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Cytotoxicity of Calcium Silicate-Based Cements: Role of Bonding Time, Strategy, and Thickness in an *In Vitro* Model

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Introduction: This *in vitro* study assessed the effects of bonding application time (immediate vs. 24-hour delay) and strategy [self-etch (SE) vs. etch-and-rinse (ER)] on cytotoxicity of three calcium silicate-based cements [calcium-enriched mixture (CEM) cement, ProRoot mineral trioxide aggregate (MTA) and Biodentine] at 1mm and 2mm thicknesses. **Materials and methods:** Specimens (n=20 per group) were fabricated using CEM, MTA, and Biodentine. Scotchbond Universal was applied immediately or after 24 hours via SE or ER strategies. Cytotoxicity of cement extracts (100%, 50%, and 25% concentrations) on human gingival fibroblasts was evaluated using methyl thiazolyl tetrazolium (MTT) assay. Data were analyzed with four-way ANOVA and Tukey's test (P≤0.05). **Results:** The biomaterials showed comparable cytotoxicity (P>0.05), with toxicity decreasing progressively at lower concentrations (25% <50%<100%). At 100% concentration, ER bonding significantly improved cell viability for CEM (P<0.05), while immediate bonding of 1-mm specimens increased cytotoxicity (P<0.05). Immediate SE bonding of 1-mm MTA and Biodentine specimens also resulted in higher cytotoxicity (P<0.05). At 50% concentration, SE strategy and 1-mm thickness increased cytotoxicity, with bonding time effects significant only in ER mode. No significant differences were observed at 25% concentration (P>0.05). **Conclusion:** Delayed bonding (24 hours), ER strategy, and ≥2-mm thickness minimize cytotoxicity of calcium silicate-based cements. Clinically, immediate permanent restoration after vital pulp therapy is viable when considering these parameters, eliminating the need for interim protective layers (*e.g.*, glass ionomer bases).

Keywords: Biodentine; Calcium Derivatives; Calcium-Enriched Mixture Cement; Cell Survival; Dental Bonding; Endodontics; Mineral Trioxide Aggregate; Tooth Pulp Disease

Introduction

Considering the increasing popularity of conservative dental procedures and the tendency to preserve pulp vitality, the frequency of vital pulp therapy (VPT) is on the rise; it can be performed either directly or indirectly [1]. Pulp capping is performed for teeth with pulpitis or cases of mechanical pulpal exposure. Biocompatible, bio-interactive (the ability to release the required ions), and bioactive (the ability to form apatite) pulp capping agents are used for this purpose to help in reparative dentin formation by stimulation of pulp cells [2].

Concerning the type of materials used in VPT, calcium hydroxide-based materials were first used. However, they had drawbacks such as the inability to bond to dentin, the presence of tunnel-shaped defects, and being washed off over time [3]. Thus, they were gradually replaced with novel calcium silicate-based cements (CSCs) which are biocompatible and bioactive [4, 5]. Mineral trioxide aggregate (MTA) was the first CSC introduced, which has a long setting time. Bioactive materials such as Biodentine with a shorter setting time (~12 minutes) have gained the spotlight [6]. Calcium-enriched mixture (CEM) cement is another CSC with optimal properties such as favorable sealing ability, biocompatibility, induction of dentinal bridge formation, setting time <1 hour, and easy availability [7-9].

In VPT, after the application of a pulp capping agent, the application of one layer of glass ionomer base is recommended to protect the underlying pulp covering biomaterials i.e., CSCs. A researcher evaluated the bond strength of Biodentine to

composite mediated by a fourth-generation bonding agent with etch and rinse (ER) bonding strategy over time and found that Biodentine should be protected for a minimum of 72 hours with glass ionomer base prior to the etching process [10]. Other studies on MTA by using self-etch (SE) bond reported significantly higher bond strength compared with ER bonding of composite and showed that a sufficiently strong bond was achieved without protection by glass ionomer [11, 12].

Optimal timing for bonding is an important issue to take into account [13]. Conventionally and practically, bonding is performed immediately after the application of pulp capping materials in the clinical setting; while, immediate bonding may have a significant effect on cytotoxicity due to materials released from the CSCs. Considering the absence of a specific protocol by the manufacturers in this regard, and in an attempt to simplify the clinical procedures, this study aimed to assess the effect of time of universal bonding with SE and ER strategies on cytotoxicity of CEM cement, ProRoot MTA, and Biodentine using two different thicknesses [14].

The null hypothesis: there is no difference between materials, thicknesses etching stratrgies.

Materials and Methods

This study was conducted on CEM cement (BioniqueDent, Tehran, Iran), white ProRoot MTA (Dentsply Tulsa Denta, Tulsa, OK, USA), and Biodentine (Septodont, Saint-Maur-des-Fossés, Creteil, France) specimens. Characteristics of materials were shown in Table 1.

Specimen preparation

The sample size was calculated based on type-one error alpha=0.05, type-two error betta=0.2 (statistical power of the tests=80%), and large Cohen's effect size=0.8.Plexiglass sheets with 1 mm and 2 mm thicknesses were used to fabricate the molds. Cylinders with an external diameter of 5 mm were made with plexiglass. In the 2-mm thick upper layer, holes with 4.5 mm diameter were created for the application of composite resin. In the lower layer, holes with 4 mm depth were created. The second layer had 1 mm thickness for half and 2 mm thickness for the other half of the specimens. The two layers were attached with heat and pressure without using any adhesive.

The molds were immersed in 70% alcohol for one hour and were rinsed with phosphate-buffered saline twice for 5 minutes. After drying, each side of the mold was exposed to ultraviolet light under a biological hood for one hour.

Under sterile conditions, the CSCs were prepared according to the manufacturer's instructions and applied in the molds having 1 mm or 2 mm thicknesses. Half of the specimens made from each cement were subjected to SE strategy while the remaining half were acid etched (ER group). Scotchbond Universal (3M ESPE, St. Paul, MN, USA) was then applied and cured for 10 seconds with a LED curing unit (Woodpecker), and Z250 composite (3M ESPE, St. Paul, MN, USA) was then applied over it and cured [15]. A total of 120 specimens were evaluated (n=5 in each subgroup).

Obtaining the cement extract

The specimens were placed in a 24-well plate (three repetitions for each specimen). For sterilization, the plates were subjected to ultraviolet radiation for 30 minutes. The extract of the cement specimens was obtained using Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C and 98% humidity immediately after setting, and after 24 hours of incubation according to the 2021 version of ISO-10993-12. The volume of culture medium for each specimen was 1 mL per each cm^2 of the specimen surface (120 and 150 µL for cements with 1 mm and 2 mm thickness). The culture medium without specimens served as the control and was incubated for 24 hours similar to the intervention groups. The extracts were collected in sterile microtubes under sterile conditions [16].

Preparation of cells

Human gingival fibroblasts (HGF1 PI 1, C165) and KB cells (NCBI: C152) were obtained from the Pasteur Institute of Iran. HGFs had been isolated from the sound gingival biopsy of a 28-year-old female. The cells were thawed and cultured in 25 mL and 75 mL cell culture flasks in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% antibiotic (penicillin-streptomycin), and stored in an incubator with 95% humidity and 5% CO_2 at 37°C. After cell passage, cells in the logarithmic phase of growth were used for the methyl thiazolyl tetrazolium (MTT) assay.

Table 1. Cements used in this study and their composition

Material	Manufacturer	Composition
CEM	BioniqueDent, Iran	Powder: Calcium oxide, sulfur trioxide, phosphorus pentoxide, silicon dioxide Liquid: PBS
MTA	Angelus, Brazil	Powder: Tricalcium silicate, dicalcium silicate, tricalcium aluminate, ferroaluminate, tricalcium, calcium oxide, bismuth oxide Liquid: Distilled water
Biodentine	Septodent, France	Powder: Tricalcium silicate, dicalcium silicate, calcium carbonate, iron oxide, and zirconium oxide Liquid: Water, calcium chloride, and soluble polycarboxylate polymer

The Shapiro-Wilk and Levene tests were used to evaluate the normality of the distributions and equality of variances, and both the assumptions were confirmed.

MTT assay

The assay was performed according to ISO-10993-12-2021 and ISO-10-993-5-2009. On day one, cells in the logarithmic growth phase were counted under sterile conditions by using a hemocytometer slide and Trypan Blue stain and seeded at a density of 5000 cells per 100 µL of culture medium (containing serum and antibiotic) in each well of a 96-well plate (4 plates). The plates were then incubated at 37° C, 95% humidity, and 5% CO_2. On day two (24 hours after cell culture) the cells reached 50-70% confluence. The overlaying solution was removed, and the cells were treated with the prepared extracts (100 µL per each well). On day three (24 hours after cell exposure to cement extracts), the plates were removed from the incubator and the morphology of cells was evaluated under an inverted light microscope and digitally photographed. The overlaying medium was gently removed, and a culture medium containing 10% MTT dye was added (100 µL per each well). The plates were then incubated for three hours until the formation of purple formazan crystals. Next, the medium was removed, and 100 µL of dimethyl sulfoxide solvent was added to each well to dissolve the formazan crystals. The optical density (OD) of each well was read by an ELISA Reader. The following formula was used to calculate the percentage of cell viability.

$$Cell\ \text{Viability}\ (\%) = \frac{Abs_{treatment\ group}}{Abs_{control\ group}} \times 100$$

Where $Abs_{treatment\ group}$ is the mean OD of the group treated with cement extract and $Abs_{control\ group}$ is the mean OD of the negative control group. According to ISO-10993-5-2009, a material causing over 30% reduction in cell viability compared with the control group (100% viability) is considered cytotoxic.

Statistical analysis

The mean and standard deviation (SD) of OD values were calculated. Statistical analysis was carried out using GraphPad Prism version 9 by four-way ANOVA and Tukey's post-hoc test at 0.05 level of significance.

Results

The cytotoxicity of CSCs (CEM, MTA, Biodentine) was significantly influenced by bonding strategy, application time, and thickness. Table 2 presents the viability of HGFs following exposure to different cement extracts.

At 100% extract concentration, ER bonding markedly improved cell viability compared to SE, particularly for CEM cement (P<0.05), with 2-mm specimens showing 80.7±4.1% viability (vs. 61.7±8.8% for 1-mm immediate bonding). Immediate bonding of 1-mm specimens consistently increased cytotoxicity across all materials (P<0.05), with MTA and Biodentine exhibiting the lowest viability in SE mode (49.0±4.1% and 45.0±1.7%, respectively). Delayed bonding (24 hours) enhanced biocompatibility, as seen in MTA's viability improvement from 49.0±4.1% (immediate SE) to 79.3±4.8% (P<0.05).

Table 2. Viability of HGFs following exposure to different cement extracts

% HGF cell viability (Mean (SD), n=5) after 24 h treatment with 24 h extract						
Concentration	Cement	Bonding time	ER		SE	
			1 mm	2 mm	1 mm	2 mm
100%	CEM	1 s	61.7 (8.8)	80.7 (4.1)	44.5 (5.1)	55.6 (5.1)
		24 h	89.5 (5.5)	90.8 (5.3)	56.0 (4.0)	68.0 (2.8)
	MTA	1 s	71.9 (1.4)	87.5 (7.7)	49.0 (4.1)	60.1 (4.8)
		24 h	102.8 (1.0)	106.0 (6.1)	79.3 (4.8)	104.8 (3.3)
	Biodentine	1 s	78.4 (3.5)	83.2 (4.1)	45.0 (1.7)	64.4 (0.7)
		24 h	102.1 (4.8)	100.8 (4.1)	57.8 (10.4)	79.6 (4.5)
50%	CEM	1 s	75.7 (7.8)	85.3.7 (6.6)	68.3 (8.0)	68.3 (6.9)
		24 h	90.6 (8.2)	99.9 (4.0)	93.2 (1.5)	98.0 (4.1)
	MTA	1 s	76.7 (8.7)	83.2 (4.3)	66.1 (4.4)	92.3 (8.6)
		24 h	98.2 (8.3)	107.1 (2.5)	99.0 (6.0)	108.3 (7.9)
	Biodentine	1 s	103.3 (6.2)	101.1 (3.9)	84.6 (1.8)	78.7 (7.6)
		24 h	103.7 (7.9)	100.6 (3.1)	79.1 (7.0)	86.5 (15.4)
25%	CEM	1 s	93.2 (18.5)	100.6 (9.7)	105.8 (9.2)	102.1 (11.2)
		24 h	108.8 (4.0)	103.6 (12.7)	102.8 (7.1)	100.0 (1.4)
	MTA	1 s	100.0 (8.3)	99.7 (11.4)	86.6 (9.8)	98.2 (7.8)
		24 h	107.0 (12.6)	110.7 (12.5)	99.0 (6.0)	108.3 (7.9)
	Biodentine	1 s	100.6 (8.2)	103.4 (6.9)	102.0 (3.3)	103.1 (13.8)
		24 h	100.5 (3.4)	106.0 (4.2)	103.4 (10.4)	102.0 (12.3)

ER: Etch and rinse; SE: Self-etch; SD: Standard deviation

At 50% concentration, SE strategy and 1-mm thickness continued to elevate cytotoxicity, though effects were less pronounced. For CEM, SE/immediate bonding of 1-mm specimens reduced viability to 68.3±8.0%, while delayed bonding restored it to 93.2±1.5% (P<0.05). MTA's ER groups showed significant time dependency, with immediate bonding at 76.7±8.7% viability versus 98.2±8.3% after 24 hours (P<0.05). Biodentine's SE/1-mm specimens also underperformed (84.6±1.8% vs. 101.1±3.9% for ER/2-mm, P<0.05).

At 25% concentration, cytotoxicity diminished across all groups, with no significant differences between materials or strategies (P>0.05). Viability exceeded 85%, and some groups (*e.g.*, CEM SE/1-mm immediate) even showed proliferative effects (105.8±9.2%), likely due to diluted bioactive ions.

Four-way ANOVA revealed bonding strategy (P<0.001) and time (P<0.001) as the strongest predictors of cytotoxicity, followed by thickness (P<0.001). ER bonding reduced cytotoxicity by neutralizing alkaline byproducts, while delayed bonding allowed cement maturation, stabilizing ion release. The 2-mm thickness acted as a barrier, reducing toxic diffusion by 15–30% compared to 1-mm specimens. Material type (CEM, MTA, Biodentine) had no significant impact (P>0.05), confirming their comparable biocompatibility when optimized for clinical handling. These findings underscore that ER strategy, delayed bonding, and ≥2-mm thickness are critical for minimizing cytotoxicity, enabling immediate *permanent* restorations in VPT (Table 3).

Discussion

In this study null hypothesis partially rejected. We dates the critical roles of bonding strategy, application timing, and material thickness in modulating the cytotoxicity of CSCs used in VPT. Our findings align with and extend prior research, offering novel insights into optimizing clinical protocols for immediate permanent restorations.

Normally, the bonding agent is immediately applied after glass ionomer cement application in the clinical setting; however, there might be no need for this additional step of glass ionomer application, and the bonding agent may be applied immediately to the cement. Immediate and direct application of bonding agent, however, may affect the cytotoxicity due to materials released from the cement[17]. Thus, this study assessed the effect of time of a universal bonding with SE and ER strategies on the cytotoxicity of CEM cement, MTA, and Biodentine. The results showed that in the use of CEM cement, cell viability was significantly higher in ER bonding strategy.

Also, immediate bonding and 1 mm thickness caused higher cytotoxicity. In 50% concentration, immediate SE bonding caused higher cytotoxicity in comparison with that of ER strategy. For MTA, maximum cytotoxicity was noted in immediate SE bonding with 1 mm cement thickness. In 50% concentration, the SE bonding strategy and lower thickness resulted in higher cytotoxicity, and the effect of bonding application time was only significant in the use of ER bonding strategy. Regarding Biodentine, the SE bonding strategy and 1 mm cement thickness caused higher cytotoxicity, and bonding application time had no significant effect on cytotoxicity. In 50% concentration, no parameter had a significant effect on cytotoxicity. In the 25% concentration, no significant difference was noted in cytotoxicity with respect to any parameter. In total, cytotoxicity decreased by a reduction in the concentration of cement extracts. In high concentrations of cement extracts, CEM with SE bonding strategy showed the highest cytotoxicity.

Table 3. Results of ANOVA regarding the interaction effects of variables

Source	Type III Sum of Squares	df	Mean Square	F	*P*-value
Thickness	2570.44	1	2570.44	102.85	0.000
Time	8166.42	1	8166.42	326.78	0.000
Strategy	10614.24	1	10614.24	424.73	0.000
Cement	2478.77	2	1239.38	49.59	0.000
Thickness * Time	43.86	1	43.86	1.75	0.191
Thickness * Strategy	422.43	1	422.43	16.90	0.000
Thickness * Cement	32.29	2	16.14	0.64	0.529
Time * Strategy	0.29	1	0.29	0.01	0.914
Time * Cement	879.97	2	439.98	17.60	0.000
Strategy * Cement	348.97	2	174.48	6.98	0.002
Thickness * Time * Strategy	364.50	1	364.50	14.58	0.000
Thickness * Time * Cement	68.63	2	34.31	1.37	0.263
Thickness * Strategy * Cement	229.34	2	114.67	4.58	0.015
Time * Strategy * Cement	381.71	2	190.85	7.63	0.001
Thickness * Time * Strategy * Cement	62.72	2	31.36	1.25	0.294

The evaluation of three different extract concentrations for each cement in this study was employed for better simulation of the clinical setting[18]. A 100% concentration simulates fresh exposure; over time, the body fluids neutralize the extracts and decrease their concentration in the oral environment.

Researchers compared the cytotoxicity of dentin-bonding agents used in SE and ER modes. They demonstrated higher cytotoxicity in the SE bonding strategy, which was in line with the present findings [19]. Other researchers reported that the SE bonding strategy significantly increased cytotoxicity and apoptosis, compared with ER mode [20, 21]. However, some other researchers demonstrated contrary results when evaluated the cytotoxicity of different bonding agents; they indicated higher cytotoxicity of ER technique compared with SE mode [22-24]. This controversy may be due to variations in the methodology of studies, the use of different types of adhesives (6th and 7th generations used in the majority of such studies), longer etching time, and the use of different cell lines. In our study, Scotchbond Universal was applied on cements to simulate VPT in the clinical setting. Since some pulp capping agents are alkaline, the ER bonding strategy which includes the use of 37% phosphoric acid, probably neutralizes their alkalinity and creates a neutral environment, which is ideal for cells and results in lower cytotoxicity compared with the SE strategy.

Three different types of biomaterials were evaluated in the present study; out of which, CEM cement showed the highest cell viability in 100% concentration; however, it had no significant difference with Biodentine and MTA in this regard. The difference between 100% concentrations of MTA and Biodentine was not significant either. This finding was in agreement with the results of other researchers, since they found no significant difference in the cytotoxicity of MTA and Biodentine for murine odontoblasts [25]. Other researchers found no significant difference among MTA, Biodentine, and CEM cement in cytotoxicity against human dental pulp stem cells, which was in accordance with the present findings, despite the use of a different cell type [26].

In the present study, in the use of a 100% concentration of CEM cement and MTA, cytotoxicity immediately after mixing was significantly higher than that after 24 hours. Recently, similar results were reported by researchers; they reported significantly higher cytotoxicity of freshly mixed MTA compared with 24 hours after mixing [27]. They also showed no significant difference in cytotoxicity when lower concentrations of MTA were used, which was in agreement with the present results. The concentration of released products is much higher when cements are freshly mixed. Thus, higher cytotoxicity immediately after mixing compared with 24 hours is justifiable. Higher thickness serves as a barrier against the release of toxic products. In the present study, higher cytotoxicity was noted in the use of lower cement thickness. However, it should be noted that the effect of cement thickness on cytotoxicity was lower than that of bonding application time and bonding strategy.

HGFs were used in this study since they are in close correlation with the pulp tissue and are more suitable for cytotoxicity assessment than other cell lines [2, 17, 28]. However, since HGFs used in this study were isolated from one donor, drawing a definite generalizable conclusion would be difficult. According to ISO 10993-5, a primary cell culture model can be used for the assessment of the cytotoxicity of dental materials in vitro. However, it should be noted that in vitro studies have shortcomings, and the results cannot be directly generalized to the clinical setting. Therefore, in vitro design may be considered as a limitation of this study and we recommend it for future studies.

This study has several limitations. The *in vitro* design employed static culture conditions, which do not replicate the dynamic physiological environment of pulp tissue or account for immune responses. Human gingival fibroblasts were used instead of pulp-derived cells, potentially limiting biological relevance to pulpal healing. These constraints highlight the need for future investigations using *in vivo* models, 3D pulp-mimetic systems, or pulp-specific cell lines to enhance clinical translatability.

Conclusion

The tested CSCs exhibited comparable cytotoxicity, with delayed bonding significantly reducing toxicity regardless of material type/thickness. The SE strategy consistently demonstrated higher cytotoxicity than the ER approach, particularly in thinner/1-mm specimens. Cytotoxicity diminished over time and with reduced extract concentration, reaching negligible levels at 25% dilution. These findings validate that, immediate permanent restorations in VPT, are clinically viable when paired with an ER bonding strategy and ≥2-mm cement thickness, eliminating the need for interim protective layers. By optimizing these parameters, clinicians can streamline workflows while maintaining biocompatibility.

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Conflict of interest

None.

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Authors' contributions
Conceptualization: NP, Methodology: SA/HT/MT, Formal analysis and investigation: MT/NP, Writing-original draft preparation: RA, Writing-review and editing: SA/RA, Supervision: SA/RA All authors read and approved the final manuscript.

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