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ORIGINAL ARTICLE

Composite-induced toxicity in human gingival and pulp fibroblast cells

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Abstract

Objective. The most important requirement for a material to be used in medical applications is its biocompatibility. Dental composite materials come into direct contact with oral tissues, especially gingival and pulpal cells. This study was performed to evaluate possible DNA damage in cells of human origin exposed to dental composites *in vitro* using a cytogenetic assay. ***Materials and methods.*** Two composite resins (Vertise Flow, Kalore) were tested on human gingival and pulp fibroblasts using the acridine orange/ethidium bromide viability staining and alkaline comet assay. Cultures were treated with polymerized composites in two different concentrations (20 mg/ml, 40 mg/ml) for 14 days. Chi-square and Kruskall-Wallis non-parametric test were used for the statistical analysis ($p < 0.05$). ***Results.*** Significant cytotoxicity was observed for 40 mg/ml of Vertise Flow in both cultures, while Kalore (40 mg/ml) showed cytotoxic effect only on human pulp fibroblasts. A significant level of DNA damage was detected for both materials and concentrations, in both cell cultures. ***Conclusion.*** If the two cell cultures are compared, the pulp cells were more sensitive to the cyto/genotoxic effects of dental composites. Based on the results, one can conclude that the use of tested materials may cause cellular damage in gingival and pulp fibroblasts *in vitro*.

Key Words: *cytotoxicity, comet assay, composite resins, fibroblasts, genotoxicity test*

Introduction

The use of composite materials for restoration of damaged and lost hard dental tissue is widespread in the restorative dental medicine. Despite the growing popularity, the concern about possible intrinsic toxicity is still great. Dental composites may release substances into the oral environment to a varying degree [1]. Substances are released during setting and polymerization of dental composite material and later as a result of material degradation. The amount of leachable substances depends on the chemical composition of a material, degree of monomer conversion, surface treatment and conditions within the oral cavity. Degradation of composites in the oral environment is caused by thermal changes, the components of saliva, chewing forces, chemical dietary changes and oral micro-organisms [2]. Dental monomers can be further metabolized in reactive by-products which stimulate the production of reactive oxygen species and consequent DNA, protein and cellular membrane damage, thus activating numerous transduction pathways leading to cytotoxicity, genotoxicity and tissue inflammation [3]. Triethylene glycol dimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA) induce changes in cell cycle and apoptosis caused by the reactive oxygen species, whereas bisphenol A-glycidyl methacrylate (Bis-GMA) alters the cell cycle by modifying gene expression and protein function [3].

The oral environment and dental materials interact in both ways, the material affects the biological environment and the environment affects the material. Biological reactions can be local or systematic [1]. Local effects are mostly manifested on pulp, gingival and oral mucosa cells [4,5]. Various degrees of

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inflammatory reactions and chemical inflammation have been found in the pulp of the teeth restored with composite resins [6]. Degraded or unpolymerized monomers can cause cellular and molecular cytotoxic injury; thus reduce pulp vitality. The influence of restorative materials on the pulp cells depends on the remaining dentin thickness, its permeability and the location [7]. The permeability of the gingival and mucosal epithelium allows penetration of leachable components, which indicates that there is a potential for a toxic effect with composite materials [4,5]. It has been reported that composites can cause irritation, chronic local reaction and epithelial proliferation on the gingiva and oral mucosa [8]. The mutual interaction between the material and the surrounding tissue does not always necessarily have noticeable clinical symptoms. Numerous studies have shown estrogenic [9,10], teratogenic [11], carcinogenic [11], mutagenic [12], genotoxic [4,5,13,14] and cytotoxic [15–17] effects of dental composite materials and their components.

The alkaline version of the comet assay developed by Singh et al. [18] detects DNA strand breaks and alkali-labile sites. Over the past two decades, the comet assay was developed as a quick, simple, sensitive and reliable genotoxicity test which is widely used to evaluate the genotoxic potential of dental materials [5,14,15]. To quantify DNA damage by the comet assay, the tail length (μm) and tail intensity (% DNA) are the most frequently used parameters. Tail intensity indicates the amount of DNA that migrated from the nucleoid directly indicating a proportion of genome affected by damage, whereas tail length is related to the migrating DNA size [19]. Up to now, this method has been used to evaluate genotoxicity of dental composites and resin monomers *in vitro* in human lymphocytes [15,20,21], leukocytes [13], human gingival fibroblasts [22,23], lung fibroblasts [24] and salivary gland tissue [20].

Nano-hybrid composite materials present generation of materials with good adhesion and improved polishing ability due to the combination of conventional and nano size fillers [25]. Compobond is a new class of composite materials that takes advantage of self-etching dentin adhesives and nano filled composites. These materials are easy to manipulate because they do not require pre-treatment of dental hard tissues which eliminate many errors, facilitates operation and increases the longevity of restorations [26].

The aim of the present study was to evaluate *in vitro* cytotoxic and genotoxic potential of two commercially available composite resins in primary culture of human gingival and pulp fibroblasts in correlation to the concentration of used material. The hypothesis set for this study was that a flowable self-adhering composite exhibits higher cytotoxicity and genotoxicity than a nano-hybrid non-flowable composite resin.

Materials and methods

Cell isolation

The human gingival fibroblasts were obtained from explants of healthy attached human gingiva around third molar from patients submitted to surgery for orthodontic reasons. Human pulp fibroblasts were taken from a human third molar germ of the same patients. These tissues were sampled after obtaining patient's informed consent strictly on a voluntary basis, with permission of the Ethical Committee of the School of Dental Medicine, University of Zagreb from three different donors (one male and two female; mean age (SD) of 20.66 (2.51) years; age range = 18–23 years).

To isolate cells from the tissue, samples were first minced mechanically, then incubated in Roswell Park Memorial Institute 1640 media (RPMI 1640; Gibco-Invitrogen, Carlsbad, CA, USA) with addition of 3 mg/ml of collagenase (Gibco-Invitrogen) at 37°C for an 1 h with continuous shaking. Suspensions were centrifuged at 1200 rpm for 3 min and isolated cells were washed 3-times in fresh RPMI 1640.

Human gingival and pulp fibroblast cells were grown on 75 cm^2 culture flasks in a 5% CO_2 atmosphere, 95% humidity and 37°C. The medium was RPMI 1640 (Gibco-Invitrogen) supplemented with 5% foetal bovine serum (FBS; Gibco-Invitrogen), 100 IU/ml penicillin/streptomycin (Sigma-Aldrich, Munich, Germany) and 50 IU/ml amphotericin B (Sigma-Aldrich), with medium changed every 3 days until confluent cell monolayers were formed. After the cells reached confluence, they were harvested with 0.25% trypsin (Gibco-Invitrogen) and 0.02% Ethilene Diamine Tetraacetic Acid (EDTA; Sigma-Aldrich) and seeded into a headspace vial at a density of 1×10^6 cells/vial in 5 ml growth media and were subsequently incubated for 48 h to reach confluence. Human gingival and pulp fibroblasts at passages 5–7 were used in the experiments.

Preparation of materials and cell culture treatment

In the present study, two dental composite resins were tested: Vertise Flow (Kerr Corporation, Orange, CA, USA) and Kalore (GC, Tokyo, Japan). Their compositions, as provided by the manufacturers, are presented in Table I. Under aseptic conditions, 0.1 and 0.2 g of each composite resin was taken (Sartorius BLG10S, Gottingen, Germany) for each sample. After weighing, each composite sample was mechanically pressed between two Mylar sheets to obtain a 2 mm thick layer. Resin composite samples were polymerized by the Bluephase C8 curing unit (Ivoclar Vivadent, Schaan, Liechtenstein) with a soft-start mode (650 mW/cm^2 for the first 5 s, followed by 800 mW/cm^2 until the end of illumination) for 40 s.

Table I. Resin-based composites used in the present study (manufacturers' data sheets).

Dental materials (type)	Manufacturer (LOT; color)	Resin (wt%)	Fillers (wt%)
Kalore (nano-hybrid resin composite)	GC, Tokyo, Japan (0906031; A2)	18% UDMA, dimethacrylate co-monomers, DX-511 monomer	82% Strontium glass (400 nm), lanthanoid fluoride (100 nm), strontium glass (700 nm), fluoroaluminosilicate glass (700 nm), silicon dioxide (16 nm)
Vertise Flow (self-adhering flowable composite)	Kerr Corporation, Orange, CA, USA (3439842; A2)	18-40% GPDM adhesive monomer, incorporates Kerr's Optibond adhesion technology	60–82% Barium glass (0.7 μm), barium glass (1 μm), colloidal silica (10–40 nm), ytterbium fluoride (40 nm)

GPDM, Glycerol phosphate dimethacrylate; UDMA, urethane dimethacrylate.

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 and placed in 5 ml of cell cultures, thus obtaining 20 mg/ml and 40 mg/ml mass concentrations. To examine DNA damage and cell viability the cells were incubated with dental resins for 14 days at 37°C. Negative control cells received only growth medium. Each DNA damage experiment included also positive control, which was hydrogen peroxide 60 μl for 15 min on ice.

Cytotoxicity testing

Cell viability was investigated by using the acridine orange/ethidium bromide viability staining. After 14 days of treatment, cultures were trypsinized and centrifuged at 1000 rpm for 3 min and supernatants were removed. The cells were gently re-suspended. On a microscope slide, 10 μl of suspension was mixed with 50 μl of 100 mg/ml acridine orange/ethidium bromide solution (Sigma-Aldrich). The suspension mixed with dye was covered by a cover slip and analysed under the epifluorescent Olympus BX 50 (Olympus, Tokio, Japan) microscope using 600× magnifications. For each fibroblast culture, 100 cells were analysed in duplicate. The nuclei of vital cells emitted a green fluorescence; early apoptotic emitted green fluorescence with condensed chromatin, late apoptotic cells red fluorescence with condensed chromatin and necrotic red fluorescence without chromatin condensation.

Comet assay

The alkaline comet assay was preformed according to standard protocol by Singh et al. [18]. Cell suspensions were obtained as described earlier. Aliquots of 10 μm cell suspensions were mixed with 100 μl of 0.5% low-melting point agarose, spread on microscope slides previously pre-coated with 1% normal-melting point agarose. The gels were covered with a cover slip and allowed to set at 4°C. After gelation, the cover slips were removed and the slide immersed in lysis solution (1% sodium sarcosinate, 2,5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Triton X-100 i 10% dimethyl sulphoxide). After lysis, 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. The slides were than stained with ethidium bromide (2 μm/ml, 10 min) and analysis was performed using a 40× objective of an epifluorescent microscope Olympus BX 50 (Olympus, Tokyo, Japan). Images of comets were scored using image analyser software Comet Assay IV (Perceptive Instruments Ltd., Suffolk, Halstead, UK). All steps were conducted in a dark place to prevent additional damage. Mean values of percentage DNA in tail and tail length were calculated from 100 cells per slide and two slides for each experimental point were analysed.

Statistical analysis

Kruskal–Wallis test was used to analyse the difference between mean values of tail length and percentage DNA in tail obtained for cultures treated with two dental composites (Kalore and Vertise Flow) in two different concentrations and negative controls, as well as to mutually compare results obtained for the two materials. The chi-square-test was applied to test significance between the cytotoxicity results for treated cultures and controls. The level of significance was set to 0.05. All calculations were performed using the commercial software, Statistica 7.0 (StatSoft, Tulsa, OK, USA).

Results

Cell death was quantified using the acridine orange/ ethidium bromide viability staining after 14 days of

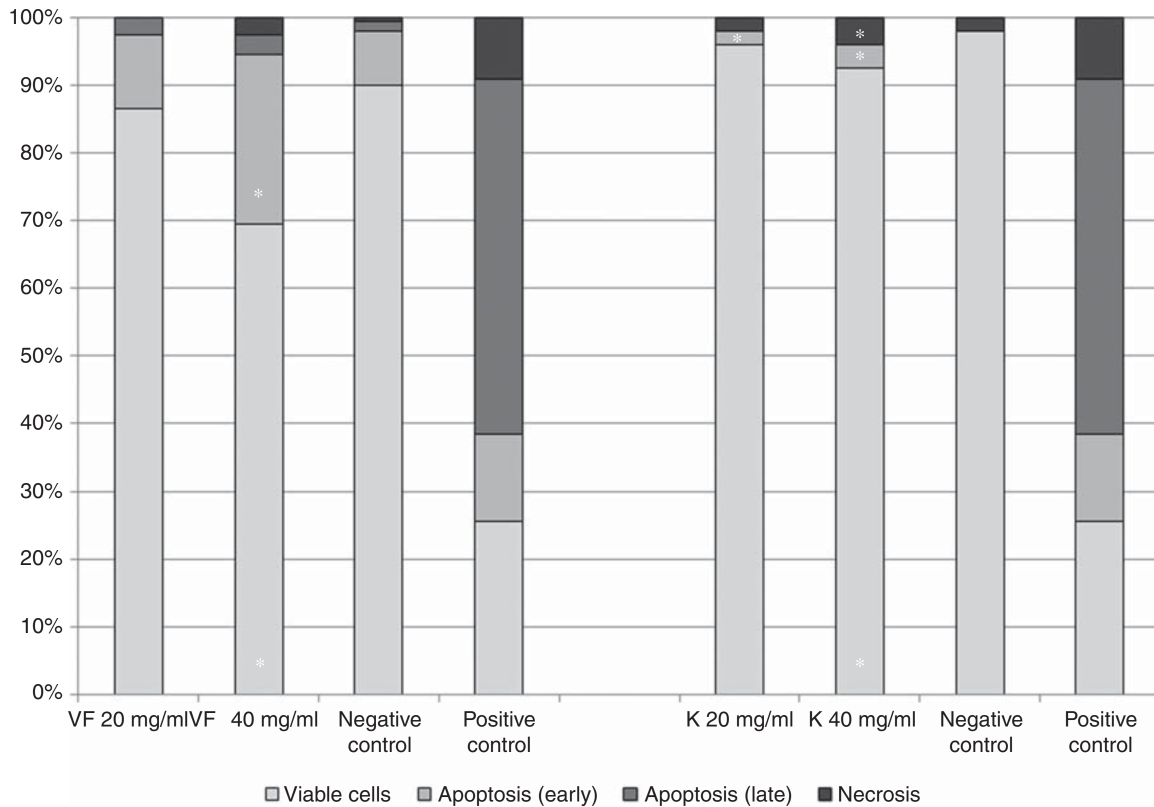

Figure 1. Cytotoxicity testing—percentage of viable, apoptotic and necrotic cells in human pulp fibroblast culture exposed to composite resins in two different concentrations. *Statistically significant values ($p < 0.05$) in comparison with the negative control. VF, Vertise Flow; K, Kalore.

treatment on primary cultures of human gingival and pulp fibroblast cells, with 20 mg/ml and 40 mg/ml dental composite resins. Vertise Flow demonstrated concentration-dependent cytotoxicity of human gingival and pulp fibroblast cells. While 20 mg/ml concentration of Vertise Flow was similar to negative control, the 40 mg/ml concentration exhibited cytotoxic activity to both human gingival and pulp fibroblast cultures inducing cell death mainly via apoptosis ($p < 0.05$) (Figures 1 and 2). Kalore (40 mg/ml) significantly decreased cell survival ($p < 0.05$), mostly inducing apoptosis in human pulp fibroblast culture (Figure 1). Regardless of testing concentration Kalore had no significant effect on the proportion of necrotic and apoptotic cells in human gingival fibroblast culture (Figure 2).

Composite resins in both concentrations and in both cultures induced a significant increase in DNA migration ability ($p < 0.05$) (Tables II and III). On culture of human gingival fibroblasts Kalore induced a significant increase in tail length ($p < 0.01$) in both concentrations compared to Vertise Flow (Table II). Also, Kalore in human pulp fibroblast cultures leads to the higher increase of tail length ($p < 0.01$) at the lower (20 mg/ml), while at the higher concentration (40 mg/ml) significantly higher damage to DNA compared to Vertise Flow was observed only in terms of the tail intensity ($p < 0.01$) (Table III).

Discussion

The purpose of this study was to examine the biocompatibility of two composite materials, Kalore and Vertise Flow, and their safety for clinical use by evaluating genotoxicity and cytotoxicity *in vitro*. The investigation was conducted using the acridine orange/ethidium bromide viability staining and comet assay. *In vitro* studies are simple and provide a significant amount of information and the results might be indicative of the effects observed *in vivo* [27].

Physiologically, gingival and pulp fibroblasts are highly exposed to resin monomers after their release from composite fillings to the oral cavity [23,28,29]. Indirect pulp capping with composite materials causes morphological changes in odontoblasts and inflammatory reaction in 22% of cases, while direct pulp capping with dental adhesive systems and composite materials leads to moderate-to-strong inflammation with abscess formation [3]. Therefore, primary cultures of human gingival and pulp fibroblasts were selected as optimal for testing of dental composite resins.

Toxicity testing of dental materials is mostly performed evaluating adverse effects of separate compounds of the final product individually, which may not be always relevant in assessing the biocompatibility of material used in restoration that is a mixture of substances that interact in formation of new

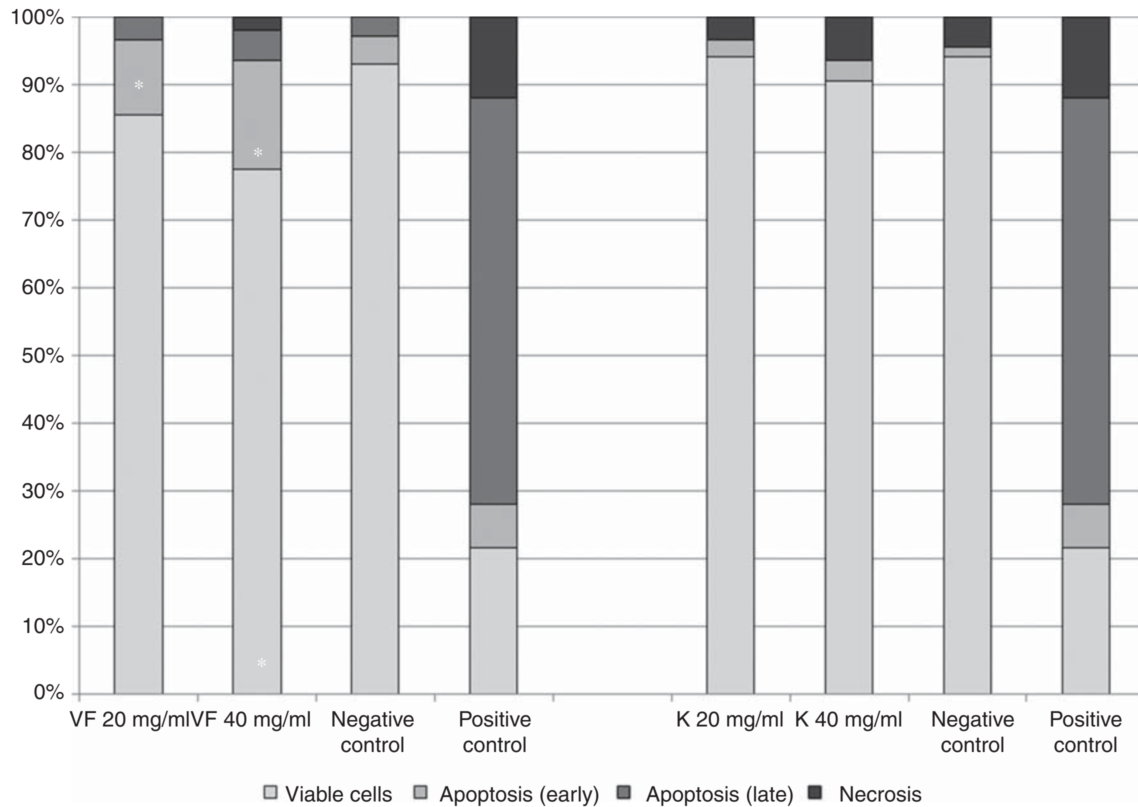

Figure 2. Cytotoxicity testing - percentage of viable, apoptotic and necrotic cells in human gingival fibroblast culture exposed to composite resins in two different concentrations. * Statistically significant values ($p < 0.05$) in comparison with the negative control. VF, Vertise Flow; K, Kalore.

chemical entities and simultaneously affect surrounding tissue. Cytotoxicity and genotoxicity of composite materials depends on released substances—unpolymerized monomers and soluble components degraded and eroded during time [30,31].

In the present study, results of cytotoxicity testing indicate that greater concentration of self-adhering composite material Vertise flow reduced cell viability and increased the number of early apoptotic cells in the pulp fibroblast culture. However, it should be mentioned that early apoptotic cells detected by differential staining do not univocally indicate cytotoxic activity that will result in cell death. The stage of early apoptosis is reversible and cells appearing as early apoptotic may recover after the removal of exogenous stressor. Nevertheless, similar cytotoxicity results were observed for the culture of gingival fibroblasts, except that, in this case, necrotic cells were also found. The only similar investigation also studying toxicity of Vertise Flow by Bektas et al. [32] has proved moderate cytotoxicity of Vertise Flow by agar diffusion test with less than 20% of lysed cells. This is not surprising knowing that Vertise Flow is based on traditional methacrylates with the addition

Table II. Tail length and percentage tail DNA (mean ± SD) in human gingival fibroblasts exposed to composite resins in different concentrations during 14 days of treatment.

Material concentration	Comet assay parameter	Vertise Flow	Kalore
20 mg/ml	Tail length (μm) ± SD	18.8 ± 3.1*	30.5 ± 5.8**##
	Tail intensity (% tail DNA) ± SD	2.3 ± 4.0*	2.4 ± 3.2*
40 mg/ml	Tail length (μm) ± SD	19.1 ± 2.6**	31.5 ± 4.5**##
	Tail intensity (% tail DNA) ± SD	2.5 ± 3.4**	1.2 ± 2.1
Negative control	Tail length (μm) ± SD	17.4 ± 3.2	24.5 ± 3.3
	Tail intensity (% tail DNA) ± SD	1.1 ± 2.7	1.5 ± 1.8
Positive control	Tail length (μm) ± SD	35.8 ± 4.1	39.3 ± 6.0
	Tail intensity (% tail DNA) ± SD	42.0 ± 11.7	51.3 ± 18.7

*$p < 0.05$; **$p < 0.01$ compared to control; ##$p < 0.01$ compared to the other material.

Table III. Tail length and percentage tail DNA (mean ± SD) in human pulpal fibroblasts exposed to composite resins in different concentrations during 14 days of treatment.

Material concentration	Comet assay parameter	Vertise Flow	Kalore
20 mg/ml	Tail length (μm) ± SD	24.1 ± 5.3	29.0 ± 4.7**##
	Tail intensity (% tail DNA) ± SD	3.4 ± 5.2**	3.0 ± 3.3**
40 mg/ml	Tail length (μm) ± SD	27.9 ± 6.5**	27.8 ± 5.1**
	Tail intensity (% tail DNA) ± SD	1.6 ± 2.4**	4.0 ± 6.9**##
Negative control	Tail length (μm) ± SD	23.0 ± 6.0	25.2 ± 3.9
	Tail intensity (% tail DNA) ± SD	0.4 ± 0.7	0.6 ± 1.0
Positive control	Tail length (μm) ± SD	39.1 ± 8.2	42.7 ± 10.4
	Tail intensity (% tail DNA) ± SD	41.0 ± 12.2	49.8 ± 11.6

*$p < 0.05$; ** $p < 0.01$ compared to control; ## $p < 0.01$ compared to the other material.

of OptiBond All-in-One (Kerr Corporation, Orange, CA, USA) self-etch adhesive system. This adhesive has in its composition acidic monomer, glycerol phosphate dimethacrylate, HEMA and Bis-GMA. Bis-GMA is a proven cytotoxic agent, which causes necrosis of human pulpal and gingival fibroblasts in lower concentrations and apoptosis in higher ones [3,33]. Concentrations higher than 0.075 mM induce apoptosis in pulpal cells, thereby releasing prostaglandins and formation of reactive oxygen species [34]. It is also demonstrated that concentrations higher than 0.1 mM are responsible for extreme depletion of reduced intracellular glutathione levels and apoptosis [33]. Cytotoxicity and genotoxicity caused by Bis-GMA is higher than that caused by UDMA, TEGDMA and HEMA [23]. HEMA also causes cytotoxic effect in human gingival fibroblasts, but this effect is a consequence of HEMA's degradation products rather than HEMA itself [35]. Similar to the present study, Szczepanska et al. [35] have found moderate, but statistically significant reduction of cellular viability in human gingival fibroblast culture and an increased number of apoptotic cells in close correlation to HEMA concentration.

Nano-hybrid composite resin, Kalore, reduced cellular viability and caused higher appearance of apoptotic and necrotic pulpal cells, but had no effect on gingival cells. Higher sensitivity of pulpal cells in comparison to gingival is in accordance to the data presented by Geurtsen et al. [17]. Kalore is based on high-molecular weight DX-511 monomer and UDMA [36], so its cytotoxicity can be related to UDMA. Chang et al. [3] have shown that UDMA causes dose-dependent reduction of cell viability on Chinese hamster ovarian cells. In human gingival fibroblasts UDMA induces mostly necrosis, whereas the number of cells in apoptosis was low [37]. Poplawski et al. [21] have suggested two possible pathways for the induction of apoptosis by UDMA: (a) UDMA can act as a genotoxic agent by disrupting the DNA physiology, which later culminates to apoptosis and (b) degradation of UDMA, which leads to the shift of redox potential on the inner mitochondrial membrane.

The comet assay results in this study have demonstrated DNA damage in all tested materials and concentrations, for both pulpal and gingival cells. Genotoxicity was positively correlated to the concentration and it was more expressed in pulpal than in gingival fibroblasts. The hypothesis that self-adhering flowable composite will have a higher genotoxic effect was rejected, since Kalore showed higher tail length than Vertise Flow ($p < 0.01$). This is an unexpected result, since the Vertise Flow contains both Bis-GMA and HEMA, which are both more genotoxic than UDMA [23]. Blasiak et al. [38] have used a neutral comet assay to show that dentine adhesives with 45% of HEMA and 55% of Bis-GMA in their composition have the ability to cause DNA damage in 0.2 mM Bis-GMA concentrations. The same study evaluated the DNA repair ability and concluded that the combination of HEMA/Bis-GMA can prevent the repair, but the exact mechanism was not explained. However, the concentrations of Bis-GMA and HEMA in Vertise Flow are probably very low and do not contribute to DNA strand breaks as much as Kalore. On the other hand, the genotoxicity of Kalore is presumably related to the above-mentioned genotoxicity of UDMA and the fact that the alkaline version of the comet assay is found to be especially sensitive for detection of alkali-labile sites caused by UDMA [21].

High quantities of methacrylates and their metabolites can be released shortly after polymerization of composite materials and they tend to decrease in time, but lower concentrations can be detected over a long time period [39,40]. Their cytotoxicity or genotoxicity can be generated by individual components or their combined effect, even if present in low or non-toxic concentrations, but in prolonged exposure. If such damage remains unrepaired, they can cause cellular death, but if their repair was inadequate, they can lead to chromosomal aberrations and genetic

instability [22]. This corroborates our previous *in vivo* study which was focused on the long-term effects of the same materials on gingival epithelial cells [5]. Genotoxic effect was the strongest after 1 month and slightly decreased after 6 months, although there was no statistical significance between them.

Both materials used in this study have demonstrated mild cytotoxic and genotoxic potential. Vertise Flow was dominantly cytotoxic, while Kalore exhibited higher DNA damage. As expected, pulp cells were more sensitive to the exposure to dental materials than gingival fibroblasts. Considering the fact that tested composite materials are in prolonged contact to the living tissue, further investigations evaluating possible toxic effects are needed.

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