

# Cytotoxicity of Zirconomer and Conventional Glass Ionomer for L929 Murine Fibroblasts Over Time

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**Objectives** This study sought to assess the cytotoxicity of zirconomer and conventional glass ionomer (CGI) for L929 murine fibroblasts over time.

**Methods** In this in vitro, experimental study, 48 discs were fabricated from FX-II CGI and Shofu zirconomer and divided into three groups (n=16) for assessment of extracts obtained after 15 minutes (group 1), 24 hours (group 2) and seven days (group 3) of incubation following their initial polymerization. L929 murine fibroblasts were cultured and after 24 hours, they were exposed to extracts of the 48 discs in 144 wells. Cell-culture plates were incubated for 24, 48 and 72 hours. Cytotoxicity was evaluated using the methyl thiazolyl tetrazolium (MTT) assay. Data were analyzed by one-way and two-way ANOVA, Tukey's test and independent sample t-test (P<0.05).

**Results** At 24 hours, the 15-minute extract of both materials showed the highest cytotoxicity while the 7-day extract of the materials showed the lowest cytotoxicity. The 15-minute extract of zirconomer showed significantly higher cytotoxicity than CGI (P<0.05). At 48 hours, the cytotoxicity of 15-minute, 24-hour and 7-day extracts of zirconomer decreased. The results for CGI at 48 hours were similar to those at 24 hours. The 15-minute extract of zirconomer had significantly higher cytotoxicity than that of CGI (P<0.05). At 72 hours, the results in both groups were the same as those at 24 hours, and all zirconomer extracts showed significantly higher cytotoxicity than CGI extracts.

**Conclusion** The cytotoxicity of both materials decreased over time. Zirconomer showed higher cytotoxicity than CGI at all time points.

**Keywords** Materials Testing; Zirconium Oxide; Cytotoxicity; Glass Ionomer; Fibroblasts

## Introduction

Despite the advances in dental science, tooth decay remains a public health dilemma worldwide. In restoration of deep carious lesions, the applied restorative material is close to the pulp chamber. Toxic compounds released from the restorative materials may adversely affect the pulp cells and cause allergic reactions or tissue injury.<sup>1</sup> Thus, assessment of the cytotoxicity of restorative materials, alongside their optimal performance and durability, is an important prerequisite for their widespread clinical use to ensure their safety.<sup>2</sup> Dental pulp stem cells play an important role in preservation of pulp vitality, and restorative materials should not have any toxic effect on these cells.<sup>3</sup> Otherwise, pulp necrosis may occur due to the release of toxic compounds from the restorative materials and would necessitate endodontic treatment, imposing high cost on patients.

A wide range of tooth-colored restorative materials are available for caries restoration. Glass ionomer (GI) cement is among the most commonly used tooth-colored restorative materials.<sup>4,5</sup> It has unique properties and can chemically bond to both enamel and dentin. Also, it releases fluoride and exerts cariostatic effects. Its modulus of thermal expansion is close to that of tooth, and it is biocompatible as well. However, it has low tensile strength and low toughness, and is therefore fragile.<sup>4,7</sup> To overcome these shortcomings, some reinforcing agents were added to

GI cement. Zirconomer, also known as white amalgam, is a recently introduced restorative material with high strength, which has been reinforced with ceramic and zirconia fillers.<sup>8,9</sup> It contains zirconium oxide, glass powder, tartaric acid (1-10%), polyacrylic acid (20-50%) and deionized water. Baddeleyite (ZrO<sub>2</sub>) is the main constituent of the zirconomer powder that has a high concentration of zirconia (96.5% to 98.5%).<sup>10</sup> The manufacturer claims that zirconomer is a type of restorative GI with the strength and durability similar to those of amalgam while benefiting from the bacteriostatic effects of GI. The manufacturer has introduced two types of zirconomers into the market namely the conventional and the improved zirconomer. Improved zirconomer contains zirconia nano-fillers and has superior translucency compared with the conventional type. It is durable and rich in fluoride and therefore, is ideal for use as a bulk-fill restorative material in patients with high risk of caries.<sup>11</sup>

Success of restorative materials is determined by their durability and biocompatibility in the oral environment.<sup>8</sup> Cytotoxicity of GI cement has been extensively evaluated in previous studies.<sup>2, 5-7, 12, 13</sup> However, a consensus has not been reached on its biocompatibility as there is evidence regarding the cytotoxic effects of GI on human gingival fibroblasts.<sup>14</sup> Evidence shows that GI cements release aluminum ions<sup>15</sup>, and the effect of released aluminum ions on pulp health is a matter of concern. On the other hand, evidence shows that when applied in deep cavities without

pulp exposure, GI cement increases the inflammation and cellular infiltrates in the odontoblastic cell layer.<sup>15</sup> Another study showed cytotoxic effects of GI cements against L929 murine fibroblast cell line.<sup>16</sup> However, some others believe otherwise and justify that the protective effect of dentin has been disregarded in such studies.<sup>5,17</sup>

The methyl thiazolyl tetrazolium (MTT) assay is a colorimetric assay that is commonly used for assessment of cytotoxicity of materials and their effect on cell viability.<sup>18</sup> This method is accurate and provides reliable results.<sup>19</sup>

Considering the recent introduction of zirconomer into the dental market, its cytotoxicity has not yet been well evaluated. Therefore, this study aimed to assess the cytotoxicity of zirconomer and conventional GI (CGI) for L929 murine fibroblasts using the MTT assay.

## Methods and Materials

This in vitro, experimental study was conducted on 48 discs fabricated from CGI (FX-II, Shofu, Japan) and zirconomer (Shofu, Japan).

**Table 1-** Composition of conventional glass ionomer and zirconomer used in this study

Material	Component	Composition
FX-II Glass Ionomer LDT:081401	Powder	Fluoroaminosilicate glass, etc. >90%
	Liquid	Polyacrylic acid less than 50% (Cas No.9003-01-4). Tartaric acid less than 5% (Cas No.87-69-4), water over 50% (Cas No.7732-18-5)
Zirconomer LDT:12151180	Powder	Fluoroaminosilicate glass, zirconium oxide, dye, etc.
	Liquid	Polyacrylic acid solution, tartaric acid

For the fabrication of CGI samples (Shofu, Japan), the capsule was compressed and placed in an amalgamator (Ultramat, SDI, Australia) operating at 4000 rpm for 10 seconds to ensure adequate mixing of powder and liquid according to the manufacturer's instructions.

For the fabrication of zirconomer samples, two scoops of the powder were mixed with one drop of the liquid on a mixing pad. First, the entire liquid was mixed with half of the powder using a plastic spatula and then the rest of the powder was added and mixed to reach a putty-like consistency at room temperature ( $23\pm 1^{\circ}\text{C}$ ). The working time was 100 seconds, and the setting time was 150 seconds.

Next, 24 discs were fabricated from each cement using a sterile stainless steel mold measuring 8 mm in diameter and 2 mm in thickness (total of 48 discs,  $n=8$  for each material for assessment at each time point). After initial setting, the discs were UV-irradiated for 10 minutes and sterilized and placed in the bottom of the wells of a 48-well plate. Samples fabricated from each material were then divided into three groups (according to the extraction period following initial polymerization) of 15 minutes (group 1), 24 hours (group 2) and seven days (group 3) and incubated at  $37^{\circ}\text{C}$  and 97% humidity (Mettmert, Germany).

Culture of L929 murine fibroblasts:

L929 murine fibroblast cell line was obtained from the Pasteur Institute of Iran and cultured in complete media containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum, 100 UI/mL penicillin, and 100  $\mu\text{g}$  /mL streptomycin. Trypsin/ethylene amine tetra-acetic acid were used for cell passage. After reaching adequate confluence, cell suspension was prepared and distributed in a 96-well plate (4000 cells in each well). The cell culture plates were incubated at  $37^{\circ}\text{C}$  and 95% humidity for 24 hours (Mettmert, Germany).

Obtaining the material extracts:

According to ISO 10993-12, (20) the amount of extract required for each group was calculated for assessment of cytotoxicity at different time points. Also, according to ISO 10993-12, a total of eight discs of each material were required for assessment at each time point.

Table 1 shows the composition of CGI cement and zirconomer used in this study.

After removal from the incubator at the designated time, according to ISO 10993-12 (20), 1 mL of DMEM (Gibco, UK) was added to each sample in a 24-well plate and incubated at  $37^{\circ}\text{C}$ , 97% humidity and 5%  $\text{CO}_2$  for 24 hours. Next, the extract was collected from the wells and filtered using a 0.2- $\mu\text{m}$  membrane filter.

Exposure of L929 fibroblasts to extracts:

First, the overlaying medium in wells containing the cultured L929 fibroblasts in a 96-well culture plate was removed and replaced with the extract plus 10% fetal bovine serum, 100 UI/mL penicillin and 100  $\mu\text{g}$ /mL streptomycin with a total volume of 200  $\mu\text{L}$  per each well. Next, the MTT assay was performed at 24, 47, and 72 hours. The positive control well contained 200  $\mu\text{L}$  of distilled water. The negative control well contained complete culture medium (DMEM, 10% fetal bovine serum, 100 UI/mL penicillin, and 100  $\mu\text{g}$ /mL streptomycin).

MTT Assay:

After the incubation time of 24, 48, and 72 hours, the plates were removed from the incubator. The medium was removed and the cells were rinsed with phosphate buffered saline (0.15M) for three times. The MTT stock solution was prepared in phosphate buffered saline at a final concentration of 5mg/mL and sterilized by using a 0.22- $\mu\text{m}$

membrane filter. This stock solution was diluted with DMEM in 1:10 ratio. Then, 200  $\mu$ L of this solution was added to each well and incubated for 2-3 hours under standard conditions. After the incubation time, the MTT solution was removed and 200  $\mu$ L of dimethyl sulfoxide was added to each well to dissolve the formazan crystals. The optical density was read at 570 nm wavelength with a 620 nm reference by ELISA reader (Anthos 2020, Austria). The percentage of cell viability was determined by the MTT assay in each group in comparison with the negative control group.

#### Statistical analysis:

Data were analyzed using SPSS version 20 (SPSS Inc., IL, USA). Normal distribution of data was assessed by the Kolmogorov-Smirnov test. Data were then analyzed and compared using independent t-test, the Kruskal Wallis test

followed by the Bonferroni post-hoc test, one-way and two-way ANOVA and Tukey's post-hoc test. Level of significance was set at  $P < 0.05$ .

## Results

Table 2 shows the percentage of cell viability in the groups at different time points. The Kolmogorov-Smirnov test showed that the data were not normally distributed in the zirconomer, GI, positive control and negative control groups. Thus, the Kruskal-Wallis test was used for comparison of the groups, which showed a significant difference among the four groups at all three time points of 24, 48 and 72 hours ( $P = 0.001$ ).

**Table 2-** Mean percentage of cell viability in the groups at different time points

Groups	Time of obtaining extract	Time of MTT assay		
		24 hours Mean (SD)	48 hours Mean (SD)	72 hours Mean (SD)
Zirconomer	15 minutes	8.12 (2.01)	3.47 (0.49)	0.92 (0.18)
	24 hours	89.90 (12.16)	72.07 (2.16)	68.52 (4.93)
	7 days	89.17 (1.60)	85.12 (9.16)	70 (5.31)
Glass ionomer	15 minutes	33.95 (2.91)	12.90 (4.22)	3.15 (0.83)
	24 hours	95 (5.43)	85.87 (13.51)	83.62 (10.51)
	7 days	85.77 (9.61)	75.92 (7.74)	75.45 (3.91)
Positive control	-	3.37 (0.35)	3.52 (0.41)	1(0.21)
	-	100 (0)	100 (0)	100 (0)

Thus, pairwise comparisons were made with the following results:

#### At 24 hours:

The positive control group had a significant difference with the CGI group in terms of cell viability ( $P = 0.025$ ) but had no significant difference with the zirconomer group ( $P > 0.05$ ). The negative control group did not have a significant difference with zirconomer or GI group in this respect ( $P > 0.05$ ). The zirconomer and GI groups were not significantly different either ( $P > 0.05$ ).

#### At 48 hours:

The zirconomer group had a significantly lower percentage of cell viability than the negative control group ( $P = 0.026$ ). No other significant differences were noted ( $P > 0.05$ ).

#### At 72 hours:

The results were the same as those obtained at 48 hours. In other words, only the zirconomer and negative control groups were significantly different in terms of cell viability ( $P < 0.05$ ). The other comparisons did not yield significant results ( $P > 0.05$ ).

Effect of extraction time and type of material on cell viability at 24, 48 and 72 hours:

Two-way ANOVA was used to assess the effect of type of material and extraction period (15 minutes, 24 hours and 7

days) on the percentage of cell viability. The results showed that the effects of both material type ( $P = 0.004$ ) and extraction period ( $P = 0.001$ ) on cell viability were significant. The interaction effect of the two was also significant ( $P < 0.05$ ). Thus, one-way ANOVA was applied to compare different extraction periods, and independent t-test was used to compare the two materials. The results were as follows:

#### At 24 hours:

The difference in the percentage of cell viability was statistically significant for both materials at different time points ( $P < 0.05$ ) such that the lowest percentage of cell viability was noted in 15-minute extract while the highest cell viability was noted in 7-day extract and 24-hour extract. The difference in this respect was not significant between the 7-day and 24-hour extracts ( $P > 0.05$ ) while the 7-day and 24-hour extracts showed significantly higher percentage of cell viability than the 15-minute extract (both  $P = 0.002$ ). Comparison of the two materials in terms of extraction period revealed that the 24-hour ( $P = 0.473$ ) and seven-day ( $P = 0.511$ ) extracts of the two materials were not significantly different in terms of cell viability while the 15-minute extract of CGI showed higher cell viability than that of zirconomer ( $P < 0.001$ ).

#### At 48 hours:

The difference among the three extracts of zirconomer was significant ( $P<0.05$ ) such that its 15-minute extract showed the lowest and its 7-day extract showed the highest percentage of cell viability ( $P<0.001$ ). Also, the 24-hour extract of zirconomer showed higher cell viability than its 15-minute extract ( $P<0.001$ ). Its 7-day extract showed significantly higher cell viability than the 24-hour extract ( $P=0.02$ ). But, for CGI, the results were the same as those of the MTT assay at 24 hours. Comparison of cytotoxicity of zirconomer and CGI extracts yielded results similar to those obtained by the MTT assay at 24 hours.

At 72 hours:

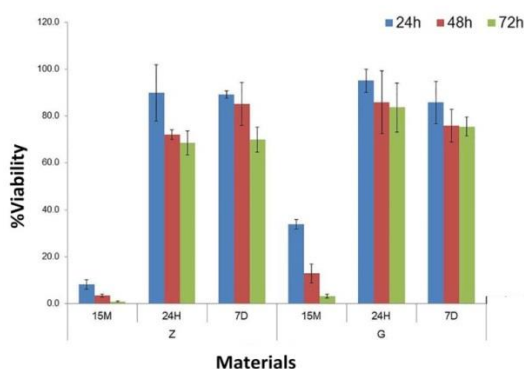
The percentage of cell viability for the 24-hour and 7-day extracts of both CGI and zirconomer was significantly higher than the 15-minute extracts of the two materials (both  $P<0.001$ ). However, the 24-hour and 7-day extracts of the two materials were not significantly different ( $P=0.454$ ). Comparison of zirconomer and CGI showed that CGI resulted in significantly higher cell viability than zirconomer ( $P<0.05$ ). Comparison of cell viability for different extracts of zirconomer and CGI:

Zirconomer:

One-way ANOVA was used to assess cell viability over time. The results showed a significant difference in cell viability at 24, 48 and 72 hours for each extraction period ( $P<0.01$  for all). Pairwise comparisons by the Tukey's test showed that the 15-minute extract caused the lowest cell viability at 72 hours while the highest cell viability was noted at 24 hours and this difference was significant ( $P<0.001$ ). Also, cell viability at 48 hours was higher than that at 72 hours and lower than that at 24 hours ( $P<0.001$ ).

The cell viability for the 24-hour extract was not significantly different at 48 and 72 hours ( $P=0.795$ ) while at both 48 ( $P=0.023$ ) and 72 ( $P=0.009$ ) hours, the percentage of cell viability was lower than that at 24 hours.

The percentage of cell viability for the 7-day extract was not significantly different at 24 and 48 hours ( $P=0.639$ ). Cell viability at 24 hours was higher than that at 72 hours ( $P=0.005$ ). Also, cell viability at 48 hours was higher than that at 72 hours ( $P=0.018$ , Figure 1).



**Figure 1:** Comparison of the percentage of cell viability at 24, 48 and 72 hours in use of different extracts of glass ionomer (G) and zirconomer (Z)

Glass ionomer:

The percentage of cell viability was not significantly different at different time points for the 24-hour ( $P=0.307$ ) and 7-day ( $P=0.143$ ) extracts. However, for the 15-minute extract, the percentage of cell viability was significantly different at different time points ( $P<0.001$ ) such that the lowest percentage of cell viability was seen at 72 hours and the highest at 24 hours ( $P<0.001$ ). Moreover, at 48 hours, the percentage of cell viability was significantly higher than that at 72 hours ( $P=0.003$ ) and less than that at 24 hours ( $P<0.001$ , Figure 1).

## Discussion

This study assessed the cytotoxicity of zirconomer and CGI and showed that at 24 hours, the 15-minute extract of both materials showed the highest cytotoxicity while the 7-day extract of the materials showed the lowest cytotoxicity. The 15-minute extract of zirconomer showed significantly higher cytotoxicity than CGI ( $P<0.05$ ). At 48 hours, cytotoxicity of 15-minute, 24-hour and 7-day extracts of zirconomer decreased, respectively. The results for CGI at 48 hours were similar to those at 24 hours. The 15-minute extract of zirconomer had significantly higher cytotoxicity than that of CGI ( $P<0.05$ ). At 72 hours, the results in both groups were the same as those at 24 hours, and all zirconomer extracts showed significantly higher cytotoxicity than CGI extracts.

In vitro assessment of cytotoxicity allows for the control of confounders, which is not often feasible in vivo. In vitro tests are affordable, cost-effective, and efficient for assessment of biological properties of dental materials and their biocompatibility (2). Chemical agents released from the restorative materials can affect fibroblasts in the dental pulp (2). Thus, L929 murine fibroblasts were used in this study according to ISO 10993-5.<sup>21</sup> This cell line resembles human cells and is easy to culture.<sup>2</sup> The MTT assay was used for assessment of cell viability in this study, which is sensitive and reliable and provides quantitative colorimetric results.<sup>3</sup> Also, ISO 10993-5<sup>21</sup> recommends its use. Moreover, both CGI and zirconomer used in our study were products of the same company and were highly similar in terms of composition. The only difference was presence of zirconia nanoparticles in zirconomer.<sup>9,11</sup> To the best of the authors' knowledge, this is the first study on the effect of extraction period of CGI and zirconomer on cell viability. Also, the cytotoxicity of zirconomer and CGI has not been previously confirmed. Thus, we compared our results with the findings of relatively similar studies.

Siqueira et al.<sup>22</sup> evaluated the cytotoxic effects of CGI on odontoblast-like cells using the MTT assay at 48 hours and showed that CGI was not cytotoxic and had effects similar to those of the negative control group. This finding was in agreement with our results regarding the CGI. Koohpeima et al.<sup>23</sup> evaluated the cytotoxic effects of CGI on human gingival fibroblasts using the MTT assay at 24 hours and indicated that it was not toxic for the cells, which was in agreement with our findings. Selimovic-Dragas et al. (6) evaluated the cytotoxic effects of CGI and resin modified GI on odontoblast-like cells and murine fibroblasts using the MTT assay at 24 hours and

reported that the CGI had the lowest cytotoxicity, which further confirmed our findings. Rodriguez et al.<sup>24</sup> evaluated the cytotoxic effects of CGI and resin modified GI on human gingival fibroblasts using lactate dehydrogenase assay at 72 hours and demonstrated that the CGI had the lowest cytotoxicity. Despite using different tests, the same results were obtained in the CGI group in our study. Tamilselvam et al.<sup>5</sup> compared the cytotoxic effects of Amalomer (containing crystalline zirconia) which is a ceramic-reinforced glass ionomer cement and CGI on L929 murine fibroblasts using the MTT assay at 24, 48 and 72 hours and showed that the cytotoxicity of Amalomer was less than that of CGI. However, our study showed higher cytotoxicity of zirconomer than CGI. This difference in the results of the two studies may be due to difference in the type of zirconia used, since zirconomer contains zirconia nanoparticles while Amalomer contains crystalline zirconia. Trumpaite-Vanagiene et al.<sup>25</sup> found that at one hour after polymerization of luting cements, they had the highest cytotoxicity, which decreased over time. This is due to the release of unpolymerized compounds during the first hour following polymerization. Our study also showed that cytotoxicity decreased over time and the 15-minute extract of both materials had the highest cytotoxicity. The 24-hour MTT assay revealed that zirconomer had the lowest percentage of cell viability with no significant difference with the positive control group. Also, the 48-hour and 72-hour MTT assays showed that zirconomer caused the lowest percentage of cell viability (significantly lower than that of the negative control group).

Lewis et al.<sup>2</sup> showed that unbound compounds in CGI may inhibit the progression of oral epithelial cell cycle and eventually result in cell death. On the other hand, cytotoxicity of GIs may be due to the presence of unpolymerized monomers such as polyacrylic acid in CGI by less than 50%. Its concentration in zirconomer has not been disclosed by the manufacturer.

Both GI and zirconomer are the same in terms of constituents with the difference that zirconomer contains zirconium oxide nanoparticles.<sup>9,11</sup> Particle size may play a role in cytotoxicity and it seems that smaller particles have higher cytotoxic effects. Siqueira et al.<sup>22</sup> compared the cytotoxicity of particles with different diameters and found that smaller particles (20 nm) had higher cytotoxicity than larger particles (80 nm to 110 nm). In the present study, zirconia nanoparticles may be the reason for higher cytotoxicity of zirconomer compared with CGI. On the other hand, it has been reported that zirconium oxide can cause cytotoxicity. For instance, Caicedo et al.<sup>26</sup>

indicated that zirconium ions in concentrations higher than 5 mM can cause apoptosis in T-helper Jurkat cells. Moreover, it was shown that zirconia with 0.6 µm particle size can induce apoptosis by increasing the release of tumor necrosis factor alpha by J744 macrophages. (27) Asadpour et al. (27) demonstrated the anti-proliferative effects of zirconium oxide nanoparticles on N2a cells (mouse neuroblastoma cell line) and PC12 (pheochromocytoma). Also, assessment of cytotoxicity of zirconium oxide using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay revealed that these effects were dose-dependent, and the cytotoxicity peak was at the first 12 hours following exposure. An association has been reported between oxidative stress and decreased cell viability. The unwanted effects of oxidative stress are due to increased lipid peroxidation, DNA damage, and induction of apoptosis. The mitochondrial protein thiol, especially glutathione peroxidase, plays an important role in cell protection against redox signaling and programmed cell death. Asadpour et al.<sup>27</sup> stated that zirconium nanoparticles decreased the activity of glutathione peroxidase and thus, oxidative stress may be a part of cytotoxic activity of these nanoparticles. This study had an in vitro design. Thus, generalization of results to the clinical setting must be done with caution. Future studies are required to assess the cytotoxic effects of zirconia, zirconomer and GI on the morphology of dental pulp cells and compare the cytotoxicity of these materials.

## Conclusion

Zirconomer showed the highest cytotoxicity at 24, 48 and 72 hours. The 15-minute extract of GI showed significantly less cytotoxicity than that of zirconomer. But, no difference was noted between zirconomer and CGI for the 24-hour and 7-day extracts. In general, the 15-minute extracts had the highest and the 24-hour and 7-day extracts had the lowest cytotoxicity.

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## Conflict of Interest

No Conflict of Interest Declared ■

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