Cytotoxicities of Different Resin Modified Glass Ionomer Cements Evaluated with MTT Test

Farklı Rezin Modifiye Cam İonomer Simanların MTT Testi ile Sitotoksisitelerinin Değerlendirilmesi

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ABSTRACT Objective: Resin-modified glass ionomer cements were developed by adding resin to conventional glass ionomer cements to improve its physical and mechanical properties. However this also produces negative properties such as poor biocompatibility. The potentially cytotoxic effects of resin cements are primarily due to the 2-hydroxyethyl methacrylate content. The purpose of this study was to determine and compare the cytotoxicity of three different resin-modified glass ionomer cements. **Material and Methods:** Three different resin-modified glass ionomer cements; Advance (Dentsply-Caulk, Milford, USA), Vitremer (3M Dental Products, St. Paul, USA), and Protec-CEM (Vivadent Ets., Schaan, Liechtenstein) were used in this study. Specimens of each type were prepared, each with 10 mm. diameter and 1mm. depth. These specimens were stored in deionized water for 10 minutes, 1 hour, 24 hours and 7 days. Eluates were collected and serial dilutions of the original (undiluted) eluates (1:2, 1:4, 1:8, 1:16) prepared. Solutions were passed through 0.22 μm (Millipore) sterile filters. Cytotoxicity levels were determined using the MTT method on L929 fibroblastic cell cultures in 96 well plates. **Results:** Evaluation of the data with the Kruskal-Wallis test revealed significant differences between the material groups and dilution subgroups were important ($p < 0.05$). Vitremer (3M) was the most cytotoxic for all four deionized water storage times. Eluates of Advance (Dentsply-Caulk) showed lower cytotoxic response than eluates of Protec-CEM (Vivadent Ets) from 10 minutes to 1 hour, ($p < 0.05$). **Conclusion:** Within the limitations of this investigation, eluates from three resin-modified glass ionomer cements (Advance, Vitremer, and Protec-CEM) were found to be cytotoxic to L-929 cell cultures.

Key Words: Cytotoxicity tests, immunologic; materials testing

ÖZET Amaç: Konvansiyonel cam ionomer simanların fiziksel ve mekaniksel özelliklerini geliştirmek için rezin ilave edilerek rezin modifiye cam ionomer simanlar üretilmiştir. Bununla birlikte biouyumsuzluk gibi negatif özellikler görülmüştür. Önceki çalışmalar 2-hidroksietil metakrilat içeriğinin olmasının sitotoksik özelliği arttırdığını göstermiştir. Bu çalışmanın amacı üç farklı rezin modifiye cam ionomer simanın sitotoksisitelerinin saptanarak karşılaştırılmasıdır. **Gereç ve Yöntemler:** Bu çalışmada 3 farklı rezin modifiye cam iyonomer siman; Advance (Denstply-Caulk, Milford, ABD), Vitremer (3M Dental Products, St. Paul, ABD), Protec-CEM (Vivadent Ets., Schaan, Liechtenstein) kullanılmıştır. Her gruptan 10 mm çapında ve 1 mm derinliğinde örnekler hazırlanmıştır. Bu örnekler deionize suda 10 dakika, 1 saat, 24 saat ve 7 gün süresince bekletilmiştir. Her bekletme süresi sonunda örneklerin bekletildiği sudan örnek sıvılar alınıp 1:1, 1:2, 1:4, 1:8, 1:16 oranında dilüsyonlar hazırlanmıştır. Seyreltilmiş bu solüsyonlar 0.22 mikrometre milipor steril filtrelerden geçirilmiştir. Örneklerin sitotoksisiteleri 96 kuyucuklu platelerde fibroblastik hücre kültürlerinde (L-929) MTT testi kullanılarak değerlendirilmiştir. **Bulgular:** Elde edilen değerler Kruskal Wallis yöntemi ile istatistiksel olarak analiz edilmiştir. Buna göre bütün grup ve alt gruplar arasında belirgin fark bulunmuştur ($p < 0.05$). 10 dakika, 1 saat, 24 saat ve 7 günlük değerlerin hepsinde Vitremer en toksik değerlere sahip olmuştur. Advance simanın 10 dakika ve 1 saat değerleri Protec-CEM'e göre daha az toksik değerler göstermiştir. **Sonuç:** Bu çalışmanın sınırları içerisinde kullanılan Advance, Vitremer, Protec-CEM rezin modifiye cam iyonomer simanlardan elde edilen solüsyonları L-929 hücre kültürü üzerinde sitotoksik etki göstermiştir.

Anahtar Kelimeler: Sitotoksisite testleri, immünolojik; malzemelerin denenmesi

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Glass-ionomer cements (GICs) are a type of bioactive dental material introduced in the early 70s. GICs are esthetic dental materials widely used as restorative, luting, lining, basing, fissure sealant and core build-up materials due to their ease of manipulation, fluoride release, chemical adhesion to enamel and dentin and their biocompatibility.[1,2] However; GICs have several inherent shortcomings; they have short working and long setting times; they show consistent sensitivity to dehydration, especially before maturation; they are susceptible to early moisture contamination; and they are brittle.[1, 2] To overcome these disadvantages of conventional GICs, resin-modified glass ionomer cements (RMGICs) were developed by combining conventional glass ionomer with resin composite components. RMGICs have improved physical and mechanical properties and handling characteristics yet retain the advantages of conventional GICs. RMGICs contain poly(acrylic)acids, photocuring monomers 2-hydroxyethyl methacrylate (HEMA) or a photocuring side chain grafted onto the poly(acrylic)acid, and ion-leaching glass.[3] RMGICs bond to tooth structure by both ion exchange reaction and micro-mechanical interlocking.[4] However, the resins are known to release free monomers such as 2-hydroxyethyl methacrylate. These monomers diffuse through dentin to the pulp space because of their hydrophilicity and low molecular weight.[4,5] Such resins have direct toxic effects on pulpal cells in vivo and can cause allergic responses in patients and dental workers.[2, 6] Finally, previous in vitro studies have shown that both fluoride ions or resin monomers can exhibit toxicity to cultured cells.[7] Cell culture tests are frequently used to evaluate the cytotoxic effects of dental materials. MTT (tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphnyltetrazolium bromide) assay is a good indicator of cell viability and was first described by Mosmann.[8] The MTT assay involves the reduction of water-soluble tetrazolium salt to water-insoluble formazan; after which formazan is dissolved in ethanol and quantified spectrophotometrically.[9-11] The purpose of this in vitro study was to evaluate the cytotoxicity potentials of the three different resin-modified glass ionomer cements .

MATERIAL AND METHODS

SAMPLE PREPARATION

Cylindrical samples of the three different resin-modified glass ionomer cements, Advance (Dentsply-Caulk, Milford, USA), Vitremer (3M Dental Products, St. Paul, USA), and Protec- CEM (Vivadent Ets., Schaan, Liechtenstein) were prepared in a special jig (10X1mm), allowing the preparation of standard samples. The powder/liquid ratio of each material was as follows (according to the manufacturers' directions): Protec-CEM 2/6, Vitremer 1/1, and Advance 1/3. Each sample was then placed in a test tubes containing 5 ml. of de-ionized double distilled water and stored for 10 minutes, 1 hour, 24 hours and 7 days. Eluates were collected at the end of each time interval and serial dilutions of the original (undiluted) eluates (1:2, 1:4, 1:8, 1:16) were prepared and sterile-filtered (Millipore 0,22 µm). The test groups are summarized in Table I.

CELL PROLIFERATION

The cells used for the experiment were L929 mouse fibroblastic cell culture. These were grown as monolayer cultures in 25T-flasks (Corning, Lowell, MA, USA) in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) (Sigma Chemical Co. St. Louis, MO, USA) containing 10% fetal calf serum (Biochrom AG, Berlin, Germany) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. After the second passage, the L929 fibroblastic cell line was plated in 96-well culture plates at an initial density of 30000 cells/ml and incubated in the same medium and the same humidified atmosphere. Cells cultured without test material were used as a control group. One sample was used for each well. At the end of each period, the culture medium was removed, and the cells were collected from the surface of the culture dish using 0.25% trypsin-EDTA (Sigma Chemical Co., St. Louis, MO, USA). L929 cells were counted with trypan blue (Sigma Chemical Co., St. Louis, MO, USA) and examined under a light microscope. Each experiment was repeated three times for each test material and control group.

TABLE 1: Glass ionomer cements used in the study.

Material	Chemical Composition	Type	Clinical Application	Manufacturer
Advance	**Powder:** Strontium alumino flurosilicate glass **Liquid:**oxiethyl methacrylate acid monomer	Resin modified glass-ionomer cement	Luting	Denstply-Caulk Milford, U.S.A.
Vitremer	**Powder:** Fluroalumino silicate glass **Liquid:** Methacrylate modified Policarboxylate acid, hydroxyethyl methacrylate	Resin modified glass-ionomer cement	Luting	3M Dental Pruducts, St. Paul ,U.S.A.
Protec-Cem	**Powder:** Ba-Al-Fluorosilicate glass, Ytterbium trifluoride, highly dispersed silicon dioxide, pigments **Liquid:** Deionized water, hydroxyethyl methacrylate, Dimethylacrylate, Methacrylate modified Polyacrylic acid	Resin modified glass-ionomer cement	Luting	Vivadent Ets, Schaan, Liechtenstein

CYTOTOXICITY (MTT ASSAY)

L929 fibroblastic cell line was plated in 96-well culture plates at an initial density of 30.000 cells/ml with test materials and incubated in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) (Sigma Chemical Co., St. Louis, MO, USA) containing 10% fetal calf serum (Biochrom AG, Berlin, Germany) in a humidified atmosphere of 95% air and 5% CO_2 at 37º. After 24 hours, the medium was removed from the wells and equal volumes of eluate were added. Cells cultured without the test material were used as the control group. The culture medium was removed from the wells and 100 µl RPMI-1640 without phenol red (Sigma Chemical Co. St. Louis, MO, USA) containing 12.5 µl MTT (tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphnyltetrazolium bromide) was filtered through a 0.22 µm filter and were added to each well. Culture plates were covered with aluminum foil and the cells were incubated in darkness for four hours at 37ºC in a CO_2 incubator. The MTT solution was then removed from the wells and 100 µl dimethyl sulfoxide (DMSO) was added to each well. Succinic dehydrogenase activity was measured as absorbance at 540 nanometer using an Ultra Violet visible spectrophotometer.

Statistical analysis

The data were analyzed with the SPSS 10.0 statistics program for Windows. The mean, median, minimum, maximum and standard deviations were calculated. Results were analyzed by Kruskal-Wallis test. The differences between the groups and the dilutions were significant ($p< 0.05$). The results of Duncan's test were also statistically significant ($p< 0.05$).

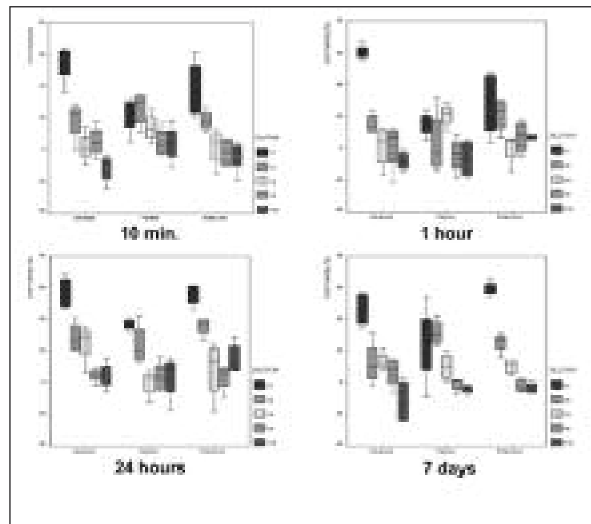

FIGURE 1: The biocompatibility of materials at different time intervals (letters show statistical differences between groups).

RESULTS

The results of the MTT assay showed that resin-modified glass ionomer cements are cytotoxic to L 929 cell cultures. Eluates from three resin-modified glass ionomer cements were cytotoxic to L 929 cell cultures when disks were collected from eluates after 10 minutes, 1 hour, 24 hours and 7 days exposure times ($p< 0.05$). Vitremer was the most cytotoxic for all four exposure times ($p< 0.05$). Eluates of Advance showed a lower cytotoxic response than Protec-CEM after 10 minutes and 1 hour

exposure times ($p< 0.05$). The leaching of toxic substances was markedly diminished in the 24-hour exposure conditions. In general, the rank order with respect to cytotoxicity at 7 days was as follows: Vitremer, Advance, Protec-CEM. The result showed that eluates from disks of Vitremer produced a significantly greater decrease in viable cell numbers than other eluates from 10 minutes to 7 days ($p< 0.05$) (Figure 1).

Samples were investigated under light microscope. For the Advance cement, eluates exposed for 10 minutes and 7 days at 0.5 dilution, and eluates exposed for 1 hour and 24 hours at 0.25 dilution had a toxic effect on cell culture. For the Vitremer cement, eluates exposed for 10 minutes, 1 hour, and 7 days at 0.5 dilution and eluates exposed for 24 hours at 0.25 dilution showed toxic effect on cell culture. For the Protec-CEM, the greatest toxic effect was observed from eluates exposed for 7 days, in which even a 0.0626 dilution had a toxic effect on cell cultures. Eluates exposed for 10 minutes at 0.125 dilution, for 24 hours at 0.25 dilution, and for 1 hour at 0.5 dilution showed toxic effects on cell culture. For the negative control group, even dilution of 0.00048828 had no toxic effect on cell cultures.

DISCUSSION

RMGICs have demonstrated reduced moisture sensitivity, improved mechanical strength, extended working time, and ease of clinical handling; however, those RMGICs containing low molecular weight substances such as HEMA, have been found more cytotoxic than GIC.[12, 13] Resin-based restorative materials can constantly release substances after extended exposure to an aqueouos environment, possibly causing moderate cytotoxic reactions and contributing to pulpal irritation. However, the cytotoxicity of resin-based restorative materials depends on the product tested, especially on the quality of leachable components. Therefore, optimum polymerization is necessary for those materials. Furthermore, the extractable amounts of leachable components should be reduced.[14] HEMA is a necessary component in RMGIC formulation as it enhances water solubility. HEMA release from RMGICs can diffuse dentinal tubules and exhibit cytotoxic effects on dental pulp tissue and osteoblasts and cause cell death by inducing apoptosis in cultured fibroblasts.[2,15]

The current study was designed to assess the possibility that leachable components from resin-modified glass ionomer cements might diffuse through dentin and cause cytotoxic effects on pulpal cells. Although the dynamics of pulpodentinal complex cannot be reproduced under in vitro conditions, it is probable that results from the indirect technique better reflect the risk for pulpal irritation when resin-modified glass ionomer cements are used.[16]

There are a variety of differences between in vitro test models for the screening of biomaterials for cytotoxicity. In direct contact tests, the biomaterial contacts the cell system directly, without barriers; in indirect contact tests, there is a barrier between the biomaterial and the cell system; while, in extract tests, eluates from materials are exposed to the cells.[17]

Differences in toxicity patterns at the various elution times may depend upon the degree of setting. This would be reflected in the rate of component leaching. Thus, the measurement of extracts taken after different exposure times could help to determine the long-term cytotoxicity of resin-modified glass ionomer cements.[14] In this study, the cytotoxicity of three resin-modified glass ionomer cements was evaluated by MTT assay using extracts in L929 fibroblastic cell culture. The MTT assay has several advantages; it is optimized in the 96-well format, complete dose response curves and greater sample comparisons can be made rapidly, and the method is economical in time and cost. Furthermore, the MTT method is based on intracellular biochemical changes, measuring cell viability rather than cell morbidity. This assay measured the reduction of MTT by those cells that remained viable after exposure and incubation with a test chemical or device.[9,18]

Exposure time may significantly influence the biocompatibility of dental resins. In this study, a variety of extract concentrations of L929 mouse fibroblasts were exposed for different periods of time. It has been demonstrated in laboratory situations

that leaching is essentially complete after 24 hours. However, while initial leaching may occur quickly, slower continued release is possible.[19] As other investigations have determined the quantities of leached monomers under specific in vitro experimental conditions with different elute types, sample sizes, and preparations, using different cytotoxicty test methods, an in vitro comparison between the cytotoxicity findings for RMGICs is not possible.

Nevertheless, although the cell systems, materials, and methods have differed, the cytotoxic nature of these materials was clearly shown.

Previous studies have shown that RMGICs are more cytotoxic than GICs.[20-22] Lan et al.[22] have found Protec-CEM, Compoglass, and Fuji II LC the most toxic to dental pulp cells. Kan et al.[1] found one RMGIC (Vitremer) highly cytotoxic, while a conventional GIC showed intermediate levels of toxicity and a second RMGIC (Fuji II LC) and a resin composite were not cytotoxic.[1] Previous studies found that resin-modified glass ionomers cytotoxic to cultured cells.[13] The results of this study were in agreement with previous studies, showing that RMGICs are cytotoxic to L929 fibroblastic cell culture. Eluates from three resin-modified glass ionomer cements were cytotoxic to L-929 cell cultures when collected in disks from eluates at 10 minutes, 1 hour, 24 hours and 7 days time periods ($p< 0.05$). Vitremer was the most cytotoxic at 10 minutes, 1 hour, 24 hours and 7 days.

However, the formulation of RMGICs usually includes a vinyl-modified polyalcenoic acid water-soluble metacrylate, such as hydroxyethyl methacrylate, an ion-leaching glass, and water. HEMA may be a major contributor to pulp toxicity. HEMA can diffuse through the dentin, especially if it is thin or acid-treated and this may be relevant to the risk of adverse pulp reactions.[10,21,23]

In vitro screening tests are very helpful in assaying the biologic effects of dental materials but may be limited in their ability to simulate clinical conditions. RMGICs could constantly release substance after exposure to an aqueous environment for extended periods, possibly causing cytotoxic reactions and contributing to pulpal irritation. However, the cytotoxicity of RMGICs varies depending on the product tested and the types of leachable components. Optimum polymerization is necessary for those materials to reduce the extractable amounts of these components.

CONCLUSION

Within the limitations of this investigation, eluates from three resin-modified glass ionomer cements were cytotoxic to L 929 cell cultures when collected from eluates at 10 minutes, 1 hour, 24 hours and 7 days time periods. Vitremer was the most cytotoxic at 10 minutes, 1 hour, 24 hours and 7 days.

REFERENCES

1. Kan KC, Messer LB, Messer HH. Variability in cytotoxicity and fluoride release of resin-modified glass-ionomer cements. J Dent Res 1997;76(8):1502-7.
2. Souza PP, Aranha AM, Hebling J, Giro EM, Costa CA. In vitro cytotoxicity and in vivo biocompatibility of contemporary resin-modified glass-ionomer cements. Dent Mater 2006;22 (9):838-44.
3. Goldberg M. In vitro and in vivo studies on the toxicity of dental resin components: a review. Clin Oral Investig 2008;12(1):1-8.4.
4. Xie D, Park JG, Zhao J. Synthesis and preparation of novel 4-arm star-shape poly(carboxylic acid)s for improved light-cured glass-ionomer cements. Dent Mater 2007;23(4):395-403.
5. Aranha AM, Giro EM, Souza PP, Hebling J, de Souza Costa CA. Effect of curing regime on the cytotoxicity of resin-modified glass-ionomer lining cements applied to an odontoblast-cell line. Dent Mater 2006;22(9):864-9.
6. Mazzaoui SA, Burrow MF, Tyas MJ, Rooney FR, Capon RJ. Long-term quantification of the release of monomers from dental resin composites and a resin-modified glass ionomer cement. J Biomed Mater Res 2002;63(3):299-305.
7. Bouillaguet S, Assaf, Y, Virgillito, M, Holz, J, Meyer, JM. In vitro cytotoxicity of resin reinforced glass ionomer cements. Acta Med Dent Helv 1999(4):14-9.
8. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65(1-2):55-63.
9. Bean TA, Zhuang WC, Tong PY, Eick JD, Chappelow CC, Yourtee DM. Comparison of tetrazolium colorimetric and 51Cr release assays for cytotoxicity determination of dental biomaterials. Dent Mater 1995;11(5):327-31.
10. Kaga M, Noda M, Ferracane JL, Nakamura W, Oguchi H, Sano H. The in vitro cytotoxicity of eluates from dentin bonding resins and their effect on tyrosine phosphorylation of L929 cells. Dent Mater 2001;17(4):333-9.
11. Lönnroth EC, Dahl JE. Cytotoxicity of dental glass ionomers evaluated using dimethylthiazol diphenyltetrazolium and neutral red tests. Acta Odontol Scand 2001;59(1):34-9.

12. Chen CC, Chen RC, Huang ST. Enzymatic responses of human deciduous pulpal fibroblasts to dental restorative materials. J Biomed Mater Res 2002;60(3):452-7.

13. Xie D, Yang Y, Zhao J, Park JG, Zhang JT. A novel comonomer-free light-cured glass-ionomer cement for reduced cytotoxicity and enhanced mechanical strength. Dent Mater 2007;23(8):994-1003.

14. Huang FM, Chang YC. Cytotoxicity of resin-based restorative materials on human pulp cell cultures. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002;94(3):361-5.

15. Koulaouzidou EA, Economides N, Beltes P, Geromichalos G, Papazisis K. In vitro evaluation of the cytotoxicity of ProRoot MTA and MTA Angelus. J Oral Sci 2008;50(4):397-402.

16. Hanks CT, Wataha JC, Parsell RR, Strawn SE, Fat JC. Permeability of biological and synthetic molecules through dentine. J Oral Rehabil 1994;21(4):475-87.

17. Cao T, Saw TY, Heng BC, Liu H, Yap AU, Ng ML. Comparison of different test models for the assessment of cytotoxicity of composite resins. J Appl Toxicol 2005;25(2):101-8.

18. Can G, Ates İ. [The effect of metal ions on viability of L 929 mouse fibroblasts]. Turkiye Klinikleri J Dental Sci 2009;15 (3):167-72.

19. Issa Y, Watts DC, Brunton PA, Waters CM, Duxbury AJ. Resin composite monomers alter MTT and LDH activity of human gingival fibroblasts in vitro. Dent Mater 2004;20(1): 12-20.

20. Consiglio R, Rengo S, Liguoro D, Riccitiello F, Formisano S, Palumbo G, et al. Inhibition by glass-ionomer cements of protein synthesis by human gingival fibroblasts in continuous culture. Arch Oral Biol 1998;43(1):65-71.

21. Bakopoulou A, Mourelatos D, Tsiftsoglou AS, Giassin NP, Mioglou E, Garefis P. Genotoxic and cytotoxic effects of different types of dental cement on normal cultured human lymphocytes. Mutat Res 2009;672 (2):103-12.

22. Lan WH, Lan WC, Wang TM, Lee YL, Tseng WY, Lin CP, Jeng JH, et al. Cytotoxicity of conventional and modified glass ionomer cements. Oper Dent 2003;28(3):251-9.

23. Xie D, Chung ID, Wu W, Lemons J, Puckett A, Mays J. An amino acid-modified and non-HEMA containing glass-ionomer cement. Biomaterials 2004;25(10):1825-30.