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Evaluation of residual monomer release and toxicity of self-adhesive resin cements

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The aim of this study was to evaluate the amount of leached residual monomers from self-adhesive resin cements and evaluate their toxicity *in-vitro*. A total of 60 disk-shaped specimens (5 mm in diameter and 0.5 mm in thickness) were prepared from each cement (RelyX U200, SpeedCEM, G-Cem) (n=20). Specimens were immersed in artificial saliva and the amount of released monomers [urethane dimethacrylate (UDMA) and triethyleneglycol dimethacrylate (TEGDMA)] was identified. Then, the cytotoxicity and genotoxicity effect on cells were evaluated using the defined amounts of released monomers from cements. The highest monomer release was detected in G-Cem (p<0.05). The highest cytotoxicity value was identified from SpeedCEM (p<0.01) and the highest genotoxicity values were calculated from RelyX U200 (p<0.05). Released UDMA and TEGDMA from self-adhesive resin cements induced cytotoxicity and genotoxicity effect on cells.

Keywords: Self-adhesive resin cement, Monomer release, Cytotoxicity, Genotoxicity

INTRODUCTION

Self-adhesive resin cements are hybrid materials based on filled polymers and are designed to adhere to tooth structure without requiring a separate adhesive or etchant. These cements were introduced to dentistry within the past decade and combine the features of composite restoratives, self-etching adhesives and dental cements[1]. The currently available self-adhesive resin cements are two-part materials that require either hand mixing, capsule trituration or delivery by an auto-mixing dispenser. One of the components within these cements is composed of conventional mono-, di- and/or multi-methacrylate monomers that are used in a variety of resin-based dental materials: bisphenol-A-glycidyl methacrylate (Bis-GMA), urethane oligomers of Bis-GMA, urethane dimethacrylate (UDMA), 2-hydroxyethyl methacrylate (HEMA), glycerol dimethacrylate (GDMA), triethyleneglycol dimethacrylate (TEGDMA) and trimethyloylpropane trimethacrylate (TMPTMA), and many others[1,2].

Ideally, a dental restorative resin would have all of its monomer content converted to a polymeric form during the polymerization reaction. However, the conversion of monomer into polymer is not complete during the polymerization process of resins, so varying amounts of free and unreacted monomers remain in the polymerized resin[3,4]. Unreacted monomers could be present as residual monomers, or they could be bound to the polymer backbones at one end, leaving the other end free. The release of these residual monomers from dental resins has been widely studied in the literature[4-7], with the main concerns being the amount of leachable monomers and the time needed for complete elution. However, little information is available regarding the role of the monomer size and chemical characteristics in this elution. Information about the time needed for the complete elution of the extractable unreacted monomers is also contradictory: Some studies have indicated that the elution is complete in 1 to 7 days, while other studies have found that it continues for a longer time[3]. The contribution of resin cements as a potential source of unreacted monomers to oral and other exposed tissues is therefore of considerable research interest.

UDMA is commonly used as a base monomer in self-adhesive resin cements, but its high viscosity necessitates the use of a diluent comonomer, such as TEGDMA[1]. The leaching of UDMA and TEGDMA from dental resin materials has been suggested to cause cytotoxic and genotoxic effects in cells[8]. An evaluation of the cytotoxicity of 35 monomers or additives used in dental resin materials showed a relationship between their amounts and the degree of cytotoxicity; TEGDMA and UDMA were both found highly cytotoxic[9]. The molecular mechanisms underlying this cytotoxicity have been suggested to include reactive oxygen species (ROS) production as a key factor leading to cell apoptosis[10]. Kleinsasser *et al.*[11] also demonstrated the capability of TEGDMA and UDMA to cause DNA damage. Very few studies have examined the toxicity of self-adhesive resin cements. Schmid-Schwap *et al.*[12] reported that adhesive resin cements were less cytotoxic

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than self-adhesive resin cements. Ulker and Sengun[13] documented that self-adhesive resin cements could modify pulp cell metabolism when the materials were used in deep cavities or in direct contact with pulp tissue. However, the actual amounts of potentially toxic residual monomers in these cements and their long term biological effects were not determined.

The very limited information available concerning residual monomer release from commercial self-adhesive resin cements prompted the present study. The specific aims were (a) to use a high-performance liquid chromatography (HPLC) to determine the substances eluted into artificial saliva from three self-adhesive resin cements and (b) to investigate the cytotoxic, genotoxic and apoptotic potential of these eluted materials and to correlate these effects with the released compounds. The first research hypothesis was that no differences would occur in elution of residual monomers (UDMA and TEGDMA) into artificial saliva after polymerization of three commercially available self-adhesive resin cements (G-Cem, GC, Tokyo, Japan; SpeedCEM, Ivoclar Vivadent, Schaan, Liechtenstein; RelyX U200, 3M ESPE, Seefeld, Germany). The second research hypothesis was that the amount of residual monomer released would not increase over time (1, 24 or 72 h). The third research hypothesis was that the three commercially available self-adhesive resin cements do not induce cytotoxic and genotoxic effects on living cells.

MATERIALS AND METHODS

Three commercially available self-adhesive resin cements (G-Cem, GC; SpeedCEM, Ivoclar Vivadent; RelyX U200, 3M ESPE) were tested in this study (Table 1).

Monomer release analysis

A total of 60 disk-shaped specimens (5 mm diameter and 0.5 mm thickness) were prepared from each material using a Teflon mold (n=20). The molds were filled with uncured material and covered with a Mylar strip to protect the resin cement from the oxygen inhibition zone. The materials were polymerized using LED light curing units (Optima-10, BA International, Northampton, UK) with an output irradiance of 1,200 mW/cm^2. The exposure times were chosen as 20 s. based on the manufacturers' recommendations. The irradiance was periodically checked with a dental radiometer during the specimen preparation (Hilux Radiometer, Curing Radiometer, Benlioglu, Ankara, Turkey).

Cured samples were detached from the Teflon molds and immediately immersed in light-proof vials containing 1.5 mL artificial saliva and held at 37°C. The light-proof vials had been cleaned by distilling with ethyl acetate twice and heating at 100°C for at least 12 h before use. Contamination from other polymer-based materials and plastics was prevented by avoiding the use of gloves. Artificial saliva was composed of NaCl

Table 1 Composition of self-adhesive resin cements used in this study

Material	Composition *
G-Cem Automix, Lot: 1401141 GC, Tokyo, Japan	Past A: UDMA, glass fiber, 2-Hydroxy-1,3 dimethacryloxypropane, Silanamine, 1,1,1-trimethyl-N-(trimethylsilyl)-, hydrolysis products with silica, y-Methacryloxypropyltrimethoxysilane, Ethyl 4-dimethylaminobenzoate Past B: UDMA, camphorquinone, hydroperoxide, quartz, 2-Hydroxy-1,3 dimethacryloxypropane, Silanamine, 1,1,1-trimethyl-N-(trimethylsilyl)-, hydrolysis products with silica, tert-butyl hydroperoxide, Distilled water, y-Methacryloxypropyltrimethoxysilane, 2,4,6-trimethylbenzoyldiphenylphosphineoxide
SpeedCEM, Lot: 627590 Ivoclar Vivadent, Schaan, Liechtenstein	Base: UDMA, TEGDMA, polyethylene glycol dimethacrylate, silicon dioxide, titanium dioxide, diiron trioxide, iron hydroxide oxide, triiron tetraoxide, ethyl p-dimethylaminobenzoate, campherquinone Catalyst: UDMA, TEGDMA, Methacrylated phosphoric acid ester, polyethylene glycol dimethacrylate, dibenzoyl peroxide
RelyX U200 Automix, Lot: 564623 3 M ESPE, Seefeld, Germany	Base: Silane treated glass powder, 2-propenoic acid, 2-methyl-, 1,1'-[1-(hydroxymethyl)-1,2-ethanediyl] ester, reaction products with 2 hydroxy-1,3-propanediyl dimethacrylate and phosphorus oxide, triethyleneglycol dimethacrylate (TEGDMA), silane treated silica, sodium persulfate, glass powder, tert-butyl peroxy-3,5,5-trimethylhexanoate Catalyst: Silane treated glass powder, substituted dimethacrylate, 1-benzyl-5-phenyl-barbic-acid, calcium salt, silane treated silica, sodium p-toluenesulfinate, 1,12-dodecane dimethycrylate, calcium hydroxide, methacrylated aliphatic amine, methacrylated aliphatic amine, titanium dioxide

* Manufacturer supplied.
TEGDMA, triethyleneglycol dimethacrylate; UDMA, urethane dimethacrylate

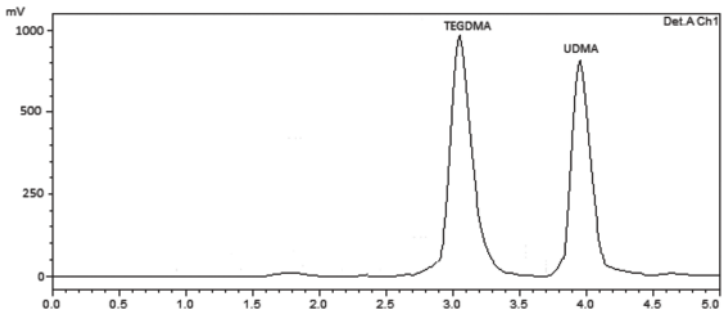

Fig. 1 A representative chromatogram of 5 μM of TEGDMA and UDMA standard solutions.

(0.4 g), KCl (0.4 g), $CaCl_2 \cdot 2H_2O$ (0.795 g), $NaH_2PO_4 \cdot 2H_2O$ (0.78 g), $Na_2S \cdot 9H_2O$ (0.005 g), urea (1.0 g) and distilled water (1,000 mL), at a neutral pH, as previously described[14]. At each sampling point (1, 24 and 72 h), the extraction media were removed and transferred to new vials for HPLC analysis. Fresh 1.5 mL artificial saliva was then added to each sample vial and the incubation was continued until the next sampling point. The cumulative amount of monomer for 24 h is the sum of the monomers amount measured at 1 and 24 h; the cumulative amount of monomer for 72 h is the sum of the monomers amount measured at 1, 24 and 72 h.

All measurements were performed three times for each extract. Calibration curves were made by relating the eluted peak area to the peak areas of known concentrations of monomers (UDMA and TEGDMA, Sigma-Aldrich, Steinheim, Germany). Standard chromatograms of monomers were obtained (Fig. 1). The concentrations of the monomers leached from the tested self-adhesive resin cements over time were calculated using the coefficients obtained from a linear regression analysis of the results obtained from the standard series. The residual monomers of resin material in solution were identified by HPLC with ultraviolet detection. Identification was confirmed with reference substances.

The analysis of extracts from the resin material, as well as reference solutions of the monomers in water/acetonitrile (35:65%), was carried out by HPLC (Shimadzu, Kyoto, Japan) under the following conditions:

—Column: steel column (Intertsil ODS-3), 150 mm length, 4.6 mm diameter, and particle size 5 μM
—Mobile phase: 65% CH_3CN (Acetonitrile)/35% H_2O
—Flow speed: 1 mL/min
—Detection: UV, 215 nm
—Injection: 10 μL loop at constant room temperature (25°C)

Toxicity analysis

Ethidium bromide (EB) (Sigma-Aldrich, Seelze, Germany), 2′,7′-dichloro-dihydrofluorescein-diacetate (DCFH-DA) (Sigma-Aldrich), acridine orange (AO) (Sigma-Aldrich), penicillin-streptomycin (Sigma-Aldrich), low-melting-point agarose (LMPA) (Sigma-Aldrich) and normal melting point agarose (NMPA) (Sigma-Aldrich) were prepared. Eagle's Minimum Essential Medium (EMEM), horse serum (HS), penicillin/streptomycin, and trypsin were purchased GIBCO (Grand Island, NY, USA). All other reagents were analytical grade, unless otherwise stated.

Stock solutions containing the same amounts of the monomers determined from HPLC results in the 72 h samples were prepared in dimethyl sulfoxide (DMSO). Each stock solution was diluted with EMEM (containing no HS). The final concentration of DMSO in the monomer solution was <1%. Prior to the start of experiments, we confirmed that this level of DMSO, as well as the serum-free medium, did not induce any DNA damage in the cells. Other reagents were prepared fresh before each experiment.

A standard mouse fibroblast cell line (L-929) was obtained from the American Type Cell Culture Collection (ATCC). The cells were cultured at 37ºC in EMEM under a 5% CO_2 atmosphere. The medium was supplemented with 10% HS, 10,000 U/mL of penicillin and 10,000 μg/mL of streptomycin. The number of viable cells was estimated using the trypan blue exclusion test.

1. Cytotoxicity assay

The cytotoxic effects of monomers on L-929 cells were determined by measuring ATP levels using a luminescence test (Cell-Titer-Glo Luminescent Cell Viability Assay, Promega, Madison, WI, USA). Cells were seeded onto 96-well plates at a density of 1×10^4 cells per well and incubated overnight at 37ºC in 5% CO_2. The medium was then replaced with fresh complete medium containing various concentrations of the monomer stock solutions (1:8, 1:4, 1:2, 1, 2, 4, 8). Control cells were treated with 1% DMSO. All the cells were incubated in a humidified 5% CO_2 and 95% O_2 atmosphere at 37ºC for 24 h. The cells were then rinsed with the culture medium and ATP levels were determined. Each sample was supplemented with 100 μL of the prepared reagent (CellTiter-Glo Luminescent Cell Viability Assay, Promega), mixed for 2 min and incubated for 10 min at room temperature. The results were read using a luminometer (Varioskan Flash

Multimode Reader, Thermo, Waltham, MA, USA). The light emitted due to the presence of ATP was quantitated in relative light units (RLU). Cell viability was expressed as a percentage of the light emitted by untreated control cells. All experiments were repeated three times.

2. Measurement of ROS generation
Generation of ROS was assessed using a cell-permeable fluorescent signal CM-DCFH-DA (2,7-dichlorodihydrofluorescein) indicator for ROS[15]. After a 24 h incubation, the 2´,7´-dichlorofluorescein (DCF) fluorescence intensity was measured using a fluorescence plate reader (Varioskan Flash Multimode Reader, Thermo) at Ex./Em.=488/525 nm. The values were expressed as % relative fluorescence compared to the control.

3. Morphological evaluation by fluorescence microscopy
Morphological changes in cells were studied by acridine orange/ethidium bromide (AO/EB) double staining, as described by McGahon *et al.*[16]. This technique distinguishes cells undergoing apoptosis from the viable cells by the morphological changes of apoptotic nuclei. Cells were treated with various concentrations of the stock solution (1:8, 1:4, 1:2, 1, 2, 4, 8). After a 24 h incubation, the AO/EB solution was added to the cell suspension and the nuclear morphology was evaluated by fluorescence microscopy (Leica DM 1000, Solms, Germany). DMSO (1%) was used as a negative control. Multiple photos were taken at randomly-selected areas. This method distinguishes live cells with normal green nuclei, apoptotic cells with green nuclei and fragmented chromatin, and dead cells with orange/red nuclei. Tests were run in triplicate.

4. Genotoxicity assay
Genotoxic effects of monomers on L-929 cells were evaluated using an alkaline single cell gel electrophoresis assay (Comet Assay), according to Singh *et al.*[17], with slight modification. Cells were seeded onto 6-well cell culture plates (approximately 2×10^5 cells per well) with cell culture medium and incubated at 37ºC in 5% CO_2 for 24 h to allow cell establishment. Various concentrations of the stock solutions (1:8, 1:4, 1:2, 1, 2, 4, 8) were then added to the cells and incubated for another 24 h at 37ºC. DMSO (1%) was used as a negative control and 50 M H_2O_2 was used as a positive control. After incubation, the cells were washed with phosphate buffered saline (PBS), harvested using trypsin/EDTA and collected by centrifugation at 400×g for 5 min at 4ºC. The supernatant was decanted and the cell density was adjusted to 2×10^5 cells/mL using cold PBS. Ten milliliter of resuspended cells were placed into centrifuge tubes for the comet assay. All experiments were repeated in triplicate. The tail intensity in DNA was analyzed as a sign of DNA damage. This quantity was correlated with the negative control as a percentage.

Statistical analysis
Differences between the groups were statistically analyzed by one-way analysis of variance (ANOVA) and Tukey HSD tests. The groups were compared to verify the differences at a significance level set at $p<0.05$ using SPSS 14 for Windows (SPSS, Chicago, IL, USA).

RESULTS

Monomer release analysis
The mean values of the residual monomers released from each self-adhesive resin cement are indicated in Table 2. The amounts of monomers eluted from all three

Table 2 Mean value of the residual monomers released from each self-adhesive resin cements

Material	Period (h)	Substances released (Mean values±SD)	
		TEGDMA	UDMA
G-Cem	1	n.d	16.78±3.72 [A]
	24	n.d	46.72±6.05 [B]
	72	n.d	65.62±10.58 [C]
SpeedCEM	1	4.22±0.70 [a]	3.200±0.46 [D]
	24	5.93±0.79 [b]	8.332±0.96 [E]
	72	6.18±0.79 [b]	10.903±1.74 [E]
RelyX U200	1	2.08±0.80 [c]	n.d
	24	2.90±0.94 [d]	n.d
	72	3.04±0.97 [d]	n.d

n.d: These values were under the quantification's limit. The concentrations were calculated as μM (Micro Molarity)
Different lowercase letters represent statistically significant differences between amount of eluted TEGDMA per storage time periods.
Different uppercase letters represent statistically significant differences between amount of eluted UDMA per storage time periods.

self-adhesive resin cements were calculated per storage period; the lowest amounts of leached monomer were found for the 1 h sampling point ($p<0.05$). The TEGDMA release from G-Cem was not detectable for all periods, however UDMA release was fairly high. In RelyX U200, released amount of UDMA was not detectable, whereas the TEGDMA release was quantitated. Higher cumulative amounts of TEGDMA were eluted from SpeedCEM than from RelyX U200, whereas the UDMA elution was higher from G-Cem than from SpeedCEM ($p<0.05$). The highest total cumulative amount of residual monomer was eluted from G-Cem ($p<0.05$) (Fig. 2). Figure 3 summarizes the HPLC data in order to compare the release profile of the related monomers. It is obviously seen that UDMA release is the highest from G-Cem and it is fairly high from SpeedCEM, TEGDMA release is higher from SpeedCEM than Relyx U200.

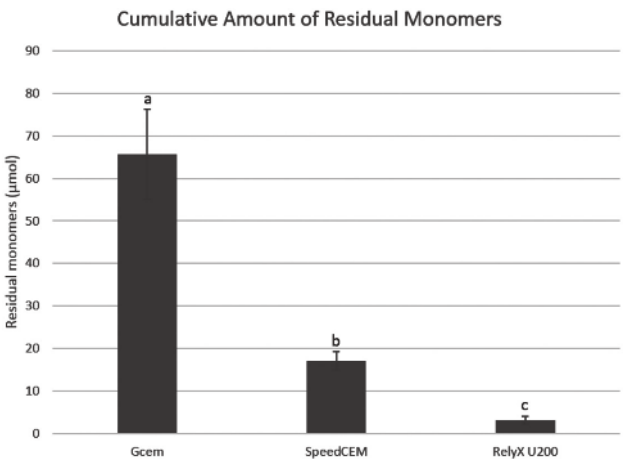

Fig. 2 The cumulative amount of total eluted residual monomers from self-adhesive resin cements.

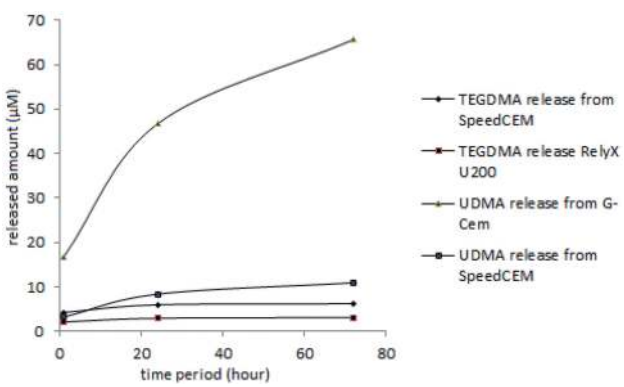

Fig. 3 Releasing profile of TEGDMA and UDMA from different resin cements for different time periods.

Toxicity analysis

1. Cytotoxicity assay

Cytotoxicity was higher for all cements than for the control group ($p<0.01$). No statistically significant differences were noted between cell viability values for G-Cem and RelyX U200 ($p>0.05$); however, the SpeedCEM gave the lowest cell viability values ($p<0.01$) (Fig. 4).

2. Measurement of ROS generation

The ROS values for all cements were not significantly different ($p>0.05$) and all cements showed higher ROS values when compared to the control group ($p<0.01$) (Fig. 5).

3. Morphological evaluation by fluorescence microscopy

AO/EB staining revealed morphological features typical of apoptosis in all groups (Fig. 6).

4. Genotoxicity assay

All cements showed increased DNA damage values when compared to the control group ($p<0.01$). Among the self-adhesive resin cements, the highest DNA damage values were obtained with RelyX U200 ($p<0.05$) (Fig. 7).

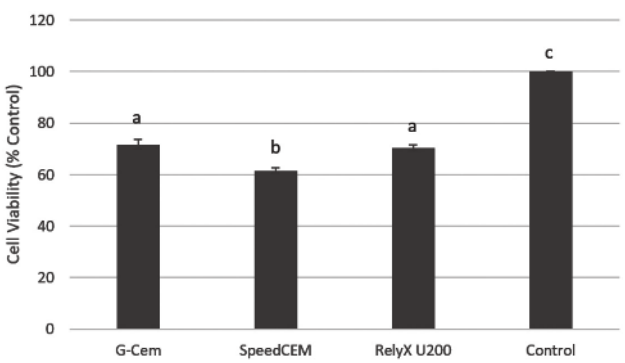

Fig. 4 Cell viability values after exposure to release monomers of self-adhesive resin cement.

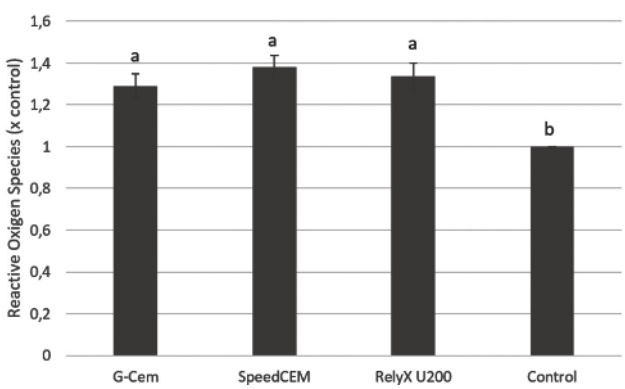

Fig. 5 Production of ROS in L-929 mouse fibroblasts after exposure to release monomers of self-adhesive resin cement.

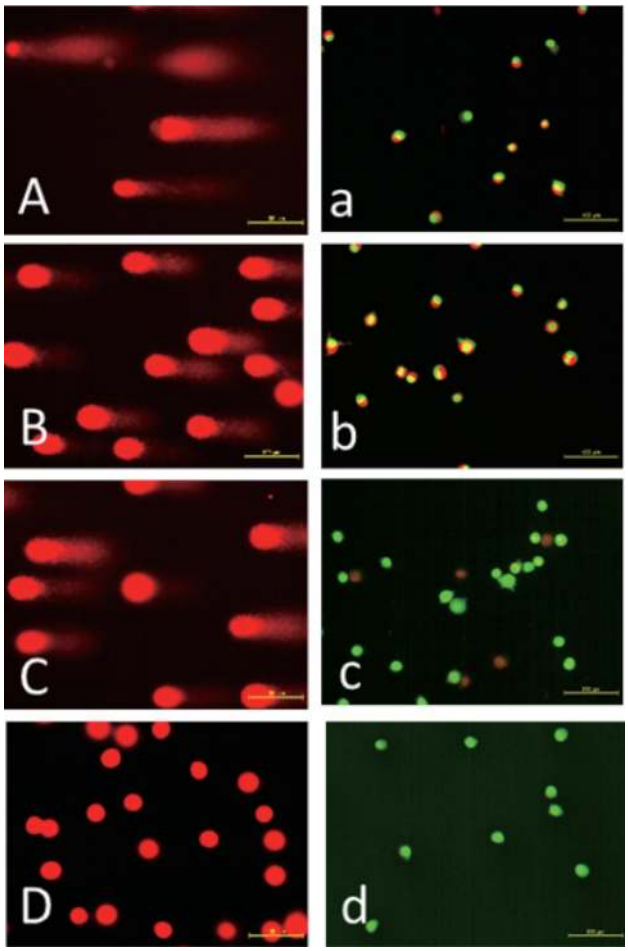

Fig. 6 Uppercase letters indicate genotoxic effect while lowercase letters shows apoptosis in L-929 mouse fibroblast cells after exposure to release monomers of self-adhesive resin cement and the control group. A and a: G-Cem, B and b: SpeedCEM, C and c: RelyX U200, D and d: Control. Comet formation pattern showed that self-adhesive resin cement groups induces DNA damage, *i.e.*, refers to occur the genotoxic effect (A–C) while the control group showed no comet formation (D). Different morphological patterns of apoptosis induced by release monomers of self-adhesive resin cement determined by AO/EB staining. Cells were treated with release monomers of self-adhesive resin cement for 24 h and, subjected to AO/EB staining. The control group showed intact nuclei with green fluorescence (d). Cells showed that apoptotic bodies with yellowish orange nuclei in self-adhesive resin cement groups (a–c)

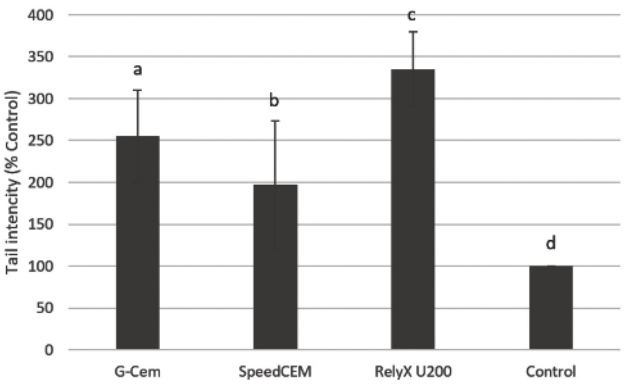

Fig. 7 Induction of DNA damage in L-929 mouse fibroblasts after exposure to release monomers of self-adhesive resin cement.

DISCUSSION

Resin-based materials are known to show toxic reactions in cell cultures and various studies have shown that toxicity is primarily induced by monomers released from the material[3,6,12,13,18-20]. In this study, the elution of UDMA and TEGDMA from self-adhesive resin cements in artificial saliva with time is evaluated and biological effects of the cumulative monomer amounts are determined. The results obtained for the elution of residual monomers from self-adhesive cements into artificial saliva solution did not support the first and second research hypotheses, which were that: 1) polymerized resin cements would show no elution of residual monomers (TEGDMA and UDMA) into an artificial saliva solution over time, and 2) the amount of residual monomers eluted would not increase with time. Instead, residual monomers clearly eluted into the artificial saliva solution and the amounts of residual monomers eluted increased with time. Moreover, monomer released from self-adhesive resin cements increased the cytotoxicity and genotoxicity, which led to rejection of the third research hypothesis: that the three commercially available self-adhesive resin cements do not have cytotoxic and genotoxic effects on cells.

Monomer release analysis

Adequate polymerization is crucial for obtaining the optimal physical properties and clinical performance of dental resin materials. Ideally, a dental resin should have all monomers converted to polymers during the polymerization reaction. Dual-cure materials tend to be more effective in the early stages of polymerization because they contain both photo initiators and components for a chemically activated reaction[19]. Kawahara *et al.*[7] used HPLC to investigate the elution of residual monomers at 1, 3, 6, 12, and 24 h and 3, 7, and 14 days. The early elution of residual monomers from dual-cured self-etch resin cement was noted at 1 and 24 h and 3 days. Therefore, in the present study, to measure early elution of monomers from dual cured resin cement, the time periods were determined as 1 and 24 h and 3 days. Krishnan *et al.*[21] studied the effect of diluents on the properties of a visible light-cured dental composite at specific intervals of 1, 7, 14, 21, 28, and 30 days and reported that the solubility of a visible

light-cured dental composite increased with time. Lee *et al.*[6] concluded that the elution of monomers from the dental resin composites specimens stored for 7, 14, and 30 days increased as a function of time, and the amounts of monomers in 30 days storage were higher than those produced after 7 days storage. These results are in accordance with the result of current study which concluded that the cumulative amount of residual monomers was the lowest after 1 h of storage.

Crosslinked dimethacrylate resins are virtually insoluble but are capable of swelling in good solvents[18,22]. The degradation of resins in the oral cavity depends on salivary enzymatic reactions, acidic conditions and erosive factors caused by food and drinks[23]; therefore, organic solvents such as ethanol, methanol, or mixtures of these solvents with water are especially preferred when simulating oral conditions[18,22]. Organic solvents have the ability to penetrate and swell the polymer network, facilitating the liberation of unreacted and leachable monomers. Penetration of the solvent into the matrix and the resultant expansion of the openings between polymer chains cause oligomers to diffuse out[22]. The intraoral fluids represent solvents with characteristics probably somewhere between the more aggressive organic solvents and water; the US FDA recommends using a 75% ethanol-water solution as a food/oral simulating liquid with clinical relevance[18,24]. However, the use of an ethanol/water immersion has been abandoned, as this would lead to the expansion of the polymer network and an excessive diffusion of residual monomers[24]. In this study, artificial saliva is used as extraction media to measure the monomer release. The saturation of the resin with the extraction medium takes weeks or months to complete, due to the slow diffusion of substances into the cross-linked matrix of the resin materials. In contrast to aggressive organic solvents (*e.g.* ethanol/water, methanol, *etc.*), artificial saliva penetrates less aggressively into the resin matrix and this could explain the increased release of residual monomers as the storage period progresses[25].

The RelyX U200 resin contained no UDMA and G-Cem contained no TEGDMA, based on the information provided by the manufacturers. G-Cem showed the highest cumulative amount of released residual monomers, whereas RelyX U200 showed the lowest. The amount of TEGDMA eluted was higher from SpeedCEM than from RelyX U200 and the amount of UDMA eluted was higher from G-Cem than from SpeedCEM. These differences in the amounts of residual monomers eluted from the three resin cements might arise from differences in filler amount and size in the resin material; the size, amount and chemical composition of the monomers; and the degree of completion of the polymerization reaction in the resin cements[24].

The design of this *in vitro* study has several limitations that complicate the comparison of the results with clinical conditions. From a clinical viewpoint, limitations arise concerning the correlation between *in vitro* and *in vivo* tests as well as clinical usage. However, the use of HPLC for *in vitro* residual monomer measuring is valuable for understanding the leaching ability of monomers from these self-adhesive resin cements at different times[4,18]. The *in vitro* elution of residual monomers from resin materials is related to their degree of polymerization, the properties of the resin composition, and the chemistry of the organic solvents used[24], whereas the *in vivo* elution is further affected by several other factors. One of these factors pertains to the dental personnel who apply the resin materials. From this point of view, following the manufacturers' instructions for the application and polymerization of resin materials is particularly important. Human oral fluids can also differ from person to person according to their chemical composition, enzyme activities and oral stresses. The present study also did not consider the monomer conversion values for self-adhesive resin cements applied and light-cured alone. The chemistry of the analyzed interface also changed when the resin was applied and diffused into the dentinal tubule, so this precluded the direct comparison of conversion values between the self-adhesive resin cement alone and in dentin liquid; thus, the significance of the observed differences is not known[26].

Toxicity analysis

Generally, information about the toxicity of self-adhesive resin cements is obtained by comparison with other resin cements[27-31]. However, the effects of monomer release by self-adhesive resins on cellular functions, such as cell viability and morphology, ROS generation, and DNA damage, have not been well studied.

1. Cytotoxicity assay

Uncured monomers released from resin-based materials were acknowledged to have cytotoxic effects on cell cultures for more than 10 years. For example, Geurtsen *et al.*[9] demonstrated a cytotoxicity effect of TEGDMA and UDMA on permanent 3T3 and three human primary fibroblast cultures in their study of the cytotoxicity of 35 dental resin monomers. Wisniewska-Jarosinska *et al.*[8] reported that a combination of both UDMA at 1 mM and TEGDMA at 5 mM did not produce a significant increase in cytotoxic effects on Chinese hamster ovary cells when compared to each compound applied individually. Durner *et al.*[32] found that mixtures of TEGDMA at 1.60 mM and UDMA at 0.04 mM had a synergistic interaction on human gingival fibroblasts. In contrast, Ratanasathien *et al.*[5] evaluated the cytotoxic effects of TEGDMA and UDMA in Balb/c 3T3 mouse fibroblasts and determined an antagonistic interaction between them. Several factors may play a role in the evaluation of monomer cytotoxicity, *e.g.* the assessed cell type and the amount, type, and combination of monomers; therefore, the results of these studies could have been affected by any of these factors. These factors suggest that the presence of TEGDMA at 6.18 mM and UDMA at 10.90 mM in SpeedCEM may have caused synergistic cytotoxicity effects in the present study.

2. Measurement of ROS generation and morphological evaluation by fluorescence microscopy

The molecular mechanisms of cytotoxicity could be important in the assessment of the potential of self-adhesive resin cement to cause adverse effects. ROS generation is known to trigger apoptosis as a major cause of cytotoxicity due to monomers leached from dental resin[33]. TEGDMA and UDMA both increased ROS levels[33,34] and apoptosis[8,35] in cells. In the present study, the amounts of TEGDMA and UDMA were sufficient to trigger apoptosis in fibroblast cells (Fig. 6 a–d). Even though an increase in ROS generation for the control group was observed, it was not found statistically significant among cement as distinct from the cytotoxicity results (Figs. 4 and 5). This could be related to different molecular mechanisms accompanying ROS generation that affect monomer induced cytotoxicity[36,37].

3. Genotoxicity assay

The monomers leached from resin cements can cause genotoxic as well as cytotoxic effects, even at very low concentrations. The genotoxic effects of monomers may have long latent periods; therefore, some time may elapse before the appearance of serious effects, *i.e.* tumorigenesis[33]. Several studies have reported genotoxic effects of TEGDMA and UDMA in different cell types using different assays[8,11,38-41]. For example, Wisniewska-Jarosinska *et al.*[8] used comet assays to show that both UDMA and TEGDMA induced genotoxicity when applied singly to Chinese hamster ovary cells, but a combination of both did not produce a significant increase in these effects. Kleinsasser *et al.*[38], who also used comet assays, observed a greater genotoxic effect for TEGDMA than for UDMA in human lymphocytes. The results of these studies agree with those presented in the current study. By contrast, Urcan *et al.*[39] and Arossi *et al.*[40], using the sensitive γ-H2AX DNA repair focus assay and somatic mutation tests, demonstrated a greater genotoxic effect for UDMA than for TEGDMA in human gingival fibroblasts and *Drosophila melanogaster* cells, respectively. These discrepancies in monomer genotoxicities may have arisen due to the use of different assay systems and cell types. The genotoxicity results also contrasted with the cytotoxicity results, where greater toxicity was observed with SpeedCEM than with either RelyX U200 or G-Cem. This may indicate that multiple mechanisms are involved in the toxicities of self-adhesive resin cements.

All self-adhesive resin cements showed increasing cytotoxicity and genotoxicity, according to the results of the current study. However, the toxicity assays used here have several limitations. For example, the *in vitro* toxic effects of TEGDMA and UDMA were studied in L-929 mouse cells instead of human cells. The use of human cells would have been a better choice for simulation; nevertheless, several studies have confirmed that various concentrations of TEGDMA and UDMA provoke cytotoxicity and genotoxicity in human cells[30,33,38] and that both L-929 mouse cells and human cells show comparable responses[41,42]. Another limitation is that self-adhesive cements may contain toxic monomers other than TEGDMA and UDMA that could also be released. Interactions could also occur between TEGDMA, UDMA and other released monomers that may be present at levels below a measurable limit. Therefore, assessment of the toxic potential of a self-adhesive resin cement requires knowing more than just the toxicities of the monomers that can be observed above the measurable limit. A third limitation is that these cements, in the clinical setting, are in close contact with the gingival tissue during cementation; therefore, the concentration of the monomers in saliva may be reduced due to the volume of the oral cavity and the tissue may not experience prolonged exposure to the monomers. *In vitro* and *in vivo* studies which can simulate better the clinical condition are needed to confirm the results of the current study.

CONCLUSIONS

Within the limitations of this study, the following conclusions can be made:

1. The highest total cumulative amounts of residual monomers were eluted from G-Cem.
2. Assessment of leaching in the three self-adhesive resin cements for 1, 24 and 72 h revealed that the lowest amount of monomer was leached after 1 h.
3. Residual monomers released from self-adhesive resin cements induced *in vitro* cytotoxicity and genotoxicity in fibroblast cells.
4. The release of TEGDMA and UDMA from self-adhesive resin cements are available in artificial saliva at all the periods measured. These monomers induce cytotoxic and genotoxic effects which may have clinical adverse effects. However long-term *in vitro* and *in vivo* studies are needed to confirm these results.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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