Cytotoxic effects of different self-adhesive resin cements: Cell viability and induction of apoptosis

Soner Şişmanoğlu[1], Mustafa Demirci[2], Helmut Schweikl[3], Gunes Ozen-Eroglu[4], Esin Cetin-Aktas[5], Serap Kuruca[6], Safa Tuncer[2], Neslihan Tekce[7]

[1]Department of Restorative Dentistry, Faculty of Dentistry, Altınbaş University, Istanbul, Turkey
[2]Department of Restorative Dentistry, Faculty of Dentistry, Istanbul University, Istanbul, Turkey
[3]Department of Operative Dentistry and Periodontology, University of Regensburg Medical Centre, Regensburg, Germany
[4]Department of Molecular Medicine, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey
[5]Department of Immunology, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey
[6]Department of Physiology, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkey
[7]Department of Restorative Dentistry, Faculty of Dentistry, Kocaeli University, Kocaeli, Turkey

PURPOSE. The effects of four different self-adhesive resin cement materials on cell viability and apoptosis after direct and indirect exposure were evaluated using different cell culture techniques. **MATERIALS AND METHODS.** Self-adhesive cements were applied to NIH/3T3 mouse fibroblasts by the extract test method, cell culture inserts, and dentin barrier test method. After exposure periods of 24 h and 72 h, the cytotoxicity of these self-adhesive materials was evaluated using the MTT assay (viability) and the Annexin-V-FITC/PI staining (apoptosis). **RESULTS.** The lowest cell viability was found in cells exposed to BeautiCem SA for 24 h in the extract test method. Cell viability was reduced to 70.6% compared to negative controls. After the 72 h exposure period, viability rate of cell cultures exposed to BeautiCem SA decreased more than 2- fold (29.5%) while cells exposed to RelyX U200 showed the highest viability rate of 71.4%. In the dentin barrier test method, BeautiCem SA induced the highest number of cells in apoptosis after a 24 h exposure (4.1%). Panavia SA Cement Plus was the material that caused the lowest number of cells in apoptosis (1.5%). **CONCLUSION.** The used self-adhesive cements have showed different cytotoxic effects based on the evaluation method. As exposure time increased, the materials showed more cytotoxic and apoptotic effects. BeautiCem SA caused significantly more severe cytotoxic and apoptotic effects than other cements tested. Moreover, cements other than BeautiCem SA have caused necrotic cell death rather than apoptotic cell death. ***[J Adv Prosthodont 2020;12:89-99]***

KEYWORDS: Apoptosis; Biocompatibility; Cell culture; Cytotoxicity; Self-adhesive resin cements

Corresponding author:
Soner Şişmanoğlu
Department of Restorative Dentistry, Faculty of Dentistry, Altınbaş University, Bakırköy, TR-34147 Istanbul, Turkey
Tel. +902127094528: e-mail, soner.s@hotmail.com
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INTRODUCTION

Resin-based materials are extensively used in a wide variety of dental applications.[1] These materials consist of cross-linking methacrylates (organic matrix), filler particles, and molecules that promote polymerization.[1] The ratio of these components varies depending on the clinical use. Resin cements generally contain a lower proportion of filler particles due to the need for lower viscosity.[2] The organic matrix of a resin cement is a combination of high molecular weight monomers such as bisphenol-A-diglycidylmethacrylate (Bis-GMA) and urethane dimethacrylate (UDMA) and low molecular weight monomers including triethylene glycol dimethacrylate (TEGDMA) and hydroxyl ethyl methacrylate (HEMA).[3] The proportion of monomers in the composi-

tion affects the conversion rate, the polymerization shrinkage, the viscosity and mechanical properties of the material.[4] In addition to cross-linking methacrylates, self-etching functional monomers are also incorporated into the chemical composition of the resin cements to considerably reduce the pH and to demineralize tooth structures, promoting micromechanical adhesion.[5] Commonly used functional monomers in the composition of self-adhesive resin cements (SARCs) are 4-methacryloyloxyethy trimellitate anhydride (4-META), 10-methacryloyloxydecyl dihydrogen phosphate (MDP), glyceroldimethacrylate dihydrogen phosphate (GPDM), and 2-methacryloyloxyethyl phenyl phosphoric acid (Phenyl P).[6] Ionic and covalent interactions occur between enamel/dentin and SARC through functional monomers, thereby ensuring chemical bonding.[5]

Self-adhesive resin cements (SARCs) simplify the cementation procedure by eliminating the multiple steps required for multi-step adhesive cementation.[7] These materials routinely used by clinicians are extensively investigated *in vitro* for bond strengths, durability, microleakage, and biocompatibility, including cytotoxicity and induction of apoptosis. Biocompatibility is defined as the organism's response to external materials.[8] The organic matrix of composite resins is a composition of components that cause adverse effects on the living organism. It has been reported that the degree of conversion (DC) varies from 55% to 70% for most composite resins.[9] Thus, there are plenty of unreacted monomers trapped in the highly cross-linked polymer network after the polymerization reaction.

The high viscosity of a luting material may predispose to inadequate penetration of the material into the dentin surface. Therefore, the inorganic phase ratio of the SARCs is reduced to decrease the viscosity and to provide a suitable film thickness for cementation.[7] Thus, these materials contain a higher amount of monomers in their chemical compositions.[10] It is well documented that dental adhesives and composites release components from their organic matrix. Two significant factors are suggested for the cause of this release: (1) the release of residual monomers even after polymerization into the oral fluids, especially in the first 24 h, (2) the release of monomers because of degradation and erosion over time.[11] Cytotoxicity may be caused by other components than monomers, such as initiators or ions present in the inorganic phase.[12] For instance, the fluoride has been incorporated into the SARCs composition due to its cariostatic properties.[7] The amount of fluoride release is directly related to the fluoride source, the fluoride-containing filler concentration, and the organic matrix structure of the resin-based material.[13] Some studies have reported that high concentrations of fluoride may cause cytotoxicity.[14,15] Pagano *et al.*[16] suggested adding nanohydroxyapatite, antibiotic and a mucosal defensive agent to conventional glass ionomer cement to reduce the overall cytotoxicity. Considering these facts, the increase in the organic phase and the degradation of dental materials over time, as well as the proximity to oral tissues, render their biocompatibility crucial issue. Besides, SARCs modify the smear layer by incorporating it into the hybrid layer rather than to remove it.[7] Due to this incorporation, the use of such materials on the deep dentin may cause pulp injuries, and the occurrence of cytotoxicity cannot be ruled out.

Despite all its ease of use and popularity, there are reservations about the biocompatibility of SARCs recently.[17-21] Thus far, the cytotoxicity of calcium- and fluoride-releasing contemporary SARCs has not been studied. Therefore, the aim of this *in vitro* study was to investigate the effects of four different SARCs on cell cultures using various testing methods: (1) extract testing, (2) cell culture inserts, and (3) dentin barrier testing. Cell viability and the induction of apoptosis were considered as parameters of material-induced cell responses after 24- and 72 h exposure periods. The tested null hypotheses were that 1) no differences between cytotoxic effects of the different SARCs would be detected, and 2) the exposure period would not have any effect on cytotoxicity.

MATERIALS AND METHODS

Four SARCs including BeautiCem SA Cement (BC; Shofu, Kyoto, Japan), Panavia SA Cement Plus (PA, Kuraray Noritake Dental Inc., Okayama, Japan), RelyX U200 (RU; 3M ESPE, St. Paul, MN, USA), and TheraCem (TC, Bisco Inc, Schaumburg, IL, USA) were tested together with a resin-modified glass-ionomer cement (VB, 3M ESPE, St. Paul, MN, USA) as a positive control.[22] Details of the materials are listed in Table 1, and the study design is given in Fig. 1.

NIH/3T3 mouse fibroblasts (CRL-1658, American Type Culture Collection, Rockville, MD, USA) were incubated in DMEM (Dulbecco's modified Eagle's medium, Biosera, East Sussex, UK) and supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 10 µg/mL streptomycin using a humidified 37°C incubator with 5% CO_2. Cells from semi-confluent monolayers were collected and viable cells were determined with trypan blue staining.

Specimens were fabricated as discs in Teflon molds (5 mm in diameter, 2 mm height) under sterile conditions using a laminar flow hood. The molds were filled with cement, and excess material was discarded by compression between two microscope slides. Light-curing was performed following the manufacturer's instructions using an LED curing unit (Elipar Deep Cure, 3M ESPE) at 1250 mW/cm^2. After light curing, the specimens were left in the dark for a complete setting (5 min for BC, PA, and TC; 3 min for RU). Thirty-three specimens per SARC were transferred into a sterile falcon tube and specimens were covered with a 14 mL culture medium for each group and incubated for 24 h. In this way, extracts were prepared at a ratio of 92.4 mm^2 specimen surface area per milliliter culture medium.[23,24] Then, extracts were sterile filtered, and stock extract solutions (1 : 1) were serially diluted in a cell culture medium. NIH/3T3 cells were seeded (10^5 cells/well) and incubated for 24 h. The culture medium was replaced by 1 mL of each of the serially diluted material extracts[24,25] and incubated

Table 1. The brand names, group codes, abbreviations, lot numbers, material types, compositions, and manufacturers of the materials used in the study

Materials #LOT	Composition	Manufacturer
BeautiCem SA Cement (BC) #121650	Fluoro-alumino-silicate glass, zirconium silicate filler, urethane dimethacrylate, 2-hydroxyethyl methacrylate, carboxylic acid monomer, phosphonate monomer, polymerization initiator.	Shofu, Kyoto, Japan
Panavia SA Cement Plus (PA) #7J0013	Bisphenol A diglycidylmethacrylate, triethylene glycol dimethacrylate, 2-hydroxyethyl methacrylate, sodium fluoride, silanated barium glass filler, silanated colloidal silica, 10-methacryloyloxydecyl dihydrogen phosphate, hydrophobic aromatic dimethacrylate, hydrophobic aliphatic dimethacrylate, dl-Camphorquinone, peroxide, accelerators, catalysts, pigments.	Kuraray Noritake Dental Inc., Okayama, Japan
RelyX U200 (RU) #591481	Base: glass powder treated with silane, 2-propenoic acid, 2-methyl 1,10 -(1-[hydroxymetil]-1,2-ethanodlyl), triethylene glycol dimethacrylate, ester dimethacrylate, silica treated silane, glass fiber, sodium persulfate and per-3,5,5- trimethyl hexanoate t-butyl. Catalyst: glass powder treated with silane, substitute dimethacrylate, silica-treated silane, 1-benzyl-5-phenyl-acid barium, sodium p-toluenesulfonate, calcium, 1,12- dodecane dimethacrylate, calcium hydroxide, and titanium dioxide.	3M ESPE, St. Paul, MN, USA
TheraCem (TC) #1700000390	Base: Calcium base filler, glass filler, bisphenol A diglycidylmethacrylate, dimethacrylates, 2-hydroxyethyl methacrylate, ytterbium fluoride, initiator, amorphous silica. Catalyst: Glass filler, 10-methacryloyloxydecyl dihydrogen phosphate, silica amorphous.	Bisco Inc., Schaumburg, IL, USA
Vitrebond (VB) #N616612 #N597334	Powder: Calcium fluoro-alumino-silicate glass. Liquid: Polyacrylic acid, hydroxyl ethyl methacrylate, water.	3M ESPE, St. Paul, MN, USA

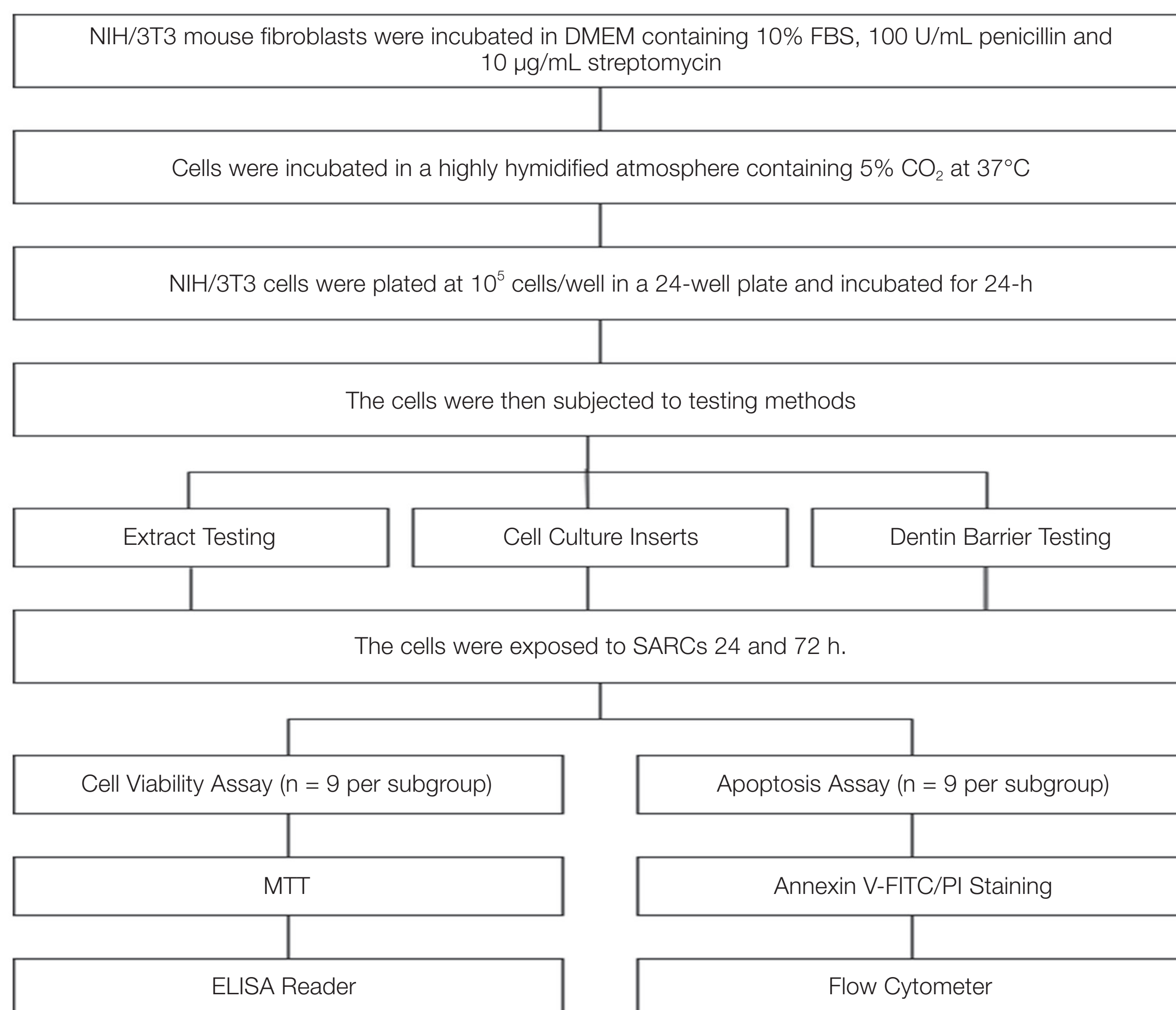

Fig. 1. Study design.

again for 24- and 72 h. Wells containing medium only were used as negative controls.

NIH/3T3 cells were seeded into a 24-well plate and incubated for 24 h. After the incubation, the fabricated specimens as described above (see 2.2) were placed into a cell culture insert (0.4 µm pore size; 24-Well SPLInsert Hanging; SPL Life Sciences Co. S. Korea), which was then transferred to the 24-well plate. Each specimen was covered with a 1 mL culture medium and was incubated for 24- and 72 h.

Human third molars were collected after the ethical clearance (2016/60) of the Ethical Committee of the Istanbul University Faculty of Dentistry. Dentin slices with a thickness of 500 ± 50 µm were cut above the pulp horns and below the dentin-enamel junction using a precision saw (Isomet, Buechler, Lake Bluff, IL, USA). The smear layer on the pulpal side of the dentin disc was removed by etching with 50% citric acid for 30 s.

A custom-made testing device was used for the dentin barrier testing. This device consists of two parts that can be screwed to each other: an outer part with a 5 mm opening at the base and an inner part with a hole in 5 mm diameter at its center. A polyvinylsiloxane "light body" impression material was applied to the outer part for isolation purposes before the dentin disc placement. After the dentin disk placement, the inner part was gently screwed into the outer part. Thus, sufficient sealing was provided to prevent direct contact of the culture medium with test material when the testing device was placed into the 24-well plate (Fig. 2).

NIH/3T3 cells were seeded into wells at a density of 10^5 cells with 1 mL culture medium and were incubated for 24 h. SARCs (0.4 mL in volume) were precisely injected with an insulin syringe onto the dentin surface and light-cured. A silicon impression material served as a negative control as recommended by ISO 7405.[26] Dentin barrier testing devices were transferred into the wells and hanged with the help of their metal rods. In this way, cell cultures were indirectly exposed to test material through the culture medium. The testing devices were incubated for 24- and 72 h.

Exposed culture medium was discarded and replaced by a combination of 900 µL fresh culture medium and 100 µL of MTT solution (5 mg/mL MTT, Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Cells were incubated for 3 h at 37°C. Formazan crystals were solubilized with dimethyl sulfoxide. Formazan formation was quantified at 570 nm using a spectrophotometer. Each material was tested in triplicate and three independent experiments were performed. The absorbance of the negative control group was set to 100%, and the percentage of viability in experimental groups was calculated according to the negative control group.

NIH/3T3 cells were washed with phosphate-buffered saline twice after the exposure, collected by centrifugation, and then resuspended in flow cytometry binding buffer. A 100-µL of cell suspension containing 10^5 cells was left in the dark with 5 µl apoptosis detection kit (Annexin V-FITC/PI, BD Biosciences, San Diego, CA, USA) for 15 min at room temperature. Then, the induction of apoptosis was determined using a flow cytometer (FACSCalibur, BD Biosciences, San Diego, CA, USA).[22] Each material was tested in triplicate and three independent experiments were performed.

Individual values obtained with each test material and testing method were recorded as percentages. The cell viability and the induction of apoptosis data were analyzed using a software (SPSS WIN 20.0, SPSS, Chicago, IL, USA) at the 5% level of significance. Differences between the mean values of the various groups were statistically analyzed with the Kruskal-Wallis test. If statistical significance between mean values was found, the pair wise comparison of the values was performed with the Mann-Whitney U test.

RESULTS

In extract testing, the lowest cell viability was found in cells exposed to BC and TC for 24 h. Cell viability was reduced to 70.6% and 69.2%, respectively. No statistically significant

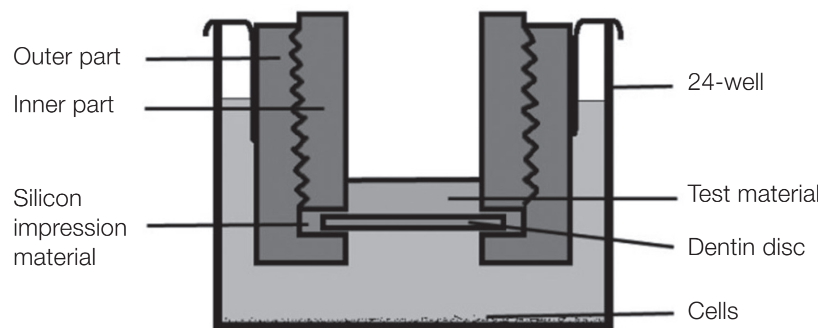

Fig. 2. Dentin barrier test device.

differences were found between mean values obtained for BC and TC (P = .247). Cell viability was reduced 92.1% by PA, whereas RU decreased to 80.7%. Within the different exposure periods, there were significant differences among the SARCs regarding cell viability (P < .05). BC reduced the cell viability to 29.5% (P < .05), followed by TC (49.3%) and PA (48.7%). On the other hand, cell viability was slightly reduced by RU to 71.4% compared to 24 h of exposure (P < .05). Extracts of VB, which was used as a positive control material, reduced the cell viability to about 10% during both exposure periods (Fig. 3).

In culture inserts, the baseline (24 h of exposure) measurements revealed that the lowest cell viability was found in cultures exposed to TC (68.6%) and BC (73.8%), without a statistically significant difference (P = .247). Cells exposed to PA showed the highest viability rate (98.4%), followed by RU (97.1%). There was no statistically significant difference among the negative controls, PA, and RU (P > .327). After 72 h of exposure, the lowest cell viability was found in cultures treated with TC (37.5%). This material was followed by BC (54.8%) and PA (67%). RU treated cells showed the highest viability rate of 95.5% (P < .05). VB, the positive control material, reduced cell viability to 43.8% and 23% after 24- and 72 h of exposure periods, respectively (Fig. 4).

BC was the most adversely effective SARC in the dentin barrier testing because it reduced the cell viability to 79.4% at the baseline measurements (24 h), followed by TC (84%) and PA (91.4%). No statistically significant differences between the negative controls and PA and between PA and RU were detected (P > .05). Similar results were noted after 72 h exposure. The lowest cell viability was again found in cultures treated with BC (60.2%), followed by TC (68.5%) and PA (87.2%), whereas RU (90.9%) caused the highest rate of viability (P < .05). The positive control material VB reduced cell viability to 63.8% and 48.3%, after 24- and 72 h exposure periods, respectively (Fig. 5).

The effect of extracts of different concentrations on cell viability is given in Fig. 6. It is observed that placing a barrier such as cell culture inserts and especially dentin barrier between the cells and the test material approximately corresponds to half of the 1 : 1 extract concentration.

In the baseline measurements (24 h), the highest rate of apoptosis was found in cultures exposed to extracts of BC (11.2%), followed by TC (6.2%). PA (3.4%) caused the lowest induction of apoptosis (P < .05), followed by RU (4.6%). Similar results were detected after a 72 h of exposure. The highest induction of apoptosis was again identified in cultures treated with BC extracts (24.1%). This material was followed by TC (9%), with statistically significant difference (P < .05). PA was the material that induced the lowest number of cells in apoptosis (4.5%), followed by RU (6%) (P < .05). The positive control material (VB) caused the highest apoptosis rate of 54.4% and 62.6% after 24- and 72 h exposure periods, respectively (Fig. 7).

In cell culture inserts, the highest number of cells in apoptosis was again found in cell cultures exposed to BC (7.3%) after a 24 h exposure (P < .05), followed by TC

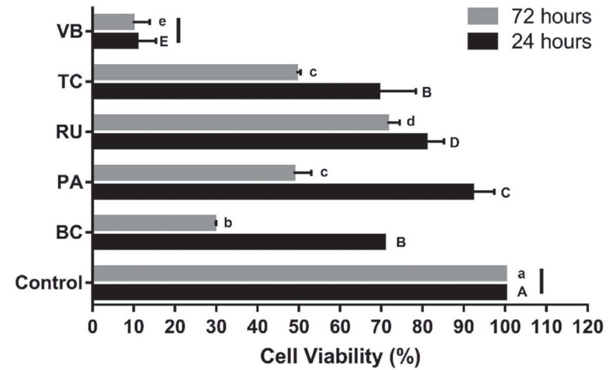

Fig. 3. Cell viability in cell cultures exposed to SARCs extracts with 1:1 concentration. Mean values represented with same uppercase letters (24 hours) or lowercase letters (72 hours) were not significant (P > .05). Vertical line implies non-significant difference between exposure periods.

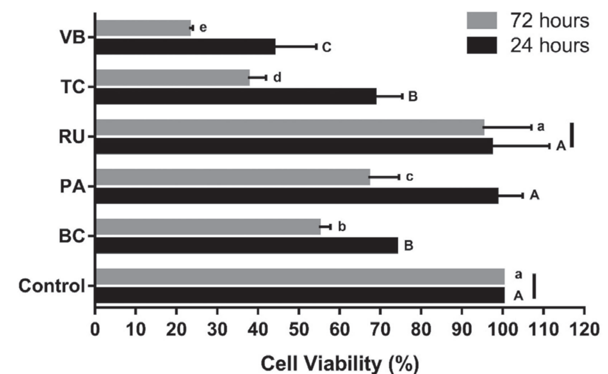

Fig. 4. Cell viability in cell cultures exposed to SARCs using an insert test device. Mean values represented with same uppercase letters (24 hours) or lowercase letters (72 hours) were not significant (P > .05). Vertical line implies non-significant difference between exposure periods.

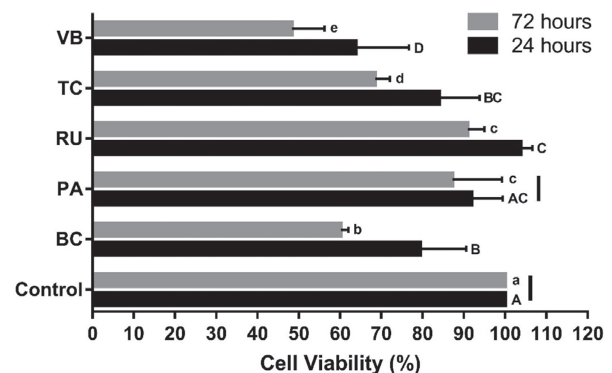

Fig. 5. Cell viability cultures exposed to SARCs using a dentin barrier test device. Mean values represented with same uppercase letters (24 hours) or lowercase letters (72 hours) were not significant (P > .05). Vertical line implies non-significant difference between exposure periods.

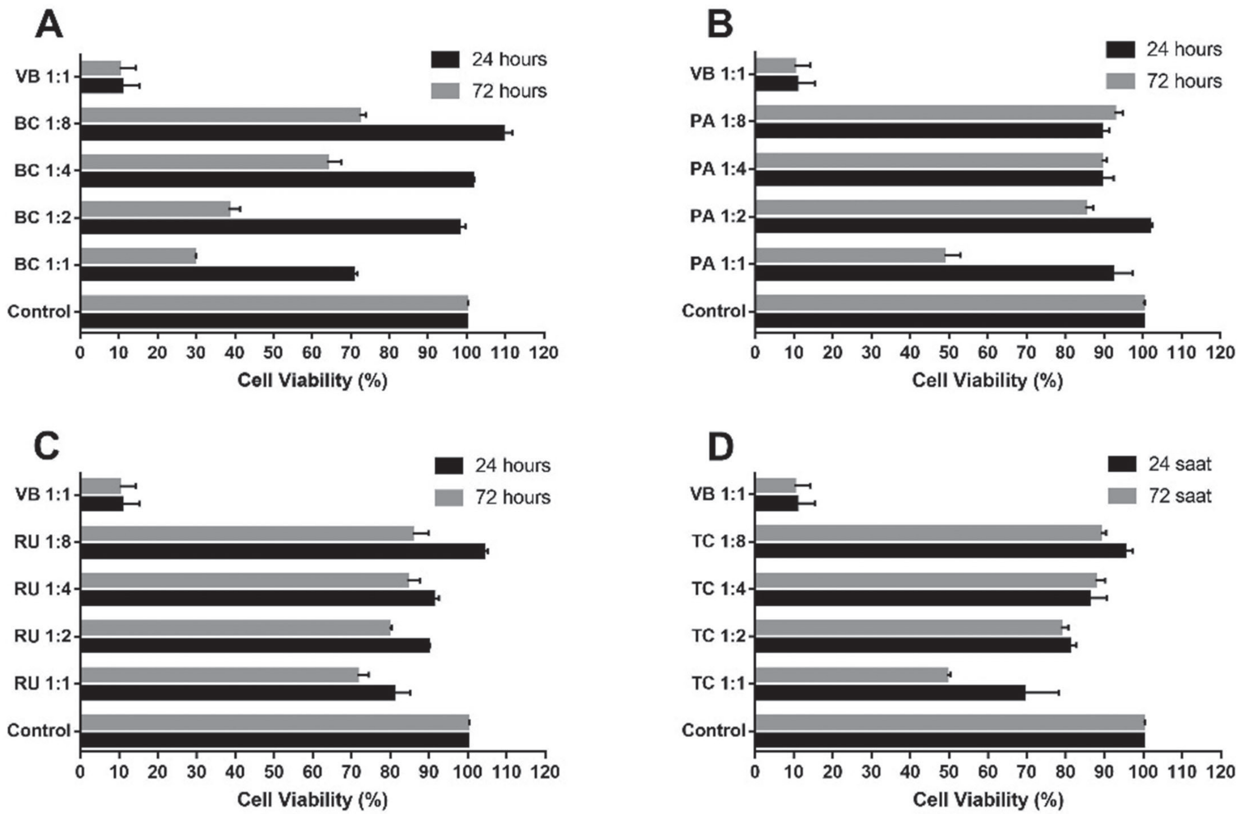

Fig. 6. Comparison of cell viability rates regarding different extract concentrations (1:1, 1:2, 1:4, 1:8). (A) BeautiCem SA, (B) Panavia SA Cement Plus, (C) RelyX U200, (D) TheraCem.

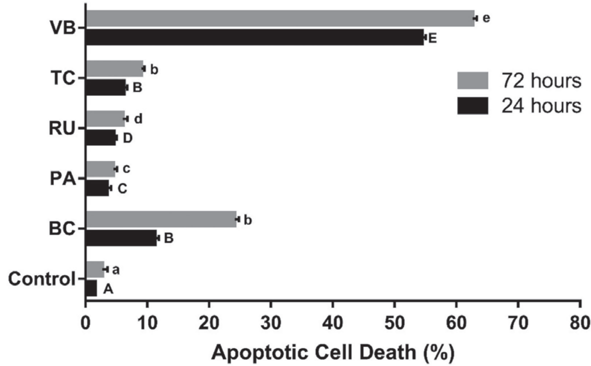

Fig. 7. Apoptosis in cell cultures exposed to SARCs extracts with 1:1 concentration. Mean values represented with same uppercase letters (24 hours) or lowercase letters (72 hours) were not significant ($P > .05$).

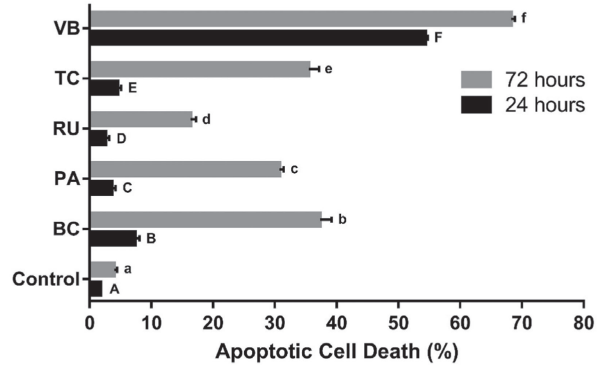

Fig. 8. Apoptosis in cell cultures exposed to SARCs using an insert test device. Mean values represented with same uppercase letters (24 hours) or lowercase letters (72 hours) were not significant ($P > .05$).

(4.5%). RU (2.5%) caused the lowest apoptosis rate ($P < .05$), followed by PA (3.5%). Within the different exposure periods, there were significant differences among the SARCs regarding the apoptosis rate ($P < .05$). BC induced the highest rate of apoptosis (38.2%) ($P < .05$), followed by TC (34.5%) and PA (30.7%). In addition, RU (16.3%) caused the lowest apoptosis ($P < .05$). VB caused the highest cell apoptosis rates of 54.3% and 68.2% after 24- and 72 h exposure periods (Fig. 8).

In dentin barrier testing, BC induced the highest number of cells in apoptosis after a 24 h exposure (4.1%) ($P < .05$). BC was followed by RU (2.2%) and TC (2.1%), without a statistically significant difference ($P = .623$). PA (1.5%) caused the least induction of apoptosis ($P < .05$). Besides,

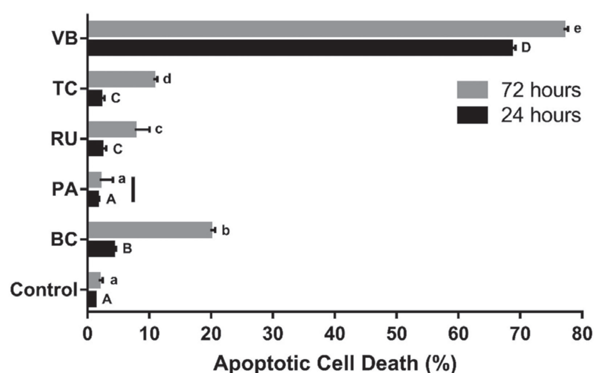

Fig. 9. Apoptosis in cell cultures exposed to SARCs using a dentin barrier test device. Mean values represented with same uppercase letters (24 hours) or lowercase letters (72 hours) were not significant ($P > .05$). Vertical line implies non-significant difference between exposure periods.

there was no significant difference between the negative controls and PA ($P = .108$). The highest number of cells in apoptosis was also found in cultures exposed to BC after a 72 h exposure (19.8%). BC was followed by TC (10.6%) and RU (8.3%). PA again led to the least apoptosis rate (1.3%). Likewise, there was no significant difference between the negative controls and PA ($P = .118$). Positive control material VB induce the highest apoptosis rate of 68.4% and 77%, after 24- and 72 h incubations, respectively (Fig. 9).

DISCUSSION

In the present study, the cytotoxic effects of four different SARCs on the NIH/3T3 cell cultures were investigated using three different testing methods at two different exposure periods. Materials that cause more than 30% reduction in cell viability are considered cytotoxic according to ISO recommendations.[27] According to the study findings, it was clear that the SARCs caused varying degrees of cytotoxicity, which increased with the exposure time. Therefore, the null hypotheses of the authors were rejected.

In spite of the limitations, the methodology we used in the present study enabled us to simulate cell responses with different types of exposures to SARCs. Pagano *et al.*[25] suggested that the extract testing was a reliable method compared with direct contact testings. Schmalz *et al.*[28] suggested that dentin barrier testing can be used, at least partially, as an alternative to animal experiments. In addition, the cytotoxicity of dental adhesives has been reported to decline with increasing dentin thickness,[29] and vice versa. In the current study, it was also found that the cytotoxic effects vary according to the testing method. While the most cytotoxic effects were observed for the extract testing, an evident reduction was observed for the cytotoxic effects of the dentin barrier testing. In line with a previous study,[25] the cytotoxic effect of tested materials was reduced with increasing dilutions with a dose-dependent manner. In the present study, it was observed that placing a barrier such as cell culture inserts or dentin between the cells and the test material approximately corresponded to half of the 1:1 extract concentration in terms of the cell viability (Fig. 6). This finding indicated that the use of a 500 µm thick dentin disc as a barrier had a protective effect on NIH/3T3 cells. On the other hand, various cell types are used for cytotoxicity studies. In our study, we preferred the permanent NIH/3T3 cell line, like many other studies,[19,22] as recommended by ISO 7405,[26] which showed similar cytotoxicity response to pulp-derived cells.[30] Moreover, NIH/3T3 cells can be easily reproduced and used for a large scale testing.[22]

In the findings of the present study, the cytotoxic effects were significantly elevated as the exposure time increased. After 72 h of exposure, more severe cytotoxic effects were observed for all three testing methods compared to those after 24 h of exposure ($P < .05$). In the extract testing results, BC and PA caused twofold more cytotoxicity at 72 h than did at 24 h. After the photopolymerization, the remaining free monomers can be released into pulp tissues after diffusing across the dentin tubules or into the saliva resulting in inflammation and necrosis.[31] In agreement with our findings, other researchers also reported elevating cytotoxic effects with increasing exposure time to SARCs.[32-34] In a recent study, the cytotoxic effects of RU and BisCem (Bisco Inc.) SARCs on NIH/3T3 cells were examined, and it was detected that more cytotoxicity was encountered after 7 days of exposure than at the end of 24 h.[19] Besides, Pagano *et al.*[35] reported that the cytotoxicity risk of a material was strongly related with the contact time, in agreement with the present study. Therefore, it may be suggested that resin-based restorative materials should be tested in both short- and long-term exposure periods.

In the present study, RU showed the least cytotoxicity on cell cultures after 24- and 72 h exposure periods. However, it was only found to be slightly more cytotoxic than PA (92.1%) in the extract testing at a 24 h exposure (80.7%). After 72 h of exposure, RU exhibited slight cytotoxicity compared to the negative control in all three testing methods ($P < .05$). Kwon *et al.*[33] found that RU showed a slight reduction in cell viability following a short duration of exposure (1.5-, 3-, and 6 h) and it maintained the cell viability in long-term (72 h), which was consistent with the present study. A transdentinal cytotoxicity study revealed that RU exhibited less cytotoxicity than a resin-modified glass-ionomer cement (RMGIC, RelyX Luting 2) and a conventional resin cement (RelyX ARC), and that the cell viability of RU was similar to the negative control group.[36] In a different study, in agreement with our results, the cytotoxicity of five SARCs was found in the following descending order: MaxCem Elite > G-Cem LinkAce > Bifix SE > PA > RU.[6]

RU contains monomers shown to be toxic to fibroblast cells at certain concentrations such as TEGDMA and hydroxyethyl methacrylate (HEMA),[6,37] which may reduce levels of glutathione, cause oxidative stress, and induce

cytotoxicity, apoptosis, or necrosis.[37] Kurt *et al.*[37] reported that RU caused oxidative stress, DNA damage, and cytotoxicity compared to the negative control group. In contrast, RU did not show considerable cytotoxicity in other studies[6,33,36] as well as in the present investigation. Although a high biocompatibility of RU has been attributed to the absence of HEMA in its chemical composition, D'Alpino *et al*. also reported that polymerized RU samples released HEMA.[6,36] On the other hand, RU additionally performs an acid-base reaction[38] during photopolymerization as well as the chemical polymerization, which may result in higher DC that can reduce the number of unreacted monomers. The manufacturer also reported that the pH of this material rises to a neutral level with the polymerization preventing further hydrolysis due to the self-neutralizing mechanism.[39]

Despite this slight cytotoxicity, an *in vivo* study demonstrated moderate inflammatory responses in pulp tissues when onlay restorations were cemented with RelyX Unicem (3M ESPE).[39] In a recent animal experiment, researchers concluded that RU was safe to use in deep cavities, although it led to a minor reduction in odontoblast cells with a higher ratio of mononuclear cells.[17] Also, Alvarez *et al.*[21] reported that SARCs could delay reparative dentin formation in their investigation using MDPC-23 odontoblast-like cells. Considering the previous findings and induced apoptosis ratio by RU, it could be proposed that the cytotoxicity of this material mainly occurs through necrotic pathways. RU induces lower apoptosis than other SARCs, including PA (MaxCem Elite, G-Cem LinkAce, Bifix SE, and PA) and it also exhibits no significant difference in comparison to the negative control group, in accordance with our finding.[6] Furthermore, another recent study, in accordance with our results, reported that the main mechanism of RU-induced cell death is by necrotic, but not apoptotic, pathways.[18]

PA exhibited only slight cytotoxicity in extract testing (92.1%) and culture inserts (98.4%) at a 24 h exposure period. However, the cytotoxicity of PA significantly increased by twofold after a 72 h exposure compared to a 24 h exposure ($P < .05$). In accordance with our findings, it was observed that Panavia F2.0 (previous version of PA) showed a gradual reduction in cell viability throughout three days, and it exhibited slightly higher cytotoxicity than did RU.[40] On the other hand, PA causes less apoptotic induction than other SARCs. Kraus *et al.*[41] detected that cytotoxic effects of the monomers were ranked as Bis-GMA, UDMA, TEGDMA, and HEMA from high to low after 24- and 72 h exposure periods.[42] Alkurt *et al.*[20] examined the cytotoxic effects of PA on pulp and gingival cells for 24- and 48 h exposures and found greater cytotoxicity after 48 h compared to 24 h. They also showed that the toxicity of PA depends on the extract concentration. Nevertheless, PA, which contains Bis-GMA, TEGDMA, and HEMA, exhibited relatively slight cytotoxicity in our study. Ratanasathien *et al.* also reported that the concentration of each monomer in the composition of adhesive systems can affect the toxicity and there are three types of interaction between the various monomers.[42] These interactions were defined as (1) enhancing the effect of each other (synergistic effect), (2) adding to the effects of each monomer (additional effect), and (3) reducing the effect of each other (antagonist effect). Moreover, it was found that antagonist activity is more dominant among all the different monomeric compounds at 24 h, and they found that the antagonist activity decreases as the synergistic effect increases at 72 h, indicating that this effect was very evident in the UDMA / TEGDMA, HEMA / Bis-GMA and Bis GMA / TEGDMA combinations.[42] The synergistic effects of the monomers tend to be more pronounced when cells are exposed to the SARCs for a longer exposure period. As a result, the duration of monomer exposure has been reported to have a brutal effect on toxicity.[42] Here, PA showed the highest rate of viability in extract testing after 24 h (92.1%) but was more effective in a negative way than RU after 72 h (48.7%). This can be explained by the above-mentioned mechanisms between the antagonist and synergist effects.

BC was found to induce more severe cytotoxic reaction than other test materials. However, there was no significant difference between BC and TC regarding the extract testing and cell culture inserts at 24 h measurements ($P > .05$). It was also observed that the cytotoxic effect of BC significantly increased with exposure periods. For instance, two times over cell viability reduction (from 70.6% to 29.5%) was seen in the extract testing. BC also induced more severe apoptosis than other SARCs. Regulation of intracellular and extracellular pH balance is vital for cell cultures. Changes in pH values can disturb cell metabolism and initiate processes such as the induction of the expression of heat-shock proteins, finally leading to apoptotic cell death.[43] BC is a giomer-based SARC that contains UDMA, HEMA, and carboxylic acid monomers. Previous studies reported that acidic pH leads to apoptosis by increasing caspase activity,[43] and monomers such as UDMA,[44] HEMA, and Bis-GMA are shown to induce apoptotic and necrotic cell death as well.[35] These different components in BC's chemical composition can be the reason for its low biocompatibility. Besides this, in giomer-based materials, fluoroaluminosilicate glass is added to the urethane resin containing silica filler after reacting with aqueous polyalkenoic acid. These materials have fluoride release and recharge characteristics. It has been reported that cytotoxicity of glass-ionomer cements was related to fluoride release.[15] Several studies have shown that relatively high concentrations of fluoride induce cytotoxicity effects on cells and tissues, such as production of oxidative stress, degradation of the antioxidant defense system, formation of inflammation, and induction of apoptosis.[45] Kanjevac *et al.*[15] concluded that a high concentration of fluoride ions released from restoratives leads to a severe cytotoxicity.

Our data suggest that the cytotoxicity of BC occurred via apoptotic pathways. A recent study showed that apoptosis was the primary mechanism of the cell death, induced by the extracts derived from RMGIC, whereas the extracts derived from RU induced cell death via a necrotic pathway.[18] From this standpoint, it can be assumed that BC acts as RMGIC, leading to apoptotic cell death. It could also be

thought that UDMA and fluoride in the composition of this material may be responsible for its cytotoxicity since it previously reported that UDMA[35] and fluoride[15] are more toxic agents. It was shown that the release of UDMA from SARCs was detectable in artificial saliva at 1-, 24- and 72 h, and they induced cytotoxic and genotoxic effects on cells.[37] Due to lack of studies on biocompatibility of BC, a comparison of our data is not possible, rendering further investigations are necessary.

On the other hand, TC, which is a SARC with Bis-GMA, HEMA and MDP monomers in its composition and capable of calcium and fluoride release, exhibited slightly less cytotoxicity than BC. MDP may also contribute to the cytotoxicity of SARCs. Kim *et al.*[46] observed that minimally toxic concentrations of MDP induced oxidative stress that contributed to the inflammation and inhibition of odontoblastic differentiation. They stated that the use of MDP in deep cavities might inhibit the formation of the reparative dentin. Thus, the use of an antioxidant agent before or together with MDP is suggested to prevent its adverse effects.[46] The initial cytotoxicity of luting materials can be attributed to their acidity.[47] This initial acidity involving a long period of acidic pH may cause diffusions of toxic components from SARCs. These diffusions may lead to detrimental effects, especially when preparation and cementation procedures are less than ideal. Karimi *et al.*[48] detected a 20% increase in cell viability when incorporating 5% amorphous calcium phosphate nanoparticles into RMGIC. Therefore, the difference between BC and TC may be the result of the alkaline pH and the calcium content of TC.[49] However, TC studies reported in the literature are scarce and there is no biocompatibility study to compare our results.

There are certain limitations of the present study, just as in all *in vitro* studies. Recent studies revealed that the action of methacrylic monomers is bidirectional with respect to the upregulation of interleukins and also by the formation of the NLRP3 inflammasome.[35] Therefore, knowing not only the apoptotic induction but also the differentiation of the different phases of the cell cycle is important. Further studies on the effects of SARCs on inflammatory / anti-inflammatory gene expression and cell cycle are also needed. In clinical conditions, the presence of the indirect restoration may reduce the DC by acting as a barrier between the SARC and the curing light. The absence of such a restorative material barrier in the present study may have an impact on the cytotoxicity and induction of apoptosis. For this reason, the biocompatibility of SARCs should be evaluated in later studies using a restorative material barrier.

CONCLUSION

According to the results of the present study, the cytotoxicity of the SARCs is material dependent. Different testing methods have resulted in varying levels of cytotoxicity. Therefore, it may be beneficial to use different testing methods in cytotoxicity studies. Despite the great viability reductions at the end of 72 h of exposure, the viability rates of all SARCs at 24 h have remained over the 30% viability reduction threshold of ISO recommendations. However, only RU and PA caused a reduction in viability that remained over this threshold at the end of 72 h and is considered non-cytotoxic. BC produced significantly more severe cytotoxic effects than other SARCs, and cytotoxicity occurred via apoptotic pathways.

ORCID

Soner Şişmanoğlu *https://orcid.org/0000-0002-1272-5581*
Mustafa Demirci *https://orcid.org/0000-0002-9297-6945*
Helmut Schweikl *https://orcid.org/0000-0002-2397-7282*
Gunes Ozen-Eroglu *https://orcid.org/0000-0003-3681-9336*
Esin Cetin-Aktas *https://orcid.org/0000-0002-8078-2780*
Serap Kuruca *https://orcid.org/0000-0001-7878-9994*
Safa Tuncer *https://orcid.org/0000-0003-2101-3225*
Neslihan Tekce *https://orcid.org/0000-0002-5447-3159*

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