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Evaluation of Cytotoxic Effect on Modified Glass Ionomer Cement with Calcium Carbonate: An *in vitro* Study

Kalsiyum Karbonat ile Modifiye Edilmiş Cam İyonomer Simanın Sitotoksisitesinin İncelenmesi: Bir *in vitro* Çalışma

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ABSTRACT Objective: Glass ionomers, commonly used as base materials, are also employed as restorative materials after modifications are made to their content. By altering their physical properties through additives, attempts have been made to impart antibacterial characteristics. This study aimed to assess the cytotoxic effects resulting from the addition of calcium carbonate (marble powder) to conventional glass ionomer luting cement on gingival fibroblast cells. **Material and Methods:** Conventional glass ionomer cement (Ionofil U, VOCO, Germany) was used in this study. The specimens were divided into three groups: Group A-Glass ionomer without marble particles (control). Group B-Glass ionomer with 5% wt. Calcium Carbonate (marble particles) added to the powder component. Group C-Glass ionomer with 10% wt. Calcium carbonate (marble particles) added to the powder component. Prepared samples were kept in medium for 24 hours. The cytotoxicity levels of the biomaterials used in the study were determined using the MTT (3-4.5-dimethyl-thiazolyl-2.5-diphenyltetrazolium bromide) method on gingival fibroblast cell. Statistical evaluation was performed using one-way analysis of variance and post hoc Duncan test ($p<0.05$). **Results:** Biomaterials other than those in Group A, when applied to fibroblast cells, did not exhibit cytotoxicity; instead, they stimulated fibroblast cell proliferation. Group A reduced fibroblast cell viability rates at concentrations of 100%, 50%, and 25%. **Conclusion:** Marble powder enhanced glass ionomers showed potential for clinical applications as samples did not show cytotoxic effects. Further physical tests are needed to assess the clinical suitability of the materials.

Keywords: Glass ionomer cement; cytotoxicity; fibroblast cells; marble powder; calcium carbonate

ÖZET Amaç: Cam iyonomer simanlar, başlangıçta kaide materyali olarak kullanılmakla birlikte içeriklerinde yapılan modifikasyonlarla restoratif dolgu materyali olarak da yaygın bir şekilde kullanılmaktadır. Bu modifikasyonlar sayesinde, simanın fiziksel özelliklerini değiştirme amacının yanı sıra antibakteriyel özellikler de kazandırılmaya çalışılmıştır. Bu çalışmada, geleneksel cam iyonomer simanın içerisine kalsiyum karbonat (mermer tozu) eklenmesinin, gingival fibroblast hücreleri üzerindeki sitotoksisite etkisinin değerlendirilmesi amaçlamaktadır. **Gereç ve Yöntemler:** Bu çalışma kapsamında, VOCO (Almanya) tarafından üretilen geleneksel cam iyonomer siman (Ionofil U) kullanılmıştır. Örnekler 3 gruba ayrılmıştır: Grup A-Herhangi bir partikül içermeyen cam iyonomer siman (kontrol grubu). Grup B-Toz bileşenine ağırlıkça %5 kalsiyum karbonat (mermer partikülü) eklenmiş cam iyonomer siman. Grup C-Toz bileşenine ağırlıkça %10 kalsiyum karbonat (mermer partikülü) eklenmiş cam iyonomer siman. Hazırlanan örnekler 24 saat besi yerinde bekletilmiştir. Kullanılan biyomateryallerin sitotoksisite düzeyleri MTT (3-4,5-dimetil-tiyazolil-2,5-difeniltetrazolyum bromür) yöntemi ile belirlenmiştir. İstatistiksel değerlendirmesi tek yönlü varyans analizi ve Duncan Post testleri ile yapılmıştır ($p<0,05$). **Bulgular:** Kontrol grubu (Grup A) dışındaki gruplarda fibroblast hücrelere uygulanan biyomateryallerin sitotoksik etkisi gözlenmemiştir; aksine, hücre üremesi teşvik edilmiştir. Kontrol grubunda ise fibroblast hücrelerinde canlılık oranlarında %100, %50 ve %25 konsantrasyonlarda azalma görülmüştür. **Sonuç:** Mermer tozu ile güçlendirilmiş cam iyonomer simanlar, testlerde sitotoksik etki göstermediğinden dolayı gelecekte klinik uygulamalar için potansiyele sahip olabilir. Malzemelerin klinik uygunluğunu değerlendirmek için ise daha ileri fiziksel testlere ihtiyaç duyulmaktadır.

Anahtar Kelimeler: Cam iyonomer siman; sitotoksisite; fibroblast hücresi; mermer tozu; kalsiyum karbonat

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Glass ionomer cement (GIC), developed in the 1960s by Alan Wilson and his colleagues as a replacement for dental silicate cements, is the most widely used cement in pediatric dentistry.[1,2]

GIC is a water-based cement that results from the acid-base reaction between polyacrylic acid and aluminosilicate glass powder. These materials are capable of physico-chemical bonding and releasing fluoride to the tooth structure. A biocompatible material for dental tissues, it is tooth-colored and effective in the remineralization process of dentin by fluoride charging. Due to these properties, glass ionomers are frequently used as restoration materials in pedodontics.[3-5]

However, glass ionomers are not currently sufficient as long term restorative materials. They lack sufficient durability and require better wear resistance. Therefore, many modifications have been made in recent years by adding various filling materials, which have been used to extend the clinical life of the glass ionomer.[6-11]

Marble (calcium carbonate) is a stone that forms when limestone ($CaCO_3$) and dolomitic limestones ($CaMg(CO_3)_2$) undergo metamorphism under heat and pressure, gaining a new structure as a result of recrystallization.[12] Easily polished and easily shining stones are called marble. Especially in the industrial sense, the term natural stone is also used instead of marble.[13] In the literature, we could not find a study on GIC modified by adding marble powder.

The reaction of tissues to these newly developed products should be tested before they are applied to people. Cytotoxicity tests have recently gained importance in evaluating the biocompatibility of dental materials. For this purpose, the use of fibroblast cells is recommended for investigating the cytotoxic effects of dental materials in in vitro studies.[14]

The study also aimed to evaluate the cytotoxicity effect of the addition of marble powder in conventional glass ionomer luting cement on the human gingival fibroblast (HGF) cells.

The null hypothesis was that the addition of the marble powder fillers had no cytotoxic effect on the HGF.

MATERIAL AND METHODS

In the study, cell culture experiments for the cytotoxic effects of modified GICs were carried out and their effects were studied.

In this in vitro experimental study, HGF cells were obtained frozen from the laboratories of the Department of Chemistry, Faculty of Arts and Sciences, Afyon Kocatepe University after making sure that these cells recovered from stress and returned to normal, they were processed to perform cytotoxicity assays.

Firstly, marble particles were prepared by being passed through a sieve (200 μ), then subjected to drying at 105 °C for 24 hours. They then went through a milling process for 10 minutes (ÜNALAN Engineering & Machinery Co., Türkiye). Subsequently, the size of the powder particles (<100 μm) was measured using a size analyzer (Mastersizer 2000, UK).

In this study, conventional glass-ionomer restorative material (Ionofil U, VOCO, Germany) was used. This powdered material contained calcium-alumino fluorosilicate glass, which was prepared with polyacrylic acid at a ratio of 1 scoop of powder: 1 drop of water, in accordance with the manufacturer's instructions. Then the marble particles were added to the powder component in proportions of 5% (Group B) and 10% (Group C) by powder weight (n=9). The specimens were categorized into three groups:

Group A: GIC without marble particles (control)

Group B: GIC with 5% wt. marble particles added to the powder component

Group C: GIC with 10% wt. marble particles added to the powder component

The effect of these groups on HGF cells was investigated using the extraction method. For this purpose, the medium used in the production of the HGF cells was used as the solvent. Glass ionomers, which are used as biomaterial in the analysis, were weighed 1 gram and placed in 15 sterile flasks. 10 mL of previously prepared RPMI 1640 (Sigma) medium was added to each sample. Thus, the solvent dissolved ratio during extraction was set to be 1:10. The extraction of biomaterials was performed for 24 hours

at 37 °C within the scope of ISO Standard 10993-12. After the medium obtained at the end of the extraction was passed through a 0.22 µm sterile injector filter, dilutions prepared with biomaterials and the medium in different proportions were applied to the cells. The dilution rates (biomaterial extract concentrations) used in the study are presented in Table 1.

The cytotoxicity levels of the biomaterials used in the study were determined by the MTT (3-4.5-dimethyl-thiazolyl-2.5-diphenyltetrazolium bromide) method. The cells to be planted for MTT analysis were counted using the trypan blue method. HGF cells were planted in 96 well plates with 200 µL of medium in each well. The cells were left in a 24-hour incubation to attach the seeded cells to the flask base. After incubation, the media in the wells were withdrawn without affecting the cells. In accordance with the predetermined well plan, pre-prepared biomaterial extracts and different dilutions each measured 200 µL. The same volume of fresh medium was added to the wells being used as the control group (Table 1). The cells were then left for another 24-hour incubation period.

MTT solution was prepared by dissolving MTT salt (Sigma) at a concentration of 5 mg/mL in phosphate buffer at pH 7.4 at the end of the 24 h incubation period. After incubation, 10% of the well volume (22 µL) of MTT solution was added to each well. Cells were incubated in this way for 2-4 hours. During this time, formosan crystals formed in the living cells under the influence of MTT. The liquid in the wells was aspirated with a pipette without affecting the crystals. To dissolve the formosan crystals, 200 µl DMSO was added to each well. The optical densities of the samples were determined at 540 nm using an ELISA microplate reader (BioTek, ELx800). The viability of the control group cells, which were not treated with biomaterial extracts or different dilutions, was accepted as 100% and the effect of each dose on cell viability was calculated using the formula below.[15,16] The viability rates of the cells at each dose used in the application were expressed as %.

Cell viability (%)=[(100*Absorbans sample)/(Absorbans control)]

STATISTICAL ANALYSIS

SPSS Statistics 18 (SPSS 18.0, IBM, USA) was used to analyse the data. The data obtained were defined as mean±standard deviation. First, an one way analysis of variance test was applied to the data to determine if there was a statistical difference. When a statistical difference between biomaterials and doses was observed, the Duncan post hoc test was used.

RESULTS

Table 2 shows the effects of different sample concentrations on cell viability 24 hours after use on HGF cells. Each group's cell viability increased at first, but this process slowed over time.

Figure 1 shows a control group with a decrease in cell viability values but no statistical difference. Figure 2 and Figure 3 show the cell increases in the groups to which we added calcium carbonate (marble powder). A statistically higher cell increase was found at the lower concentration in the Group B and C ($p<0.05$).

Except for Group A, no cytotoxic effect was observed in the other groups; on the contrary, it was ob-

TABLE 1: Dilution rates used in the study (biomaterial extract concentrations).

Concentrations (%)	Dilution rates (V/V)	Biomaterial extract volume used in dilution (mL)	Medium volume used in dilution (mL)
0	0	0	5
100	1:1	5	0
50	1:2	2.5	2.5
25	1:4	1.25	3.75
12.5	1:8	0.625	4.375
6.25	1:16	0.3125	4.6875
3.125	1:32	0.15625	4.84375

TABLE 2: The effect of biomaterial extracts on human gingival fibroblast cell viability.

Concentration (%)	Group A (% vitality)	Group B (% vitality)	Group C (% vitality)
0	100.5±10.6$^{+,\wedge}$	100.0±9.70*	100.0±9.75*
3.125	117.9±14.46$^{\#}$	129.1±14.27$^{\wedge}$	145.3±9.72$^{\wedge}$
6.250	117.3±11.91$^{\#}$	124.7±14.16$^{+,\wedge}$	149.2±17.05$^{\wedge}$
12.5	109.1±17.44$^{\#}$	126.7±11.67$^{+,\wedge}$	149.6±11.23$^{\wedge}$
25	91.25±10.40^{+}	116.8±8.36^{+}	145.3±9.72$^{\wedge}$
50	85.96±8.19*	119.1±11.54$^{+,\wedge}$	123.7±16.04^{+}
100	84.14±13.91*	118.8±11.94$^{+,\wedge}$	120.6±16.02^{+}

Data are given as mean±standard deviation (n=9). Statistical differences between the experimental groups are expressed as superscript with symbols ($^{*,+,\wedge,\#}$): The difference between the means carrying different exponential expressions in the same column with Duncan post hoc test ($p<0.05$).

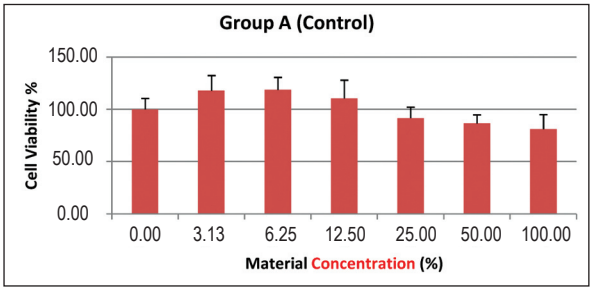

FIGURE 1: Group A effects of cell viability 24 hours after their application to human gingival fibroblast cells in different % concentrations.

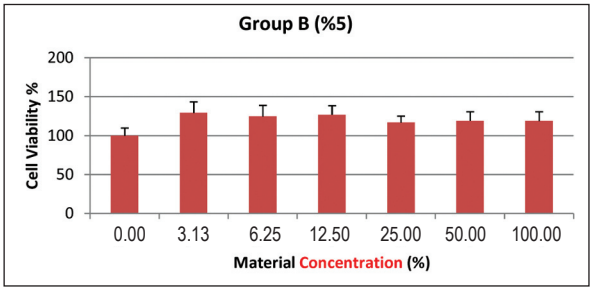

FIGURE 2: Group B effects of cell viability 24 hours after their application to human gingival fibroblast cells in different % concentrations.

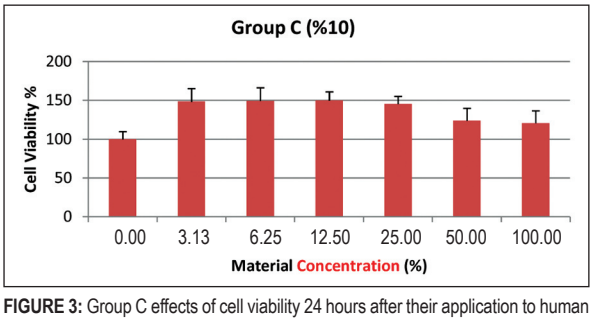

FIGURE 3: Group C effects of cell viability 24 hours after their application to human gingival fibroblast cells in different % concentrations.

served to cause HGF cell proliferation. Although HGF decreased the viability rates of HGF cells in Group A at 100%, 50%, and 25% concentrations, there was no statistical difference ($p>0.05$) (Table 2).

DISCUSSION

Biocompatible antibacterial glass-ionomer cement is essential for dentists who practice minimal intervention dentistry and alternative restorative techniques. It is significant because of its ability to promote oral health while reducing the need for invasive procedures.[3]

Because primary teeth have a shorter clinical life and a lower bite force than permanent teeth, all types of GICs are a good choice for use in children. Modifications to traditional GICs have improved their properties, including increased strength, superior handling, and increased wear resistance.[7,17]

Marble is formed when limestone undergoes metamorphism under high pressure and temperature, resulting in the formation of a new structure via recrystallization. The powders produced during marble extraction and processing are used in a variety of industries.[6,18-20] In our study we evaluated marble powder's biocompatibility in GIC. This study, it was the first study to be published in the literature that made the modification with calcium carbonate together with marble powder.

Many studies have been conducted to improve the physical properties of glass ionomers. A study using calcium-enriched eggshell discovered that it increased compressive strength.[7] We planned this study with the intention of evaluating it first in terms of biocompatibility. As a result, tests for durability were

not conducted, which we will highlight as one of the study's limitations.

The traditional cytotoxic effect has been studied. Selimović-Dragaš et al. demonstrated in their study that conventional glass ionomers are less toxic than other resin-modified GICs.[21] In this study, we used conventional glass ionomer.

de Souza Costa et al. evaluated the cytotoxicity of different GICs in their study. They found that conventional glass ionomers were less toxic than resin-containing glass ionomers. and they reported that conventional CIS showed high viability on fibroblast cells.[22] In our study, we found a decrease in vitality values in pure glass ionomers, but no statistical significance ($p>0.05$). On the other hand, a statistically significant difference was detected in marble powder added samples ($p<0.05$). When we look at the results, marble powders added to the glass ionomer did not cause cytotoxic effect, supporting our first hypothesis.

Zhu et al., showed that there was no cytotoxic effect observed of the glass ionomer modified by adding nanohydroxyapatite/polyhexamethylene biguanide. In this study, modified samples performed well improvements, and the cytotoxicity of modified GIC did not differ statistically from pure GIC.[23] In our study cell proliferation was observed as well, but no cytotoxic effect was observed.

Yu et al., in their study, modified with hexagonal boron nitride and titanium dioxide (h-BN-TiO2) discovered that viability tests were poor within 24 hours.[24] But, in our study, increased cell proliferation was observed at the end of 24 hours.

Kashyap et al., they reported that they did not detect any cytotoxic effects in a glass ionomer modified with carboxymethyl chitosan. They claimed that this newly developed material has anticariogenic and cytotoxic properties.[25] New biocompatible products can be used as a result of the non-cytotoxic properties discovered in our study.

Other modified forms of glass ionomers, such as resin-modified glass ionomers and glass carbomers, are available. The physical properties of these ionomers are comparable to those of conventional glass ionomers, but their biocompatibility is poor.[1] One more study silver reinforced glass ionomer is more toxic material than giomer- and resin-modified glass ionomer.[21] But in our modified glass ionomer study, marble added glass ionomer groups showed less toxicity effect than the control group.

Previous studies have reported that polyacrylic acid is the main cytotoxic factor in GICs. While GIC initially has a highly acidic character (pH: 1.6-3.7), its acidity decreases with the completion of the setting reaction (pH: 5.4-7.3).[21,26] In our study, we believe that the powder causes the reduced acidic effect. In a study on a glass ionomer modified with seashells, it was found to increase the pH in its content. Similar to our study, they did not investigate the cytotoxic effect of calcium carbonate reinforced glass ionomer and reported that it can be structurally incorporated into glass ionomers.[27] Our study may contribute to the literature by determining that glass ionomer modified with different calcium carbonate sources does not show cytotoxic effect.

LIMITATIONS

Similar to any in-vitro study, the limitation of the current study may be the use of static cell culture methods and cannot be created in cell culture studies to mimic the exact clinical scenario. The materials used in this study could have been better prepared in different ratios and evaluated. In addition, another limitation of the study is that only the cytotoxicity of the samples was investigated and other chemical and physical properties were not investigated.

CONCLUSION

Conventional GICs are constantly evolving in terms of durability. New materials should be tested for cytotoxicity while attempting to increase the durability of these materials. The cytotoxicity data of our samples in cell culture are promising in our study.

As a result, marble powder modified glass ionomers have the potential to be used in clinical applications. Furthermore, the clinical suitability of marble powder modified glass ionomers should be evaluated by evaluating properties such as strength and adhesion.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Mehmet Ünal;* ***Design:*** *Ömer Hazman;* ***Control/Supervision:*** *Mehmet Ünal;* ***Data Collection and/or Processing:*** *Ömer Hazman;* ***Analysis and/or Interpretation:*** *Burcu Güçyetmez Topal;* ***Literature Review:*** *Mehmet Ünal;* ***Writing the Article:*** *Mehmet Ünal;* ***Critical Review:*** *Burcu Güçyetmez Topal;* ***References and Fundings:*** *Mehmet Ünal, Ömer Hazman;* ***Materials:*** *Mehmet Ünal.*

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