μM ; CEM/SIM 0.01: Bioceramic cement containing simvastatin 0.01 μM

sensitivity and the ability to detect early phases of apoptosis.^[22,23]

Studies on calcium silicate bioactive cements have shown^[11,24] that these materials provide a suitable substrate for pulp tissue healing activity by the long-term release of calcium ions as well as creating a highly alkaline environment.^[25] Hydroxyapatite, another component of this cement, which contains calcium and phosphate, is the main constituent of enamel and dentin. Studies have confirmed its biocompatibility and stability to induce osteogenesis, growth, proliferation, and attachment

Table 3: Descriptive statistics of Annexin V

Compound	Mean (%)±SD		
	1 day	3 days	7 days
CEM	16.995±0.997	20.675±1.648	33.58±3.395
SIM 1	25.855±0.629	32.15±1.344	45.45±0.071
SIM 0.1	13.05±0.212	23.15±1.344	29.75±0.212
SIM 0.01	6.6±0.141	9.6±0.707	14.5±0.141
CEM/SIM 1	12.3±0.283	13.15±1.485	18.8±1.838
CEM/SIM 0.1	7.95±1.626	10±1.131	14.6±1.414
CEM/SIM 0.01	4.2±1.414	8.1±0.707	9.15±1.344
MTA	4.845±0.191	5.6±0.424	6.05±0.636
Control -	1.005±0.148	1.46±0.071	2.01±0.283

CEM: Bioceramic cement with full concentration, SIM 1: Simvastatin with concentration of 1 μM , SIM 0.1: Simvastatin with concentration of 0.1 μM , SIM 0.01: Simvastatin with concentration of 0.01 μM , CEM/SIM 1: Bioceramic cement containing simvastatin 1 μM , CEM/SIM 0.1: Bioceramic cement containing simvastatin 0.1 μM , CEM/SIM 0.01: Bioceramic cement containing simvastatin 0.01 μM , MTA: Mineral trioxide aggregate

of osteoblast cells.^[26,27] Strontium and silicon in the cement cause proliferation, cell differentiation, and mineralization of exposed pulp and improvement of a leakage-free seal in humid environments, respectively.^[28,29] This cement showed significantly less cytotoxicity and higher cell survival compared to MTA at most evaluation times. However, cell survival for both substances has increased over time. The higher cytotoxicity of MTA may be due to a decrease in the pH of the environment at the setting time. This acidity decreases over time.^[30]

Few studies have evaluated the biocompatibility of bioceramic cements on pulp stem cells from primary teeth.^[31] In our study, SHED were the target cells used to evaluate the cytotoxicity and induced apoptosis of the new pulp dressing cement. These cells are considered progenitor cells because they have inherent regenerative capabilities in addition to relatively easy access.^[32] Despite similar characteristics of permanent and deciduous dental pulp stem cells, SHED have shown higher proliferation and expression of growth factors and as immature mesenchymal stem cells, they express higher levels of CD 105 and CD 146 on their surface, indicating a high differentiation potential.^[32]

In this study, simvastatin was added to the bioceramic cement to benefit from its properties. *In vitro* studies have shown desirable properties of simvastatin, such as increased cell proliferation, odontoblastic differentiation, mineralization, and suppression of inflammation. This is the first time that simvastatin-induced cytotoxicity and induced apoptosis on SHED have been investigated. In previous studies, 5–40 mg per day has been recommended as the therapeutic dose of simvastatin.

Table 4: Pairwise multiple comparisons of Annexin V assay

Reference group	Comparison group	Statistical significance after (P)		
		1 days	3 days	7 day
CEM	CEM	-	0.4 ^{a,*}	<0.0001 ^{b,*}
	SIM 1	<0.0001*	<0.0001*	<0.0001*
	SIM 0.1	0.3	<0.9	0.3
	SIM 0.01	<0.0001*	<0.0001*	<0.0001*
	CEM/SIM 1	0.1	0.0003*	<0.0001*
	CEM/SIM 0.1	<0.0001*	<0.0001*	<0.0001*
	CEM/SIM 0.01	<0.0001*	<0.0001*	<0.0001*
	MTA	<0.0001*	<0.0001*	<0.0001*
SIM 1	Control -	<0.0001*	<0.0001*	<0.0001*
	SIM 1	-	0.004 ^{a,*}	<0.0001 ^{b,*}
	SIM 0.1	<0.0001*	<0.0001*	<0.0001*
	SIM 0.01	<0.0001*	<0.0001*	<0.0001*
	CEM/SIM 1	<0.0001*	<0.0001*	<0.0001*
	CEM/SIM 0.1	<0.0001*	<0.0001*	<0.0001*
	CEM/SIM 0.01	<0.0001*	<0.0001*	<0.0001*
	MTA	<0.0001*	<0.0001*	<0.0001*
SIM 0.1	Control -	<0.0001*	<0.0001*	<0.0001*
	SIM 0.1	-	<0.0001 ^{a,*}	0.002 ^b
	SIM 0.01	0.003*	<0.0001*	<0.0001*
	CEM/SIM 1	<0.9	<0.0001*	<0.0001*
	CEM/SIM 0.1	0.05	<0.0001*	<0.0001*
	CEM/SIM 0.01	<0.0001*	<0.0001*	<0.0001*
SIM 0.01	MTA	<0.0001*	<0.0001*	<0.0001*
	Control -	<0.0001*	<0.0001*	<0.0001*
	SIM 0.01	-	0.8 ^a	0.07 ^b
	CEM/SIM 1	0.01*	0.5	0.2
	CEM/SIM 0.1	<0.9	<0.9	<0.9
	CEM/SIM 0.01	<0.9	<0.9	0.03
CEM/SIM 1	MTA	0.9	0.3	<0.0001*
	Control -	0.01*	<0.0001*	<0.0001*
	CEM/SIM 1	-	<0.9 ^a	0.01 ^b
	CEM/SIM 0.1	0.1	0.3	0.2
	CEM/SIM 0.01	<0.0001*	<0.05	<0.0001*
CEM/SIM 0.1	MTA	0.0003*	0.0003*	<0.0001*
	Control -	<0.0001*	<0.0001*	<0.0001*
	CEM/SIM 0.1	-	<0.9 ^a	0.1 ^b
	CEM/SIM 0.01	0.4	<0.9	0.02*
CEM/SIM 0.01	MTA	0.7	0.1	<0.0001*
	Control -	0.001*	<0.0001*	<0.0001*
	CEM/SIM 0.01	-	0.3 ^a	<0.9 ^b
MTA	MTA	<0.9	<0.9	0.7
	Control -	0.7	0.002*	0.0006*
MTA	MTA	-	<0.9 ^a	<0.9 ^b
	Control -	0.3	0.2	0.3

^aComparison between day 3 and 1, ^bComparison between day 7 and 3, *Statistically significant, Green: Numerical superiority of the reference group, Red: Numerical superiority of the comparison group, CEM: Bioceramic cement with full concentration, SIM 1: Simvastatin with concentration of 1 µM, SIM 0.1: Simvastatin with concentration of 0.1 µM, SIM 0.01: Simvastatin with concentration of 0.01 µM, CEM/SIM 1: Bioceramic cement containing simvastatin 1 µM, CEM/SIM 0.1: Bioceramic cement containing simvastatin 0.1 µM, CEM/SIM 0.01: Bioceramic cement containing simvastatin 0.01 µM, MTA: Mineral trioxide aggregate

According to the 5% bioavailability of the drug, its systemic concentration has been estimated between

0.05 and 5 µM.^[33] Therefore, most *in vitro* studies have selected this range to evaluate the cytotoxicity and other biological properties of simvastatin.^[15,34,35] Saewong *et al.* concluded that a concentration of 0.1 µM simvastatin caused a significant increase, and concentrations of 1 and 10 µM significantly reduced cell viability over 72 h, and a concentration of 10 µM significantly increased apoptotic cells.^[35] Also, Sabandal *et al.* reported that concentrations of 0.01 and 0.1 µM simvastatin significantly increased cell viability of permanent dental pulp stem cells over 21 days compared with concentrations of 1 µM and 2 µM that significantly reduced cell viability.^[33] The results of the present research are comparable to the mentioned studies. At all evaluation times, lower than 1 µM concentrations of simvastatin caused significantly higher cell viability; however, only the exposure of cells to SIM 0.01 caused an increasing trend in cell viability. Xue *et al.* showed that simvastatin suppressed the expression of the examined cytokines, with the highest alterations in IL-1 β and TNF-α, suggesting that simvastatin could relieve the inflammatory response.^[36] Thus, this anti-inflammatory effect could be a reason for increased cell viability. The findings of cell apoptosis also confirm that decreasing the concentration of simvastatin has reduced the rate of cell apoptosis; Thus, SIM 0.01 caused significantly less cell apoptosis compared to two other concentrations, and unlike them, the apoptosis rate did not significantly increase in each evaluation time compared to the previous one. The different effects of simvastatin on cell viability have been attributed to different types of cells studied, differentiation stage, the location from which the cell was harvested, and different laboratory conditions such as dose and interval.^[35] Also, Croons *et al.* suggested that different cell sensitivity to statin-induced death was related to the activity of the cellular enzyme HMG-CoA reductase, which is affected by simvastatin.^[37]

The results from the combination of the new bioceramic cement and simvastatin indicate that the concentration of simvastatin affects cytotoxicity and induced apoptosis. CEM/SIM 1 showed a decreasing trend in cell viability, although it was not statistically significant. In addition, the decrease in simvastatin concentration reversed the trend, and lower concentrations provided favorable conditions for cell proliferation. Also, it can be concluded that lower concentrations of simvastatin can reduce the apoptosis rate. It should not be overlooked that the presence of bioceramic cement in this compound could reduce

the severity of the negative effects of simvastatin, especially at a concentration of 1 μM . In other words, each of the three compositions of bioceramic cements containing simvastatin showed significantly higher cell viability and lesser apoptosis compared to pure simvastatin at respective concentrations. For example, CEM/SIM 1 showed about 20% higher cell viability and 27% less apoptosis compared to SIM 1, respectively. This difference can be attributed to the presence of bioceramic cement as a biocompatible material that can improve the conditions of cell activity and survival in the presence of simvastatin by previously discussed properties. It should be noted that along with the reduced concentration of simvastatin in the cement, the abovementioned difference became less obvious, and cell viability took an upward trend. The bioceramic cements containing 0.1 μM and 0.01 μM simvastatin had significant superiority in terms of cell viability compared to MTA; however, as mentioned earlier, after the initial setting and along with the reduction of the acidity of the medium exposed to MTA, the cell viability increases.

Regarding concerns about the use of formocresol in pediatric dentistry and affordability of MTA, inexpensive biocompatible material with desirable biological properties might be considered as an alternative. The results of the present study are basic steps to evaluate the new material.

CONCLUSION

Considering the limitations of the study, it can be concluded that:

1. In the presence of the new bioceramic cement, the viability of SHED is not endangered. Moreover, this cement can provide an environment for cell proliferation
2. Simvastatin at concentrations of 0.1 and 0.01 μM does not threaten cell viability and may even cause desirable biological properties that lead to cell proliferation
3. Bioceramic cement containing 0.1 and 0.01 μM simvastatin has no toxic effect on SHED in comparison with MTA.

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Conflicts of interest

The authors of this manuscript declare that they have

no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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