

Micronucleus, alkaline, and human 8-oxoguanine glycosylase 1 modified comet assays evaluation of glass-ionomer cements - *in vitro*

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The purpose of this study was to evaluate the genotoxic potential of components leached from two conventional self-curing glass-ionomer cements (Fuji IX and Ketac Molar), and light-curing, resin modified glass-ionomer cements (Vitrebond, Fuji II LC). Evaluation was performed on human lymphocytes using alkaline and hOGG1 modified comet, and micronucleus assays. Each material, polymerised and unpolymerised, was eluted in extracellular saline (1 cm² mL⁻¹) for 1 h, 1 day, and 5 days. Cultures were treated with eluates using final dilutions of 10⁻², 10⁻³, and 10⁻⁴. Alkaline comet assay did not detect changes in DNA migration of treated cells regardless of the ionomer tested, polymerisation state, and elution duration. Glass ionomers failed to significantly influence micronucleus frequency. No oxidative DNA damage in treated lymphocytes was observed using hOGG1 modified comet assay. Obtained results indicate high biocompatibility of all tested materials used in the study under experimental conditions.

KEY WORDS: *biocompatibility; cytotoxicity; DNA damage; genotoxicity; lymphocytes*

The most important requirement for a material to be used in medical applications is its biocompatibility. Dental materials can lead to cell damage of the pulp, gingiva, and oral mucosa. Various tests have been applied to assess the risk of such damage (1-4).

Due to their properties, glass ionomer cements (GICs) are widely used as restorative materials in dentistry. Since their commercial introduction, GICs have been available in essentially two types: 'conventional' glass ionomers (CGICs) and resin-modified glass ionomer cements (RMGICs) (5). CGICs are composed of fine calcium fluoroaluminosilicate glass powder and aqueous solutions of polyacrylic acid and its copolymers. RMGICs are usually formulated from fluoro-

aluminosilicate glasses, photo-initiators, polyacrylic acid, water, and a water-soluble methacrylate monomer, such as hydroxyethyl methacrylate (HEMA) (6, 7).

Despite the improved mechanical properties of RMGICs their biocompatibility is still controversial (4, 5). Most of the studies suggested that CGICs revealed minimal toxicity *in vitro* or *in vivo*, whereas RMGICs showed significant cytotoxicity and genotoxicity (8, 10-12). There are sufficient data demonstrating that the liberated substances may diffuse pulp ward in high quantities (triethyleneglycol-dimethacrylate - TEGDMA, HEMA), particularly through thin dentin layers or after acid-etching of dentin. It appears that high quantities of these

components may result in pulpal inflammation (13). De Souza Costa et al. (10) reported that Vitrebond reduced viability of odontoblasts by 74.6 %, Fuji II LC by 45.5 %, Fuji IX by 29.5 %, and Ketac Molar by 32.5 %. However, Koulaouzidou et al. (14) found that Fuji II LC showed only minimal cytotoxicity and mild antiproliferative effect on three cell lines (L929, BHK21/C13, and RCP-C2A). Do Nascimento et al. (15) reported that Vitrebond could cause marked inflammatory pulpal reactions and a large necrotic zone. Six et al. (16) used Fuji IX on rats' upper molars and found that after 8 days it caused disruption of the odontoblastic layer, blood vessels' dilatation, and inflammatory reaction of the pulp with presence of bacteria in dentinal tubules. However, after 30 days, pulpal tissue was again healthy and looked normal (16).

Thus, the aim of this *in vitro* study was to comparatively evaluate possible genotoxicity of monomers released from two types of commercially available groups of glass ionomers, CGICs and RMGICs, with respect to their polymerisation state and time elapsed from the setting of the materials. To detect possible differences in their mechanism of actions two different cytogenetic techniques were applied on human lymphocyte cultures: comet and micronucleus assay. In addition, human 8-oxoguanine glycosylase 1 (hOGG1) enzyme modified comet assay was performed to evaluate possible oxidative DNA damage.

MATERIALS AND METHODS

Blood sampling

Each material was tested on lymphocytes of three young, healthy, non-smoking voluntary donors. The donors were one male and two females with mean age 29.33 ± 1.69 . According to a questionnaire filled by donors, in 12 months prior to blood sampling, they had not been exposed to any physical or chemical agents that might have interfered with the results of genotoxicity testing. Blood was drawn by antecubital venipuncture into heparinised vacutainers (Becton Dickenson, Plymouth, UK). All donors were acquainted with the study and they willingly signed permissions to use their blood samples for scientific purposes. All described procedures were in accordance with the high ethical standards, complied with the principles laid down in the Declaration of Helsinki.

Preparation of glass ionomer materials

Four different glass ionomers were tested: Fuji IX (GC Corp, Tokyo, Japan) and Ketac Molar (3M/ESPE, St. Paul, MN, USA) as conventional GICs, and Fuji II LC (GC Corp, Tokyo, Japan) and Vitrebond (3M/ESPE, St. Paul, MN, USA) as resin-modified GICs. The composition of selected materials as per their manufacturers is presented in Table 1. Ionomers were polymerised in aseptic conditions as recommended by the manufacturers, in 4 mm in diameter and 2 mm thick rounded plates. CGICs were polymerised chemically in the air, and RMGICs with the Elipar

Table 1 Glass-ionomers used in the present study (according to manufacturers' data sheets)

Glass ionomer group	Ionomer tested	Components
Resin-modified glass ionomer cements	Fuji II LC (GC, Tokyo, Japan)	<i>Powder:</i> fluoroaluminosilicate glass <i>Liquid:</i> polyacrylic acid and copolymer of acrylic and maleic acid, 2-hydroxyethyl-methacrylate (HEMA), initiator, water.
	Vitrebond (3M ESPE, St. Paul, MN, USA)	<i>Powder:</i> SiO ₂ , AlF ₃ , ZnO, Na ₃ AlF ₆ , SP-940S glass powder, disodiumhydrogenphosphate, diphenyliodoniumchloride <i>Liquid:</i> conacid-isocyanatoethyl-methacrylateacrylic acid, HEMA, bornandion, di-tert-butyl-p-cresol
Conventional glass ionomer cements	Fuji IX (GC, Tokyo, Japan)	<i>Powder:</i> fluoroaluminosilicate glass, polyacrylic acid powder <i>Liquid:</i> polyacrylic acid, polybasic carboxylic acid
	Ketac Molar (3M ESPE, St. Paul, MN, USA)	<i>Powder:</i> fluoroaluminosilicate glass, ZnO, polycarboxylic acid, pigments <i>Liquid:</i> water, copolymer acrylic calcium, maleic acid, tartaric acid, benzoic acid

TriLight halogen curing unit (650 mW cm⁻²) from a distance of 2 mm for 40 s. Polymerised ionomers were eluted for 1 h, 1 day, and 5 days in extracellular saline (ECS), consisting of 40 mmol L⁻¹ NaCl, 3 mmol L⁻¹ KCl, 1 mmol L⁻¹ CaCl₂, 1 mmol L⁻¹ MgCl₂, 10 mmol L⁻¹ HEPES, adjusted to pH 7.3–7.4 with NaOH. Material surface vs. ECS volume ratio was 1 cm² mL⁻¹ (17). The elutions of unpolymerised glass ionomers in ECS were set up in the same manner.

Initiation of cell cultures and micronucleus assay

To initiate the cell cultures, 0.5 mL of blood was introduced into a cell culture flask (Nunc GmbH, Wiesbaden, Germany) containing 5 mL of RPMI medium (Gibco, Carlsbad, USA) supplemented with 15 % foetal bovine serum (Sigma, St. Louis, USA), 10 µg mL⁻¹ of phytohemagglutinin (Murex, Dartford, UK), 2.5 g L⁻¹ of phenol red pH indicator (Sigma, St. Louis, USA), 100 IU of penicillin (Sigma, St. Louis, USA), and 100 IU of streptomycin (Sigma, St. Louis, USA). Duplicate cell cultures per donor were treated with the following four dilutions of each glass ionomer eluate: 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴. Dilutions were prepared in RPMI medium, and pH values were adjusted at 7.2. The same treatment scheme was used for each of the three elution times (1 h, 1 day, and 5 days). GIC eluates were initiated 5 days, 1 day, and 1 h prior to blood sampling. The whole blood cultures were treated for 72 h. Simultaneously, negative control cultures were treated with the same volume of physiological solution of ECS, whereas the positive controls were treated with bleomycin (Sigma, St. Louis, USA) at the final concentration of 30 µg mL⁻¹ for the 5 last hours of cultivation. Cytochalasin B (Sigma, St. Louis, Germany) was added at a final concentration of 6 µg mL⁻¹ to the cultures after 44 h of incubation. Cultures were incubated at 37 °C for 72 h. After incubation, the same culture was simultaneously subjected to analysis by all techniques.

For micronucleus assay, cells were centrifuged, pretreated with physiological solution, and fixed with 3:1 (v/v) methanol/acetic acid solution. They were then air-dried and stained with 5 % Giemsa (Sigma, St. Louis, USA) (18). For each elution time and dilution tested, 1000 binucleated lymphocytes per duplicate slide per donor were analysed scoring the number of micronuclei. We applied the scoring criteria described by Fenech et al. (19).

Cytotoxicity testing

Using the trypan blue exclusion technique, the relative cell viability was tested 72 h after the cultures were initiated. For all tested ionomers, cytotoxicity of dilution 10⁻¹ of 1-day eluates appeared to be higher than 17 %. Therefore, only the cultures treated with dilutions of 10⁻², 10⁻³, and 10⁻⁴ were analysed using micronucleus, alkaline, and hOGG1 modified comet assays for all elution periods. After the treatment of cell cultures with ionomer eluates pH indicator contained within the medium did not show any changes in pH value. After the end of cultivation period, pH of each cell culture was additionally checked by a SevenEasy pH meter (Metler-Toledo GmbH, Schwerzenbach, Switzerland) and it was continuously 7.27±0.04, which is, according to the manufacturer, the regular pH value for RPMI cell culture medium.

Alkaline and hOGG1 modified comet assay

Following the treatment, cultures were centrifuged, supernatant removed, and both alkaline and hOGG1 comet assays were performed on whole blood samples in accordance with the protocols by Singh et al. (20) and Smith et al. (21), respectively. All the chemicals were obtained from Sigma Chemical Company (Sigma-Aldrich, Munich, Germany).

Normal melting point agarose (NMP) 0.6 % was layered on slides precoated with 1 % NMP. Blood samples (8 µL) were mixed with 0.5 % low melting point agarose, placed on the slides, and immersed in standard ice-cold lysis solution (pH 10) and stored overnight at 4 °C. For the alkaline comet assay the standard procedure was followed (20). For the hOGG1 modified comet assay, following the lysis slides were rinsed in three changes of neutralisation buffer (40 mmol L⁻¹ HEPES, 0.1 mol L⁻¹ KCl, 0.5 mmol L⁻¹ EDTA, pH 7.4) for 5 min at room temperature. One parallel slide was treated with hOGG1 enzyme in neutralisation buffer (V=60 µL; final dilution 1:1000). The other parallel slide was treated with 60 µL of neutralisation buffer only. Treatment was performed in a humidified chamber for 10 min at 37 °C and followed by 15 min of denaturation (1.5 mol L⁻¹ NaCl, 1 mmol L⁻¹ Na₂EDTA, pH 12.1) and 20 min of electrophoresis at 0.7 V cm⁻¹, 300 mA. The slides were neutralised in three changes of buffer (0.4 mol L⁻¹ Tris-HCl, pH 7.5), 5 min each, stained with ethidium bromide (20 g mL⁻¹) and examined using a 250x magnification fluorescence microscope (Olympus,

Japan). A total of 100 comets per concentration tested were scored on each duplicate slide. The edges of the gel, occasional dead cells, and superimposed comets were avoided. Tail length and tail intensity were measured using Comet Assay IV analysis system (Perceptive Instruments Ltd., Suffolk, UK). Oxidative DNA damage was calculated as per the following description. For each donor, mean tail intensity was calculated separately for the slides treated with hOGG1 enzyme and for the slides treated with buffer. Further difference between those two values was obtained for each donor, indicating oxidative DNA damage. Results of hOGG1 comet assay were presented as mean tail lengths only, since in this type of assay modification percentage of DNA in tail is the most informative parameter (21).

Statistical analysis

Differences in the number of micronuclei treated and control lymphocytes were evaluated using chi-square and Fisher's LSD test. Regarding the comet

assay endpoints, to normalise the distribution and to equalise the variances, a logarithmic transformation of data was applied. Differences between the groups were tested using ANOVA followed by Duncan's test. To evaluate oxidative DNA damage, differences between the means for the slides treated with enzyme and the corresponding slides treated with buffer only were tested using ANOVA followed by Duncan's post-hoc analysis. The level of significance was set at 0.05.

RESULTS

Micronucleus assay did not show statistically increased micronucleus frequency for any of the eluates tested. A slightly increased number of micronuclei was detected in the cultures treated with 10^{-2} dilution of 1-h eluates of unpolymerised Vitrebond and Ketac Molar (Figure 1).

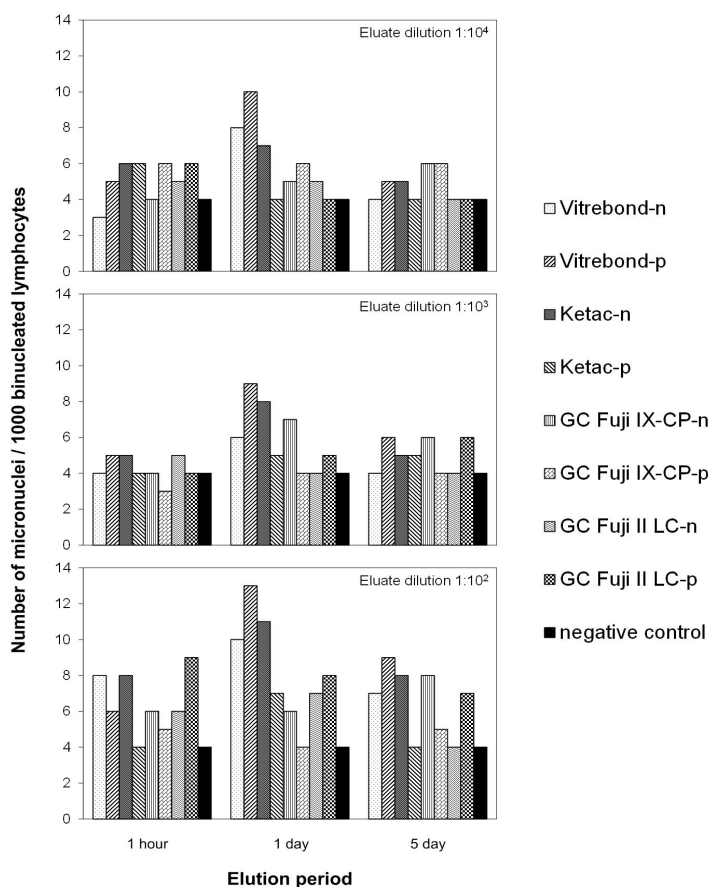


Figure 1 Number of micronuclei in lymphocyte cultures treated with different dilutions (10^{-2} , 10^{-3} , and 10^{-4}) of initial glass ionomer eluates ($1 \text{ cm}^2 \text{ mL}^{-1}$ of extracellular saline) after 1 h, 24 h, and 5 days of elution. One thousand binucleated lymphocytes were analysed per treatment on each of three blood samples
p - polymerised, n - unpolymerised

Eluates of polymerised forms of these ionomers did not influence micronucleus frequency. Further, 10^{-2} and 10^{-3} dilutions of 1-day eluates of unpolymerised Ketac Molar, and the polymerised form of GC Fuji II LC slightly, but insignificantly elevated the number of micronuclei (Figure 1).

Similarly, in the culture treated with dose eluates, a slightly increased micronucleus frequency compared to 1-h elution was found. Dilution 10^{-2} of 5-day eluates of polymerised Vitrebond and GC Fuji IX GP, and unpolymerised Ketac Molar induced further insignificant elevation of MN (Figure 1).

Neither did the comet assay show statistically increased tail length or Olive tail moment for any of the eluates of the tested glass ionomers, polymerised or unpolymerised. Again, slightly elevated DNA migration was detected in lymphocytes treated with 10^{-2} dilution of 1-h of both unpolymerised and polymerised Vitrebond, unpolymerised Ketac Molar, and polymerised Fuji II LC (Figures 2, 3). Later

eluates did not show any differences regardless of their dilution or polymerisation state.

Application of hOGG1 modified comet assay did not indicate an increase in the 8-oxoguanine formation due to possible oxidative stress induced by resin monomers released from the tested glass ionomers (Table 2). A slight increase in tail intensity was observed for all tested materials 24 h after the initial setting, and only for the lowest eluate dilution (10^{-2}). However, the increase was simultaneously observed for the slides treated with hOGG1 enzyme and the corresponding buffer treated slides. We did not observe any statistical difference in the percentage of DNA in tails due to hOGG1 treatment.

DISCUSSION

The study presents the results of genotoxicity evaluation of eluates of four glass ionomers: Fuji II LC and Vitrebond as resin-modified glass ionomer

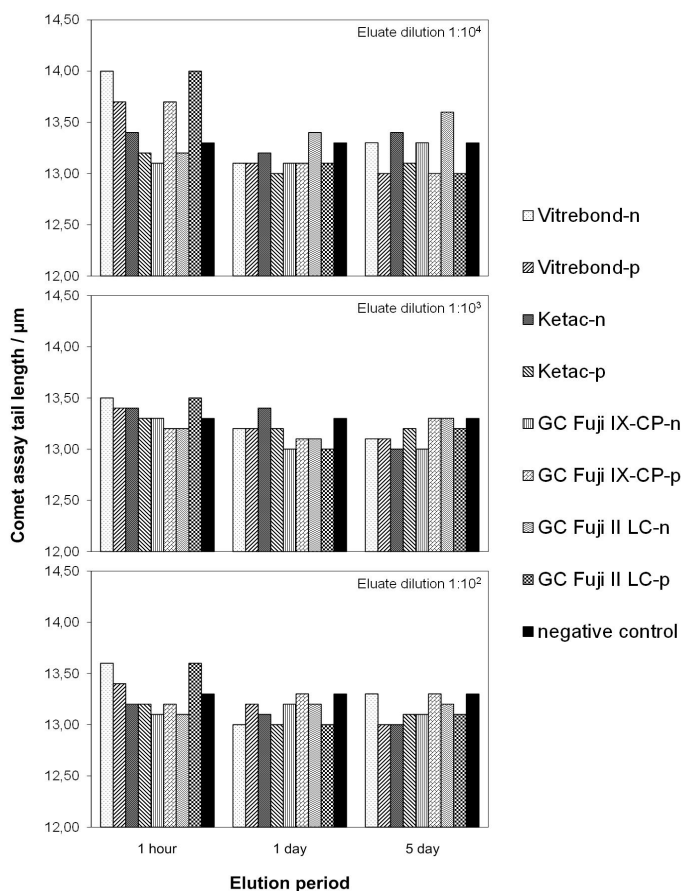


Figure 2 Tail length values for lymphocyte cultures treated with different dilutions (10^{-2} , 10^{-3} , and 10^{-4}) of initial glass ionomer eluates ($1 \text{ cm}^3 \text{ mL}^{-1}$ of extracellular saline) after 1 h, 24 h, and 5 days of elution. One hundred comets were analysed per treatment on each of three blood samples.
 p - polymerised, n - unpolymerised

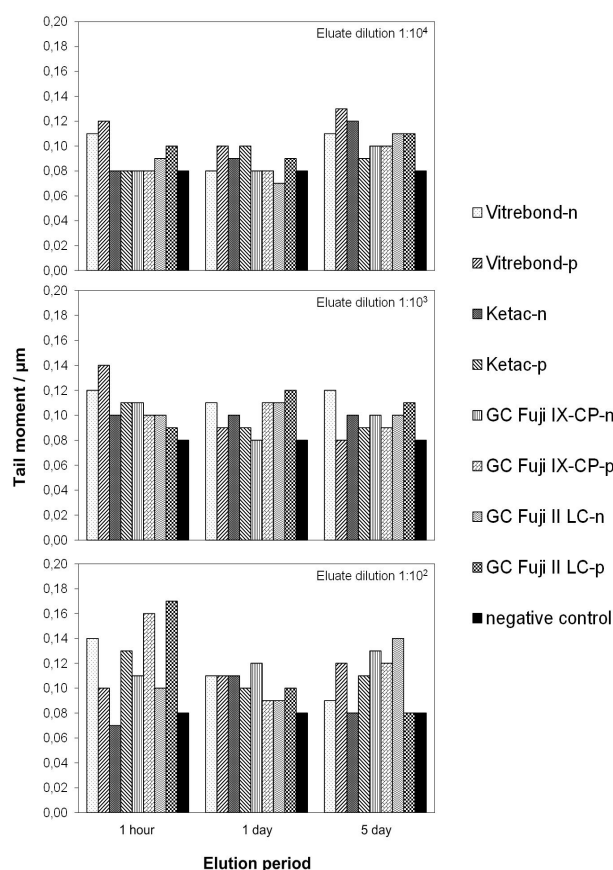


Figure 3 Mean tail moment values for lymphocyte cultures treated with different dilutions (10^{-2} , 10^{-3} , and 10^{-4}) of initial glass ionomer eluates ($1 \text{ cm}^2 \text{ mL}^{-1}$ of extracellular saline) after 1 h, 24 h, and 5 days of elution. One hundred comets were analysed per treatment on each of three blood samples.

p - polymerised, *n* - unpolymerised

cements, and Fuji IX and Ketac Molar as conventional glass ionomer cements. We aimed to detect possible differences in the mechanisms of their genotoxicity that may arise due to differences in their composition and initiation of polymerisation (chemical or photoactivation). To get more information on their biocompatibility, glass ionomer cements were tested both in their unpolymerised and polymerised form. In this assessment two cytogenetic techniques were used: alkaline comet and micronucleus assay. Comet assay detects primary DNA damage that could result from direct interaction of monomers, initiators or any other leached components with genomic DNA. That type of damage does not require cell division to be expressed and evaluated (22). Modification of the comet assay procedure by exposing the isolated DNA to hOGG1 enzyme allows to specifically detect 8-oxoguanine base that arises as a result of oxidative DNA damage. Micronucleus assay detects DNA lesions that are not repaired properly and after replication result in the formation of acentric chromosome fragments. It also detects damage of the cell spindle disabling chromosomes to be attached

properly and chromatids to be divided between the newly forming cells. Thus, contrary to the comet assay, micronucleus assay enables detection of both types of genotoxic agents, clastogens and aneugens (18).

It has been proven that due to incomplete polymerisation, residual monomers (HEMA, Bis-GMA) are leached from dental materials (23). In our study we did not detect any genome damage in the above mentioned period of time and eluate dilutions using comet assay. However, micronucleus assay indicated a slight but insignificant increase in the micronucleus frequency in lymphocytes treated with the lowest dilution (10^{-2}) of 1-day eluate of polymerised GC Fuji II LC, and 5-day eluates of polymerised Vitrebond and GC Fuji IX GP. Unpolymerised Vitrebond and GC Fuji IX GP. Unpolymerised Vitrebond and Ketac Molar insignificantly elevated micronuclei incidence as early as after 1-h elution indicating faster leaching of active components from unpolymerised materials. Unpolymerised Ketac Molar slightly influenced micronuclei induction throughout the entire period of 5-day elution indicating persistence of its leached active components, presumably ZnO that was suspected to be clastogenic and that was not

Table 2 Results of hOGG1 modified comet assay presenting mean tail intensity values (% DNA in comet's tail). The results obtained for the slides treated with buffer and the corresponding slides treated with enzyme are shown for each treatment. One hundred comets were analysed per treatment on each of three blood samples

Glass ionomer	Form	Eluate dilution	Oxidative DNA damage after elution of glass ionomers (mean±SD)		
			1 h	1 day	5 days
Vitrebond	Unpolymerised	10 ⁻²	0.70±0.32	1.98±0.94	0.12±0.06
		10 ⁻³	0.16±0.08	1.86±0.98	0.08±0.02
		10 ⁻⁴	0.14±0.06	1.02±0.86	0.06±0.00
	Polymerised	10 ⁻²	0.18±0.08	1.18±0.80	0.05±0.03
		10 ⁻³	0.12±0.04	1.33±0.82	0.06±0.01
		10 ⁻⁴	0.24±0.07	0.92±0.47	0.04±0.01
Ketac Molar	Unpolymerised	10 ⁻²	0.16±0.03	1.53±0.68	0.34±0.11
		10 ⁻³	0.02±0.00	0.95±0.23	0.13±0.05
		10 ⁻⁴	0.03±0.01	1.31±0.54	0.11±0.01
	Polymerised	10 ⁻²	0.27±0.09	1.40±0.86	0.16±0.03
		10 ⁻³	0.04±0.00	0.90±0.32	0.22±0.08
		10 ⁻⁴	0.14±0.05	0.13±0.06	0.18±0.06
GC Fuji IX GP	Unpolymerised	10 ⁻²	0.01±0.00	1.52±0.27	0.18±0.03
		10 ⁻³	0.14±0.02	1.07±0.09	0.24±0.09
		10 ⁻⁴	0.07±0.05	0.86±0.14	0.06±0.02
	Polymerised	10 ⁻²	0.14±0.04	0.97±0.07	0.11±0.03
		10 ⁻³	0.15±0.04	1.13±0.74	0.14±0.02
		10 ⁻⁴	0.01±0.01	0.98±0.55	0.12±0.02
GC Fuji II LC	Unpolymerised	10 ⁻²	0.13±0.05	0.93±0.28	0.01±0.02
		10 ⁻³	0.09±0.03	1.20±0.23	0.14±0.03
		10 ⁻⁴	0.08±0.03	0.87±0.17	0.12±0.03
	Polymerised	10 ⁻²	0.11±0.03	1.04±0.19	0.14±0.03
		10 ⁻³	0.02±0.01	1.17±0.41	0.10±0.02
		10 ⁻⁴	0.04±0.03	0.92±0.47	0.11±0.02
Negative control (22 µL extracellular saline)			0.02±0.02	0.81±0.18	0.03±0.01
Positive control (0.1 mmol L ⁻¹ H ₂ O ₂ 10 min)			16.4±1.82 ¹	28.07±1.20 ¹	15.7±1.05 ¹

¹statistically significant oxidative DNA damage, $p < 0.05$ between the corresponding slides treated with hOGG1 enzyme and buffer only. Oxidative DNA damage was calculated as difference in tail intensity values between hOGG1 and buffer treated slides.

contained in other glass-ionomers (24). However, the observed effects could not at all be considered biologically relevant. Considering the effect of Vitrebond and Fuji II LC on micronucleus frequency, it would be mainly mediated by HEMA that is known to be genotoxic (25). Contrary to Kleinsasser et al. (25) that reported increased DNA migration ability of human lymphocytes exposed to HEMA, we did not detect any effect of ionomers containing this monomer on comet assay endpoints. Thus, the observed fluctuations in the number of micronuclei, although biologically insignificant, might have been mediated by slight aneugenic effect of monomers. Vitrebond's genotoxicity could be additionally mediated by diphenyliodinechloride (DPICI) that is used as the

initiator. Leyhausen et al. (26) reported that DPICI that was leached from polymerised Vitrebond exhibited genotoxic activity. These results may suggest that even from the unpolymerised form of ionomers, after mixing powder and liquid components HEMA could not be leached in the amounts needed to induce significant DNA damage under the experimental conditions used in our evaluation. The choice of solvent vehicle used in elution might also be critical. Kleinsasser et al. (25) used organic solvent DMSO, and we used extracellular saline as the main polar solvent saline solution. Ribeiro et al. (27) published the results on genotoxic evaluations of Vitrebond and Ketac Molar on Chinese hamster ovary (CHO) and mouse lymphoma (L5178Y) cell lines

using comet assay. Unlike in our study, they tested the components separately without mixing them. The results showed that powders from assay did not show genotoxic effects. The authors observed increased DNA migration only for the highest concentration of Ketac Molar powder phase (100 $\mu\text{g mL}^{-1}$ and 1000 $\mu\text{g mL}^{-1}$), and the highest concentration of Vitrebond liquid phase (1 %). No measurable genotoxicity was found in the liquid from Ketac Molar in all dilutions tested (0.01–1 %). In the study of Bakopoulou et al. (28), 72 h-eluates of Ketac Cem significantly increased the frequency of sister chromatid exchanges and chromosomal aberrations in human lymphocyte cultures.

Stea et al. (29) evaluated genotoxicity of Vitrebond eluates in human lymphocytes cultures using sister chromatid exchange (SCE) assay. The authors reported increased SCE frequency for 10^{-2} eluate dilution of unpolymerised Vitrebond and 10^{-1} of the polymerised one. Bakopoulou et al. (28) showed Vitrebond's eluate to be very cytotoxic, as the first series of dilutions of both materials caused a complete mitotic arrest in lymphocyte cultures but the authors used ten-fold lower dilutions of eluate in culture treatment than we did in the present study. Also, the eluate derived from Vitrebond specimens (5 mm in diameter and 2 mm thick) caused extensive genotoxicity after 24 h, increasing the frequencies of sister chromatid exchange and the number of chromosomal aberrations (28). Of all the observed insignificant effects on micronucleus frequency, the one caused by Fuji IX was the lowest.

The main deficiency of *ex vivo* tests is their questionable clinical relevance. Although the effects detected in *in vitro* studies could not be directly extrapolated into *in vivo* conditions, they are informative and could indicate a potential degree of health risk that may arise from using certain chemical compounds in dental restoration. *In vitro* studies provide limited responses due to the lack of biological and physiological components that cannot be replicated in the laboratory. The largest limitations of this study were low concentrations used to reproduce the situation in the mouth and the type of cell on which we carried out the research. It would have been a better solution to have used oral cells that are the first line of defence in the oral cavity in direct contact with the tested material. We could suggest that under the conditions presented in this study, in non-toxic concentrations range, the evaluated glass ionomer cements (Vitrebond, Ketac Molar, Fuji IX GP, and

Fuji II LC) did not exhibit any biologically relevant genotoxic activity on peripheral blood lymphocytes *in vitro* measured by comet and micronucleus assays, nor were they able to induce oxidative DNA damage. With respect to such results, a slight and insignificant increase in the micronucleus number might raise suspicion of their restricted aneugenicity. Altogether, under the experimental conditions used in the study, the tested glass ionomers showed a satisfying level of biocompatibility.

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Conflict of interest

The authors declare no conflict of interest.

REFERENCES

- Schmalz G, Arenholt-Bindslev D. Biocompatibility of dental materials: Springer Verlag Berlin Heidelberg; 2009.
- Bogović A, Nižetić J, Galić N, Željezić D, Micek V, Mladinić M. The effects of hyaluronic acid, calcium hydroxide, and dentin adhesive on rat odontoblasts and fibroblasts. Arh Hig Rada Toksikol 2011;62:155-61. PMID:21705303.
- Tadin A, Galic N, Zeljezic D, Mikelic Vitasovic B, Marovic D, Kovacic I. *Ex vivo* evaluation of genotoxic effects of four dental adhesives on human leukocytes. J Dent Sci 2013;8:37-43. doi:10.1016/j.jds.2012.12.001.
- Tadin A, Galic N, Mladinic M, Marovic D, Kovacic I, Zeljezic D. Genotoxicity in gingival cells of patients undergoing tooth restoration with two different dental composite materials. Clin Oral Investig 2014;18:87-96. PMID: 23385426.
- Tyas MJ. Clinical evaluation of glass-ionomer cement restorations. J Appl Oral Sci 2006;14(Suppl):10-3. PMID:19089080.
- Berzins DW, Abey S, Costache MC, Wilkie CA, Roberts HW. Resin-modified glass-ionomer setting reaction competition. J Dent Res 2010;89:82-6. PMID:19966038.
- Coutinho E, Yoshida Y, Inoue S, Fukuda R, Snauwaert J, Nakayama Y, De Munck J, Lambrechts P, Suzuki K, Van Meerbeek B. Gel phase formation at resin-modified glass-ionomer/tooth interfaces. J Dent Res 2007;86(7):656-61. PMID:17586714.
- Aranha AM, Giro EM, Souza PP, Hebling J, de Souza Costa CA. Effect of curing regime on the cytotoxicity of resin-modified glass-ionomer lining cements applied to an odontoblast-cell line. Dent Mater 2006;22:864-9. PMID:16388848.

9. Xie D, Brantley WA, Culbertson BM, Wang G. Mechanical properties and microstructures of glass-ionomer cements. *Dent Mater* 2000;16:129-38. PMID:11203534
10. de Souza Costa CA, Hebling J, Garcia-Godoy F, Hanks CT. 2003. *In vitro* cytotoxicity of five glass-ionomer cements. *Biomaterials* 2003;24:3853-8. PMID:12818558.
11. Souza PP, Aranha AM, Hebling J, Giro EM, Costa CA. *In vitro* cytotoxicity and *in vivo* biocompatibility of contemporary resin-modified glass-ionomer cements. *Dent Mater* 2006;22:838-44. PMID:16387357.
12. Stanislawski L, Daniau X, Lauti A, Goldberg M. Factors responsible for pulp cell cytotoxicity induced by resin-modified glass ionomer cements. *J Biomed Mater Res* 1999;48:277-88. PMID:10398031.
13. Geurtsen W. Biocompatibility of resin-modified filling materials. *Crit Rev Oral Biol Med* 2000;11:333-55. PMID:11021634.
14. Koulaouzidou EA, Papazisis KT, Economides NA, Beltes P, Kortsaris AH. Antiproliferative effect of mineral trioxide aggregate, zinc oxide-eugenol cement, and glass-ionomer cement against three fibroblastic cell lines. *J Endod* 2005;31:44-6. PMID:15614005.
15. do Nascimento AB, Fontana UF, Teixeira HM, Costa CA. Biocompatibility of a resin-modified glass-ionomer cement applied as pulp capping in human teeth. *Am J Dent* 2000;13:28-34. PMID:11763899.
16. Six N, Lasfargues JJ, Goldberg M. *In vivo* study of the pulp reaction to Fuji IX, a glass ionomer cement. *J Dent* 2000;28:413-22. PMID:10856806.
17. ISO standard 10993/12. Biological evaluation of medical devices-Part 12: sample preparation and reference material. ed. G ISO: Geneva;1996. [Displayed 23 February 2013]. Available at <https://law.resource.org/pub/ie/ibr/is.en.iso.10993.12.2012.html>.
18. Fenech M. The cytokinesis-block micronucleus technique and its application to genotoxicity studies in human populations. *Environ Health Perspect* 1993;101(Suppl) 3:101-7. PMID: 8143599.
19. Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, Zeiger E; HUMAN Micronucleus project. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat Res.* 2003 Jan 10;534(1-2):65-75. PMID: 12504755.
20. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 1988;175:184-91. PMID:3345800.
21. Smith CC, O'Donovan MR, Martin EA. hOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII. *Mutagenesis* 2006;21:185-90. PMID:16597659.
22. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. *Molr biotechnol* 2004;26:249-61. PMID:15004294
23. Kawahara T, Nomura Y, Tanaka N, Teshima W, Okazaki M, Shintani H. Leachability of plasticizer and residual monomer from commercial temporary restorative resins. *J Dent* 2004;32:277-83. PMID:15053910.
24. Dufour EK, Kumaravel T, Nohynek GJ, Kirkland D, Toutain H. Clastogenicity, photo-clastogenicity or pseudo-photo-clastogenicity: Genotoxic effects of zinc oxide in the dark, in pre-irradiated or simultaneously irradiated Chinese hamster ovary cells. *Mutat Res* 2006;607:215-24. PMID: 16797222.
25. Kleinsasser NH, Wallner BC, Harréus UA, Kleinjung T, Folwaczny M, Hickel R, Kehe K, Reichl FX. Genotoxicity and cytotoxicity of dental materials in human lymphocytes as assessed by the single cell microgel electrophoresis (comet) assay. *J Dent* 2004;32:229-34. PMID:15001288.
26. Leyhausen G, Abtahi M, Karbakhsh M, Sapotnick A, Geurtsen W. Biocompatibility of various light-curing and one conventional glass-ionomer cement. *Biomaterials* 1998;19:559-64. PMID: 9645563.
27. Ribeiro DA, Marques ME, Salvadori DM. Genotoxicity and cytotoxicity of glass ionomer cements on Chinese hamster ovary (CHO) cells. *J Mater Sci Mater Med* 2006;17:495-500. PMID: 16691346.
28. Bakopoulou A, Mourelatos D, Tsiftoglou AS, Giassin NP, Mioglou E, Garefis P. Genotoxic and cytotoxic effects of different types of dental cement on normal cultured human lymphocytes. *Mutat Res* 2009;672:103-12. PMID:19028600.
29. Stea S, Visentin M, Cervellati M, Verri E, Cenni E, Savarino L, Stea S, Pizzoferrato A. 1998. *In vitro* sister chromatid exchange induced by glass ionomer cements. *J Biomed Mater Res* 1998;40:545-50. PMID:9599030.

Sažetak**Procjena *in vitro* toksičnosti stakleno-ionomernih cementa primjenom mikronukleus testa, alkalnog komet testa i komet testa modificiranog hOGG1 enzimom**

Svrha istraživanja bila je procijeniti genotoksični potencijal komponenata koje izlučuju dva konvencionalna samopolimerizirajuća stakleno-ionomerna cementa (Fuji IX i Ketac Molar) te svjetlosno polimerizirajući i smolom modificirani stakleno-ionomerni cementi (Vitrebond, Fuji II LC). Istraživanje je provedeno na ljudskim limfocitima primjenom alkalnog komet testa, komet testa modificiranog hOGG1 enzimom te mikronukleus testa. Svaki materijal, polimerizirani i nepolimerizirani, eluiran je u fiziološkoj otopini ($1 \text{ cm}^2 \text{ mL}^{-1}$) tijekom jednog sata, jednog dana i tijekom 5 dana. Kulture limfocita tretirane su eluatima u razrjeđenjima 10^{-2} , 10^{-3} i 10^{-4} . Alkalnim komet testom nisu zabilježene promjene u migraciji DNA iz tretiranih stanica bez obzira na ispitani ionomer, vrstu polimerizacije i trajanje elucije. Izloženost staklenim ionomerima nije značajno utjecala na učestalost mikronukleusa. Primjenom hOGG1 modificiranog komet testa nije zamijećeno oksidativno oštećenje DNA u tretiranim limfocitima. Dobiveni rezultati upućuju na visoki stupanj biokompatibilnosti svih testiranih materijala koji su se koristili u eksperimentalnim uvjetima.

KLJUČNE RIJEČI: *biokompatibilnost; citotoksičnost; genotoksičnost; limfociti; oštećenje DNA*

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