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Genotoxic biomonitoring of flowable and non-flowable composite resins in peripheral blood leukocytes

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Abstract

Objective. Composite restorative materials represent one of the most important groups of materials in contemporary dental practice. However, their incomplete polymerization may lead to monomer-induced genotoxicity. The objective of this study was to evaluate the genotoxicity of three flowable (Filtek Supreme XT Flow, Tetric EvoFlow, Gradia Direct Flo) and three non-flowable dental composite materials (Filtek Z250, Tetric EvoCeram, Gradia Direct Posterior). ***Materials and methods.*** Genotoxicity assessment of composite materials was carried out *in vitro* in human peripheral blood leukocytes using the alkaline single-cell gel electrophoresis technique (comet assay). Prepared materials were eluted in saline solution for 1 h, 1 day and 5 days. Thereafter leukocyte cultures were treated with different concentrations of eluates obtained from each of the tested dental composite materials. Kruskall-Wallis non-parametric test was used for statistical analysis ($p < 0.05$). ***Results.*** The tested materials did not show genotoxic effects after exposure of leucocytes to 1 h eluates. Culture treated with 1 day eluates of all tested materials, only at a highest concentration (10^{-2}), affected the measured cytogenetic parameters. Of all tested materials, only Filtek Z250 and Filtek Supreme XT Flow did not exhibit a genotoxic effect in cultures that were under the influence of 5 day eluates. ***Conclusion.*** Tested materials exhibited limited genotoxic activity in peripheral blood leukocytes. Since the effect was observed only in leukocyte cultures treated by 1-day eluates at the highest concentration (10^{-2}) and it decreases in cultures exposed to 5 day eluates, it should not pose a significant risk to the human genome.

Key Words: *biocompatibility, comet assay, composite resin, genotoxicity test, leukocytes*

Introduction

A composite is a mixture of two or more components; each of these components contributes to the overall properties of the composite and may be clearly distinguished from one another [1,2]. The major components of composites are: organic resin matrix, inorganic fillers, coupling agents, initiators and accelerators. The relative percentages and different types of monomers influence the polymerization shrinkage, viscosity and water uptake of composites. Bisphenol A-diglycidyl dimethacrylate (Bis-GMA), along with urethane dimethacrylate (UDMA), is the most commonly used oligomer in dental composites today. Because of their high viscosity, diluent monomers with low molecular weight must be added in order to reduce the viscosity to a useful clinical level. Inorganic filler particles are added to improve various physical properties of composite materials. Today, most composites contain a variety of glass fillers, including fine colloidal silica particles, lithium-aluminum silicate glass and silica glasses containing barium, strontium or zinc [1].

In the oral cavity, composites can be degraded through a variety of physical and chemical mechanisms [3]. Biodegradation in the mouth involves dissolution and disintegration in saliva, wear and erosion by food, chewing forces and bacterial activity [4,5]. Nearly all the major components of composite resins have been found to be released following curing. Elution

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experiments revealed that the substances BisGMA, 2-hydroxyethyl methacrylate (HEMA), triethyleneglycol dimethacrylate (TEGDMA) and methyl methacrylate (MMA) have the highest propensity to get eluted [6]. The amount of release depends on the type of composite and the method and degree of cure that has been achieved [3,7]. Nanohybrid composites have shown greater release of monomers than from microhybrid and ormocer composites [8]. The influence of different light sources and different light intensities is extensively studied and it is concluded that a low degree of cure will contribute to the higher monomer elution [9,10]. Besides, the elution medium, which can be water, saline solution, ethanol, culture medium or artificial saliva, also has a great impact on monomer release. Ethanol is most commonly used, but the results indicate that saliva might be more appropriate because of the low Bis-GMA values obtained from composites immersed in ethanol [11]. The amount of released substances can be detected by using high-performance liquid chromatography [8], liquid chromatography–mass spectrometry [6,12] and gas chromatography–mass spectrometry [13].

After the different degradation products have left the surface of the resin composite restoration, they may continue degrading as they are transported through the body and may participate in various biologic reactions. These reactions can cause health problems and have an impact on the biocompatibility of composite restorations [14,15]. To screen and summarize if composite dental restorative materials are biologically acceptable, a set of toxicity tests may be used [16]. Numerous *in vitro* studies revealed estrogenic [17–19], mutagenic [19], teratogenic [19], genotoxic [14,17,19,20] and cytotoxic effects [14,19–22] of composite components. DNA damage caused by methacrylate monomers is of special significance due to the toxicology and biocompatibility of these substances.

The alkaline single cell microgel electrophoresis (Comet) assay detects genotoxicity in a wide variety of human cell materials [14,20,23] and has proven to be a sensitive and valid *in vitro* method [24,25]. This technique does not require cell cultivation; it detects primary DNA damage *in situ* at the level of each individual cell. The advantages are its sensitivity for detecting low levels of DNA damage, the requirement for small numbers of cells per sample, low costs and short time of processing. Despite the advantages of the test system, limitations do exist as far as the validity of detecting genotoxic effects in situations with elevated cytotoxic effects is concerned. With higher levels of non-vital cells, either from apoptosis or necrosis, the effects may be ambiguous, since ultimately they all lead to fragmentation of the DNA. As a result of these considerations, a consensus has been reached that only tests with 75% of vital cells should be interpreted as genotoxicity alone [26]. In a comet assay, tail intensity indicates the amount of DNA fragments directly indicating a proportion of genome affected by damage, whereas tail length is related to the DNA fragment size [23].

Flowable composites contain fewer filler particles or more diluent monomers than conventional non-flowable resins, which enable them to flow more readily than conventional composite materials [2]. Due to a higher resin/filler ratio in flowable composites, higher elution of residual monomers is expected. However, contrary results were reported and more TEGDMA and Bis-GMA is released from Tetric EvoCeram than from its flowable counterpart Tetric EvoFlow [6,12]. Second generation of flowable materials with high filler amount is advertised as being able to sustain higher masticatory forces similar to conventional composites. However, in order to maintain low viscosity, they contain more low-molecular diluent monomers, such as TEGDMA [27]. TEGDMA is one of dental monomers which is considered to be most cyto- and genotoxic [28,29].

The hypothesis set for this study was that flowable composites exhibit higher genotoxicity than non-flowables. In our work we focused on six commercial materials widely used in restorative dentistry, three flowable and three non-flowable composite resins. The aim was to compare and evaluate *in vitro* genotoxic effects of polymerized flowable and non-flowable composite materials in human leukocyte cells by the single cell gel (comet) assay in correlation to the duration of elution period and the elution concentration of used material.

Materials and methods

Blood sampling

Evaluation of potential genotoxicity of dental composite resins was performed on leukocytes obtained from three young, healthy, non-smoking voluntary donors. The donors were one female and two males with a mean age of 29.7 years (SD = 2.52). According to a questionnaire, which the donors completed, they had not been exposed to any physical or chemical agent that might have interfered with the results of genotoxicity testing in the 12-month period prior to blood sampling. A peripheral blood sample (V = 5 ml) was collected under sterile conditions by venepuncture into heparinized tubes (Becton Dickenson, Plymouth, UK). All donors participated voluntarily and they signed an informed consent form, which was reviewed and approved by the Ethical committee of School of Dental Medicine, University of Zagreb, Croatia.

Preparation of materials

Six dental composite materials were tested: Tetric EvoCeram (Ivoclar Vivadent, Schaan, Liechtenstein), Tetric EvoFlow (Ivoclar Vivadent), Gradia Direct

Posterior (GC, Tokyo, Japan), Gradia Direct Flo (GC), Filtek Z250 (3M ESPE, St. Paul, MN) and Filtek Supreme XT Flow (3M ESPE). Their composition, as provided by the manufacturers, is presented in Table I. Under aseptic conditions, 0.23 (0.03) g of each composite resin was taken for each sample. After weighing, each composite sample was mechanically pressed between two Mylar sheets to obtain a 1 mm thick layer. Resin composite samples were polymerized by the Elipar TriLight (3M ESPE) halogen curing unit in standard mode (800 mW/cm^2) for 40 s. The light curing tip was flush pressed onto the Mylar sheet on top of the composite samples. Thereafter, the polymerized composites were separated from the Mylar sheets, fragmented and placed in bottles (Sartorius BLG10S, Goettingen, Germany). For each 0.1 g of composite, 1 ml of saline solution was added (NaCl 0.9%, Sigma, St. Louis, MO) for the purpose of elution. Composite samples of each material were left in saline solution for 1 h, 1 day and 5 days.

Trypan blue exclusion test

To monitor cytotoxic effects, the trypan blue exclusion test was applied. Cultures for cytotoxicity testing were set up at the end of the cultivation period. By the end of the cultivation period, the pH of each cell culture was additionally checked by a SevenEasy pH meter (Metler-Toledo, GmbH, Schwertzenbach, Switzerland) anda pH indicator in the medium did not show any changes in pH value.

Two millilitres of primary leukocyte culture was introduced into 8 mL of F-10 HAM's medium (Sigma) without serum or mitotic activator. Cultures were treated for 48 h at 37°C with 100 μl, 10 μl and 1 μl of eluates obtained from each of the tested dental composites to simulate final mass concentrations of 10 mg of material/ml, 1 mg/ml and 0.1 mg/ml (three different dilutions of eluate 10^{-2}, 10^{-3} and 10^{-4}), respectively.

Thereafter, the cultures were centrifuged at 1000 rpm for 10 min. Supernatant was removed and the precipitate was resuspended and the samples for vital staining and comet assay were taken. To control for cytotoxic effects, leucocyte viability was tested using the trypan blue exclusion test. A cell suspension was mixed with 0.4% trypan blue (Sigma) and then visually examined by using an Olympus CX 40 light microscope (Olympus, Tokyo, Japan) under 100 × magnification. For each concentration tested, 1000 lymphocytes were analysed. Blue coloured cells were considered to be non-viable and cells with clear cytoplasm as viable [30].

Comet assay

The comet assay was carried out under alkaline conditions, as described by Singh et al. [31]. All chemicals used to perform the comet assay were obtained from Sigma.

Table I. The composition of tested materials as reported by the manufacturers.

Composite resin	Manufacturer	LOT	Composite type	Resin (wt%)	Fillers (wt%)
Tetric EvoCeram	Ivoclar Vivadent, Schaan, Liechtenstein	P03092	Nano-hybrid	16.8% Bis-GMA, UDMA, Bis-EMA	82–83% barium glass, ytterbium trifluoride, mixed oxide and prepolymer
Tetric EvoFlow	Ivoclar Vivadent, Schaan, Liechtenstein	P80394	Nano-hybrid flowable	37.6 % Bis-GMA, UDMA, decandioldimethacrylat	61.4% barium glass filler, ytterbiumtrifluoride, mixed oxide, highly dispered silica, prepolymers
Gradia Direct	GC, Tokyo, Japan	1104262	Micro hybrid	23% UDMA, dimethacrylate co-monomers	77% silica, fluoro-alumino-silicate glass, prepolymerised filler
Gradia Direct Flo	GC, Tokyo, Japan	1105181	Micro hybrid flowable	25–40% di-2-methacryloyloxyethyl 2,2,4-trimethylhexamethylene dicarbamate, TEGDMA	55–70% fluoro-alumino silicate glass, silica
Filtek Z250	3M ESPE, St. Paul, MN	N321535	Micro hybrid	16% Bis-GMA, Bis-EMA, UDMA, TEGDMA	84% zirconia/silica particles
Filtek Supreme XT Flow	3M ESPE, St. Paul, MN	680931	Nanofilled flowable	35% Bis-GMA, TEGDMA and Bis-EMA, dimethacrylate polymer	65% silica nanofiller, zirconia nanofiller, zirconia/silica nanocluster

Bis-GMA, bisphenol A-glycidyl methacrylate; TEGDMA, triethylene glycol dimethacrylate; UDMA, urethane dimethacrylate; Bis-EMA, Bisphenol A polyethylene glycol diether dimethacrylate.

After 48 h, the culture medium was carefully removed and 5 μL of the sediment containing leukocytes was suspended in 100 μL of 0.5% low melting agarose to obtain 10 000 leukocytes per slide. This agarose layer was sandwiched between a layer of 0.6% normal melting agarose and a top layer of 0.5% low melting agarose on fully frosted slides. The slides were coded and kept on ice during the polymerization of each gel-layer. After the solidification of the 0.5% agarose layer, the slides were immersed in a lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Triton X-100 and DMSO 10%) at 4°C. After 1 h, the slides were placed in an electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH 13) at 4°C for 20 min to allow the DNA to unwind. Electrophoresis was conducted in a horizontal electrophoresis platform in fresh, chilled electrophoresis buffer for 20 min at 300 mA and 19 V. All of these steps were conducted under dimmed light to prevent the occurrence of additional damage. After electrophoresis, the slides were taken out of the tank and neutralized with Tris-HCl buffer (pH 7.5) 3-times for 5 min. Each slide was stained with 10% ethidium-bromide for 10 min.

For visualization of DNA damage, slides were examined at 1000 × magnification using a 40 × objective on a fluorescence microscope Ortoplan (Leitz, Wetzlar, Germany). Measurements were made by a computer-based imagine analysis system 'Comet assay III' (Perceptive Instruments Ltd, Halstead, UK). Images of 100 randomly selected leukocytes. i.e. 50 cells from each of two replicate slides per material/concentration/time point, were analysed. Breaks in the DNA molecule disturb its complex supercoiling, allowing liberated DNA to migrate towards the anode. Staining shows the DNA as 'comets'. The mean value of the tail length and tail intensity was calculated and used for the evaluation of DNA damage. During the analysis, the edges and eventually damaged parts of the gel as well as debris, superimposed comets, comets of uniform intensity and comets without a distinct head ('clouds', 'hedgehogs' or 'ghost cells') were avoided.

As the positive control, hydrogen peroxide (1 mmol/L) pre-treated slides were used. After layering the leucocytes in agarose gel on slides, 60 μL of hydrogen peroxide (1 mmol/L) was applied for 10 min on ice. Simultaneously, negative control cultures were treated with 100 μl of saline solution (NaCl 0.9%, Sigma). Slides were processed as described for the treated leucocytes cultures.

Statistical analysis

The comet test results (six materials/three time points/ three dilutions/100 comets) were tested by the Kruskal-Wallis test to determine the statistical significance. The level of significance was set at 0.05. All calculations were performed using commercial software, Statistica 7.0 (StatSoft, Tulsa, OK). Results are expressed as mean (SD).

Results

Cell viability, as tested using trypan blue exclusion of each treated group, was more than 85%. The pH value in the cell cultures was always between 7.18–7.31.

The DNA damage is expressed as tail length and tail intensity in the leukocytes following *ex vivo* 1 h, 1 d and 5 d treatment with three different dilutions of dental composite eluates is given in Figure 2. Representative images show cells with no damage—negative control (Figure 1A), positive control (Figure 1B) and cells with increasing tail length and intensity, previously treated with 1 day eluates of Tetric EvoCeram in 10^{-2} dilution (Figure 1C).

According to the data obtained from three separated experiments, there was no difference among materials after exposure to 1 h eluates compared to negative control (Figure 2A). Tail intensity increased after 1 day elution only for the highest concentrations, i.e. the lowest dilutions (10^{-2}) of Tetric EvoCeram, Gradia Direct and Gradia Direct Flow, as compared with negative control. The tail intensity increased above the negative control values at highest concentrations of all tested materials (Figure 2B). After 5 days of exposure to eluates none of the tested composite materials exhibited an increase in tail length. Only the highest concentrations, i.e. the lowest dilutions (10^{-2}) of Tetric EvoCeram, Tetric EvoFlow, Gradia Direct and Gradia Direct Flow revealed an increase in tail intensity. No effect of Filtek Z250 and Filtek Supreme XT Flow on the comet assay end-point was observed (Figure 2C).

The tail length values of the positive control reached 33.7(1.27) and tail intensity was 15.72 (0.72), whereas tail length for the negative control was 13.3 (0.15) and the tail intensity was 0.08 (0.02).

Discussion

The present study was aimed to investigate the differences of genotoxicity of flowable and non-flowable dental composites using comet assays *in vitro*. Based on the results of this study, the materials with a higher amount of resin were not those which were most genotoxic. The extent of genetic damage was dose-dependent and only the highest concentrations of eluates (10^{-2}) exhibited higher tail intensity.

The results indicate that not the amount of resin, but rather the type of resin in the material composition could influence the genotoxic potential of the materials. In this study, groups of composite resins from the same manufacturer had similar effects. Generally, Filtek Z250 and Filtek Supreme XT Flow were the least genotoxic materials with 16 and

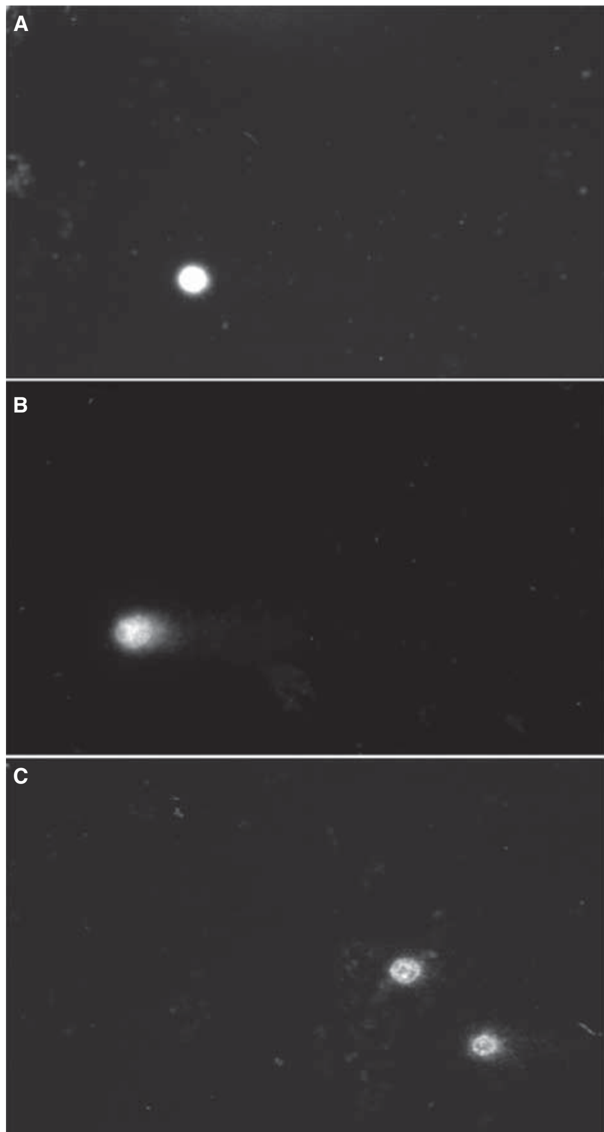

Figure 1. Comet assay assessment of composite resins genotoxicity in peripheral blood leukocytes: (A) Representative comet images of negative control (100 μl of 0.9% NaCl 4 h), (B) representative comet images of positive control (60 μl of 1 mM H_2O_2 10 min), (C) representative comet images of leucocyte cell treated for 1 day with an eluate of Tetric EvoCeram in 10^{-2} dilution.

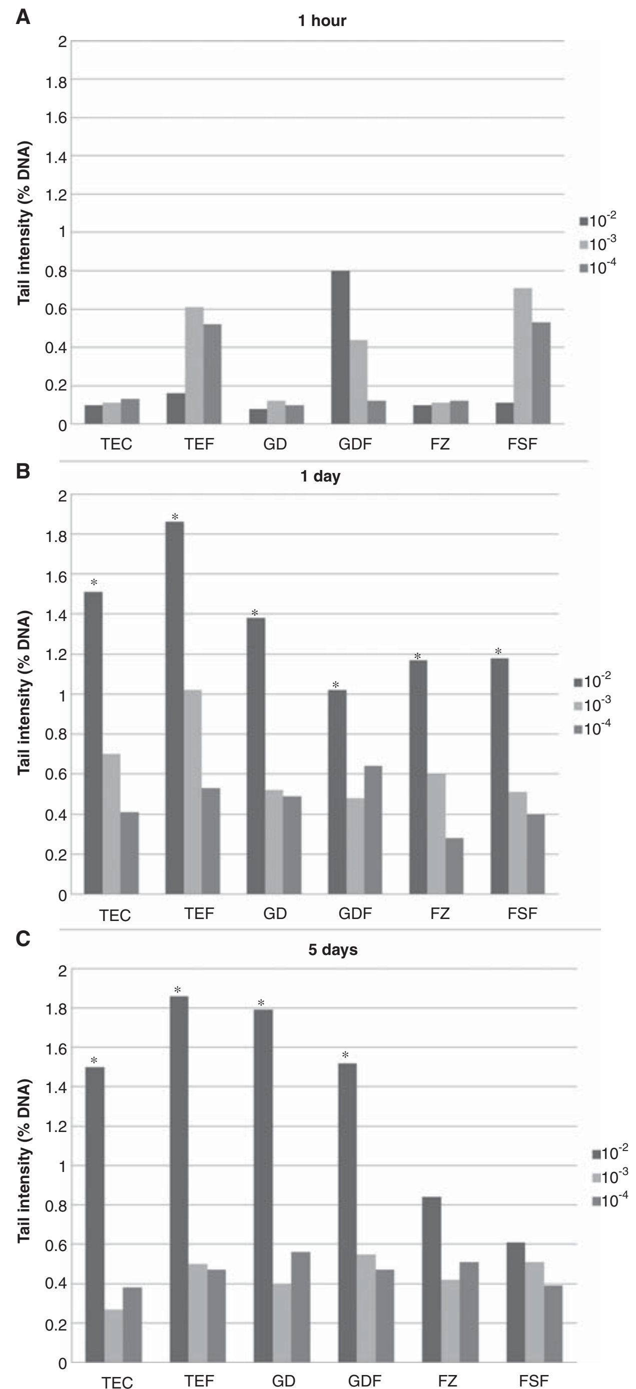

Figure 2. Percentage of DNA strand breaks (tail intensity) of leucocytes exposed to eluates from all tested composite resins: (A) 1 h eluates; (B) 1 day eluates; (C) 5 day eluates. Statistically significant differences are marked with * ($p < 0.05$). TEC, Tetric EvoCeram; TEF, Tertic EvoFlow; GD, Gradia Direct Posterior; GDF, Gradia Direct Flo; FZ, Filtek Z250; FSF, Filtek Supreme XT Flow.

35 wt% of resinous part, respectively. Their extracts from composite samples which remained in the saline solution for 5 days did not have a negative effect on the leukocyte culture. Besides Gradia Direct Flo, they also showed the least DNA damage in leukocytes after exposure to 1 day extracts. These results are in line with the findings of Al-Hiyasat et al. [32], where the smallest amounts of bis-GMA, TEGDMA and UDMA were eluted and detected for Filtek Z250 in comparison to all other tested materials.

Tetric EvoFlow and Tetric EvoCeram exhibited the highest genotoxic influence after treating the culture of human leucocytes with 1 day and 5 day eluates. The manufacturer claims that these materials do not contain TEGDMA and HEMA, but there are some conflicting results published in the independent studies [8,13]. These monomers are considered to be one of the most cyto- and genotoxic in dental resin-based materials [19,28]. However, the evidence of Bis-GMA-induced genetic damage is accumulating and it is known to demonstrate the highest toxicity, higher than UDMA, TEGDMA and HEMA [33]. Another study reported the greatest elution of UDMA

and BisGMA from Tetric EvoCeram and the least from Filtek Z250 [8], which is in agreement with our results. The flowable material, which was the material with the highest amount of resin, was more toxic when compared to the conventional counterpart from the same manufacturer. Besides, even though Tetric EvoCeram was advertised as having a small resinous part, it contains 34% of pre-polymerized filler particles which are also consisted of resin and fillers. It is known that there is no composite resin which can be 100% polymerized, regardless of which method of polymerization is used. Therefore, additional monomer release is expected also from pre-polymers [8]. This is the possible explanation of better results obtained for Filtek composites, since both groups contain a similar resin/filler ratio. Contrary to Filtek materials, where the genotoxic effect is decreasing, there was no difference among 1 and 5 days of elutions for both Tetric resins.

As claimed by the manufacturer, high genotoxicity of Bis-GMA was the reason why this monomer was excluded from the Gradia composite materials and replaced by UDMA. However, the urethane-based polymers are more prone to water absorption than the aromatic-based materials. Absorptions of aqueous solvents may cause passive and enzymatic hydrolytic degradation of the polymer matrix [34,35]. In our study, interesting results were obtained for both flowable and non-flowable Gradia materials. The genotoxic effect was visible in cultures exposed to 1 day and 5 day eluates. With 5 day eluates treatment, the genetic damage was significantly higher than for 1 day. The reasoning for these results could be the gradual release and biodegradation of UDMA. This monomer hydrolyses into methacrylic acid and epoxides, which are well known toxic and possibly mutagenic and carcinogenic agents [35]. In this manner, the genotoxicity is also increased, as demonstrated by the results of this study.

In the present study, polymerized composite samples remained in the saline solution for 1 h, 1 day and 5 days in order to simulate the effect of the release of unbounded monomers into saliva. It was anticipated that the greatest genetic damage will occur in leucocytes exposed to 5 day eluates, when the highest amount of eluted monomers is extracted. However, this was not the case, except for Gradia materials, and the behaviour patterns were different for the materials from different manufacturers. The Tetric group retained the same level of damage as after exposure to 1 day eluates, whereas the Filtek group decreased the genotoxicity. It is shown that the peak of the release of unbounded monomers is a couple of hours after polymerization [19]. Thus, the explanation for this phenomenon could be the arrestment of monomer release after the first day. Also, some of the eluted methacrylates tend to be hydrolysed into less harmful degradation products [36].

It is important to note that all the DNA damage for all the materials in this study was obtained only in the highest concentrations of 10^{-2}. This is in line with the study by Demirci et al. [37], who studied the cytotoxicity and genotoxicity of dental adhesives. A dose-dependent genotoxic behaviour of dental monomers is well-known and extensively studied [19,28,29,37].

The genotoxicity of dental composites is strongly related to their degree of conversion [19,38]. The composites with higher degree of conversion will generally have lower genotoxicity [19]. Under the same curing intensity, flowable composites exhibit higher degrees of conversion than their corresponding more viscous composites [39]. However, flowable composites usually contain more monomers of small molecular weight and are, therefore, more susceptible to elute into immersion medium [40] and cause cytotoxic and genotoxic effects [28,29]. In the present study, all the materials were polymerized in very thin layers to obtain a high degree of conversion. Although the degree of conversion was not measured, the same conditions for preparation of the samples should be sufficient to ensure the comparability of the data.

Within the limitations of the present study, it is concluded that the higher amount of resin found in flowable composite resin materials do not implicate higher genotoxicity. Therefore, the hypothesis of this study was not confirmed. The resin type and the released monomer species were found to have a greater influence on the genetic damage in human leukocytes *in vitro*. Since only the greatest concentrations of eluates demonstrated genotoxicity, tested materials should not pose a significant hazard to human genome. However, further *in vitro* and *in vivo* tests are necessary to confirm these findings.

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