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Cytotoxicity of resin modified glass ionomer cements on dental pulp stem cells

Rezin ile modifiye edilmiş cam iyonomer simanların diş pulpası kök hücreleri üzerindeki sitotoksisitesi

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ABSTRACT

Objective: The aim of this study was to observe the cytotoxic effect of Resin modified Glass Ionomer Cement (RMGIC) on human dental pulp stem cells (DPSCs) proliferation by using xCELLigence®, a device that measures real-time cell viability and evaluates cytotoxic effects, and to determine the CC_{50} value on these cells for 72 hours.

Methods: DPSCs obtained from the American Type Culture Collection were seeded on E-Plates®. After 24 hours, three different dilutions (100%, 10% and 1%) of the elution obtained from RMGIC were added to three wells. DMEM solution was used as the control group. Real time cell index data were acquired by using xCELLigence® device for 72 hours. In order to compare cell index values, repeated-measures analysis of variance and linear regression analysis were used.

Results: In contrast to the 100% dilution of RMGIC which exhibited toxic effect on DPSCs, its 1% dilution showed proliferative effect. And 10% dilution was similar to the control group. While the coefficient of determination was above 80% in all groups, it was found to be lower only in the RMGIC 100%-dilution group by 0.7%. Also, the regression coefficient was found to be significantly different from zero in all equations except RMGIC 100%-dilution group ($P < .001$). CC_{50} values of RMGIC at the 24th, 48th and 72nd hours were 5.07%, 5.07% and 5.08%, respectively.

Conclusion: In order to provide more reliable results, CC_{50} values determined in our study will guide the further studies to improve RMGICs by adding different molecules to its structure for reducing its cytotoxicity.

Keywords: Glass ionomer cements, dental pulp stem cell, mesenchymal stem cell, cytotoxicity, xCELLigence

ÖZ

Amaç: Bu çalışmanın amacı, gerçek zamanlı hücre canlılığını ölçen ve sitotoksik etkileri değerlendiren bir cihaz olan xCELLigence® kullanarak rezin ile modifiye cam iyonomer simanın (RMCİS) insan dental pulpa kök hücrelerinin proliferasyonu üzerindeki sitotoksik etkisini gözlemlemek ve 72 saat boyunca bu hücreler üzerindeki CC50 değerini belirlemektir.

Yöntemler: American Type Culture Collection'dan elde edilen diş pulpası kök hücreleri E-Plates® üzerine ekildi. 24 saat sonra RMCİS'dan elde edilen elüsyonun üç farklı dilüsyonu (%100, %10 ve %1) üçer kuyucuğa eklenmiştir. Kontrol grubu olarak DMEM solüsyonu kullanılmıştır. 72 saat boyunca xCELLigence® cihazı kullanılarak gerçek zamanlı hücre indeks verileri elde edildi. Hücre indeks değerlerini karşılaştırmak için tekrarlayan ölçümlerde varyans analizi ve lineer regresyon analizi kullanılmıştır.

Bulgular: Diş pulpası kök hücreleri üzerinde toksik etki sergileyen RMCİS %100 dilüsyonunun aksine, %1'lik seyreltme proliferatif etki gösterdi. %10 seyreltme ise kontrol grubuna benzerdi. Bütün gruplarda varyasyon açıklama katsayısı %80'in üzerinde iken sadece RMCIS 100% grubunda %0,7 olarak daha düşük tespit edilmiştir. Ayrıca, regresyon eşitliklerindeki eğimi veren regresyon katsayısı RMCİS 100% dışındaki bütün denklemlerde sıfırdan anlamlı derecede farklı bulunmuştur ($P < .001$). Rezin ile modifiye cam iyonomer simanın 24., 48. ve 72. saatlerde CC_{50} değerleri sırasıyla %5.07, %5.07 ve %5.08 idi.

Sonuç: Daha güvenilir sonuçlar sağlamak için çalışmamızda belirlenen CC_{50} değerleri, RMCİS'lerin sitotoksisitesini azaltmak için yapısına farklı ajanların katılarak RMCİS'leri iyileştirmeye yönelik bundan sonraki çalışmalara rehberlik edecektir.

Anahtar Kelimeler: Cam iyonomer siman, diş pulpası kök hücresi, mezenşimal kök hücre, sitotoksisite, xCELLigence-

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INTRODUCTION

Glass ionomer cements (GICs), first discovered in 1972 by Wilson and Kent under the name ASPA (Alumino silicate polyacrylic acid), are often preferred as restorative materials due to their advantages such as fluoride release, dentin adhesion and esthetic color.[1] In addition to all these advantages, their fragile structures are disadvantageous, thus resin modified glass ionomer cements (RMGICs) were developed in the late 1980s in order to improve their mechanical properties.[2]

While conventional GICs have been suggested to have minimal toxicity, RMGICs have been shown to be cytotoxic and genotoxic.[3,4] 2-hydroxyethyl methacrylate (HEMA) monomer, which is the main component of RMGIC may cause various biological side effects such as cytotoxicity, recurrent infections, respiratory problems, apoptosis and contact dermatitis.[5] Reactive oxygen species (ROS) production, intracellular glutathione consump-

tion and increase of cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) expression were found to be effective in inducing HEMA's cytotoxicity by triggering oxidative stress in some in vitro studies.[6-9] Furthermore, HEMA genotoxically induces mutant number and micronucleus formation.[10, 11] Other important factors that play a significant role in RMGIC cytotoxicity are its acidity or ingredient ions such as Zn^{+2}, Al^{+3}, Fe^{+2}.[12,13] Also, glass ionomers with high levels of fluoride release was shown to have high cytotoxic effect to DPSCs.[14]

Cytotoxicity of RMGIC lining cements was shown to be decreased with the curing time.[15] Also, successful results were obtained in reducing RMGIC cytotoxicity by adding substances such as chlorhexidine, glutaraldehyde, stannous fluoride, doxycycline hyclate to its structure.[16-18] However, CC_{50} (cytotoxicity concentration 50%) values, which have an important role in the expression of toxicity by indicating the cytotoxic concentration of extracts that kills 50% of the host's living cells, were not included in these studies. When various agents were added to the RMGIC structure, consideration of CC_{50} values would be a more reliable approach in the comparison of the changes in its cytotoxicity.[19]

The aim of this study was to evaluate the RMGIC's cytotoxic effect on dental pulp stem cell (DPSC) for 72 hours and to determine its CC_{50} value using a real-time cell viability measuring device (xCELLigence® system). The null hypothesis is that RMGIS would have no cytotoxic effect on DPSCs.

MATERIAL AND METHODS

DPSC line, obtained from the American Type Culture Collection, was used in our experiment. DPSCs were incubated with DPSC culture medium at 37°C in a high humidity, 5% CO_2, 95% air incubator (NuAire®, Plymouth, USA). Culture flasks were monitored daily and culture medium was changed once every three days. DPSCs, grown to confluency (>90%) were harvested from the tissue culture flasks on the 7th day. 5000 cells were seeded into 12 wells of an E-Plateâ (ACEA Biosciences, San Diego, USA) containing 100 µL of Dulbecco's modified essential medium (DMEM) (Lonza, Verviers, Belgium) per well and incubated for 24 hours.

RMGIC (Fuji II LC, GC Corporation, Tokyo, Japan) samples were prepared according to the ISO 10993-12:2004 [20] standards, with a diameter of 9 mm and a height of 7 mm. The samples were immersed in DMEM supplemented with 15% fetal bovine serum (BioWest®, Miami, USA) and 1% PSA (Penicillin, Streptomycin, Amphotericin B; Lonza®, Walkersville, USA) for 24 hours at a surface area-to-volume ratio of 3 cm^2 : 1 mL to generate 100% extracts. Two serial (1:10) dilutions were then prepared by adding DMEM to the resulting media containing the RMGIC eluates. Finally, solutions of RMGIC in 3 different concentrations (1:1, 1:10 and 1:100) were obtained.

After the solution in the E-Plate® was removed at the 24th hour, 100 µl of freshly prepared solutions were added to the wells with three replicates for each concentration. Also, 100 µl of DMEM was added to the remaining three wells as control group. Afterwards E-Plate® was placed in xCELLigence® device and incubated.

DPSCs were monitored every 15 min for a period of 72 hours using xCELLigence® system to observe the cytotoxic effect of RMGIC on DPSCs. As a result of the assay, time-dependent proliferation graphs were obtained using the RTCA-integrated software of the xCELLigence® system (ACEA Biosciences, Inc., California, USA).

The statistical evaluations used in this study were performed with SPSS 25.0 (SPSS Inc., Chicago, USA) at a significance level of 5%. Cell index values measured in 4 different groups at 3 different times were evaluated by repeated-measures analysis of variance. Duncan's test was used to determine the differences between the groups. Linear regression analysis of time-dependent proliferation of cells were performed in GraphPad Prism® 7.0a for Mac OS X (GraphPad Software, Inc., California, USA).

RESULTS

The impedance averages obtained from 3 different wells of each group were expressed as arbitrary units called cell index (Figure 1a). Cell index values in all wells were normalized at the 24th hour using RTCA. In Figure 1b, control group was used as a reference to observe cell index changes between the groups more clearly.

When we assessed average cell index values, we observed higher, similar and lower proliferation rates in 1%, 10% and 100% RMGIC eluate concentrations respectively, in comparison to the control group in all time periods (Table 1, $P < .001$).

Different letters in the same column indicate statistically significant differences between groups ($P < .05$).

Three different RMGIC concentrations were introduced in the wells filled with DPSC.

Linear regression analysis was used to evaluate the time-dependent shifts in cell index values for the following 72 hours (Figure 2). Obtained regression lines were given in Table-1 with the formula "y = a + bx" (y: cell index value, a: regression constant, b: regression coefficient, x: time in hours after adding RMGIC eluate).

The increase in the index value per hour in the RMGIC 1% group was greater than in all other groups by 0.1056. In addition, compared to the control group, the regression coefficient was slightly higher at RMGIC 10% and lower at RMGIC 100%. While the coefficient of determination was above 80% in all groups, it was found to be lower only in the RMGIC 100% -dilution group by 0.7%. Also, the regression coefficient, showing the slope in the regression equations, was found to be significantly different from zero in all equations, except RMGIC 100% ($P < .001$).

CC_{50} values were obtained from the dose response curves of RMGIC eluates at the 24th, 48th and 72nd hours (Figure 3). CC_{50} values of RMGIC at the 24th, 48th and 72nd hours were 5.07%, 5.07%, and 5.08%, respectively.

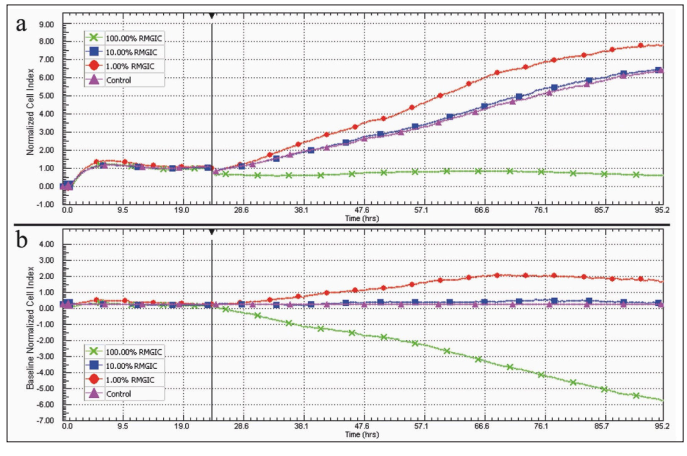

Figure 1. (a) Cell index change and (b) standardized cell index change compared to the control group observed in xCELLigence® for 72 hours following the addition of RMGIC dilutions to DPSCs at the 24th hour.

Table 1. Mean cell index values and standard deviations of experimental groups for different time intervals

Concentration of RMGIC Dilution	0-24 Hours	0-48 Hours	0-72 Hours
0% (Control)	1.66 ± 0.56[b]	2.59 ± 1.22[b]	3.59 ± 1.85[b]
1%	2.04 ± 0.84[c]	3.46 ± 1.78[c]	4.71 ± 2.36[c]
10%	1.68 ± 0.53[b]	2.67 ± 1.15[b]	3.7 ± 1.76[b]
100%	0.64 ± 0.11[a]	0.73 ± 0.19[a]	0.73 ± 0.27[a]
P	<.001	<.001	<.001

Different letters in the same column indicate statistically significant differences between groups ($P < .05$).

Table 2. Linear regression analysis of the groups.

Concentration of RMGIC Dilution	Regression Equation	R^2	*P*
0% (Control)	Y = 0.6388 + 0.0807*X	0.8352	<.001
1%	Y = 0.8447 + 0.1056*X	0.8718	<.001
10%	Y = 0.6420 + 0.0836*X	0.9899	<.001
100%	Y = 0.6817 + 0.001083*X	0.0071	0.1534

R^2: The proportion of variance explained
P: The significance of the slope of the regression line

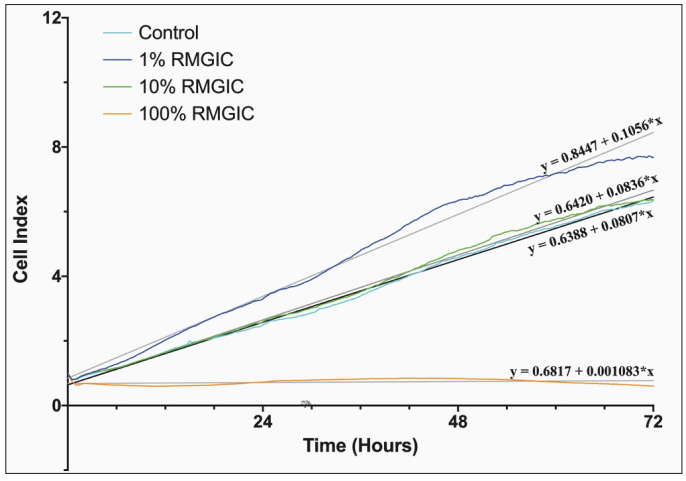

Figure 2. Distribution and regression lines of time-dependent cell indices of the study groups after adding RMGIC dilutions.

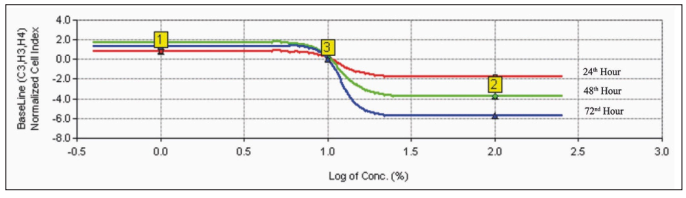

Figure 3. *Dose response curves of RMGIC at the 24th, 48th and 72nd hours.*

DISCUSSION

When selecting the most appropriate dental material in restorative procedures, its cytotoxic effect on oral mucosa and pulp should be considered in addition to the its physical properties. RMGIC are widely used in modern dentistry because of their low sensitivity to moisture, adhesion to dental hard tissues, fluoride release and esthetic colors. However, direct contact with pulp is not recommended as its toxic effect was determined in studies evaluating the efficacy of RMGIC.[21-25] Some researchers modified RMGIC by adding doxycycline hyclate, chlorhexidine, glutaraldehyde, stannous fluoride, and nanohydroxyapatite to its structure to reduce cytotoxic effect and increase its antibacterial properties.[16-18, 26] However, none of these studies included CC_{50} values. Because CC_{50} is the dose which kills 50% of the cells, higher CC_{50} values indicate lower material cytotoxicity. CC_{50} value is one of the most commonly used indicators in cytotoxicity studies and when any material is modified using another agent, more sensitive and accurate data can be obtained in cytotoxicity measurements by monitoring the changes in CC_{50} values.[17] In order to obtain reliable results in in vitro studies, the doses of both the material to be modified and the agents to be used should be selected considering the CC_{50} values. In this study, the cytotoxic effect of three different concentrations of RMGIC solution on DPSC cells was observed for 72 hours and CC_{50} values were determined after 24, 48 and 72 hours in order to serve as a reference for further studies. Based on the results, the null hypothesis that RMGIS would have no cytotoxic effect on DPSCs was rejected.

Eluates (extracts) of the RMGIC were used in the present study. Eluates allow studying the effect of materials on cells that are both distant to and in contact with them. Eluates can be prepared at different concentrations to observe a possible dose-response relationship and to determine the ideal concentration for the sensitivity of the tested cells. ISO standard 10993-12:10.3.2 requires the preparation of eluates for larger molded substances, such as RMGIC, in cytotoxicity testing in such a way that the ratio of the surface area of the test material to the volume of the extraction material is 1.25 cm^2 / ml.[20] In this study, the surface area / volume ratio used for eluate preparation was in accordance with the ISO standard. This elution was then diluted in a ratio of 1:1, 1:10 and 1:100 to produce solutions in which a value of CC_{50} could be obtained. Compliance with ISO standards allows comparing the results between different studies.

Conventional glass ionomer cements have shown low cytotoxicity in the previous studies.[21, 27, 28] Ersahan et al.[4] found that 3 out of 4 RMGIC had similar cell viability on DPSCs and L929 mouse fibroblast cells to the control group. This difference may be due to the fact that the DPSC is significantly more sensitive to the RMGIC used in our study or due to the use of RMGIC samples with a smaller surface area / volume ratio than ISO 10993-12:2004 standard[20] where the low density of toxic molecules dissolved in elution may have had a positive effect on cell viability.

In the MTT analysis, which is often used to determine the cytotoxicity of RMGIC, cell viability is usually studied with undiluted elutions.[29, 30] The CC_{50} of a material can be estimated by linear regression analysis using the MTT results of its elutions in at least three different concentrations. However, RTCA (integrated software of the xCELLigence® system) easily gives the CC_{50} value at the desired time using the cell index data in different wells containing the elutions of three different concentrations. In biocompatibility tests, with the aim of reducing the RMGIC toxicity by adding different substances, the determination of the CC_{50} values allows the results to be compared between different studies.

xCELLigence® system, which is the preferred method in novel cytotoxicity studies, was used in our study. Since this device can measure real-time cell viability by evaluating the electrical current between the gold electrodes at the base of the E-Plate®, separate cell cultivation is not required for all evaluated time intervals, as in the case for single endpoint analyzes such as XTT, MTT, WST-1 and fluorescence microscopy.[31, 32] As standardization is achieved through this feature, real-time results in cell studies can be obtained easily and reliably in a short time. Also, the integrated software of the xCELLigence® system calculates the CC_{50} values for toxicity tests.

Although there are many studies investigating the cytotoxicity of RMGICs, our data are valuable both to determine previously unreported CC_{50} values and to obtain reliable real-time measure-

ments using the xCELLigence® system.[15, 27, 33] We believe that the results of this study will guide the cytotoxicity studies in RMGIC improvement. As a result of obtaining CC_{50} values of RMGIC on DPSCs, the need for determining CC_{50} values of RMGIC prior the cell studies (ie. adding new substrats to reduce RMGIC cytotoxicity) including DPSCs and RMGIC will be eliminated or simplified.

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