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Genotoxicity and cytotoxicity of glass ionomer cements on Chinese hamster ovary (CHO) cells

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Abstract Glass ionomer cements are widely used in dentistry as restorative materials and adhesives for composite restorations. However, the results of genotoxicity studies using these materials are inconclusive in literature. The goal of this study was to examine the genotoxic and cytotoxic potential of three different glass ionomer cements available commercially (Ketac Cem, Ketac Molar and Vitrebond) by the single cell gel (comet) assay and trypan blue exclusion test, respectively. For this, such materials were exposed to Chinese hamster ovary (CHO) cells in vitro for 1 h at 37°C. Data were assessed by Kruskall-Wallis nonparametric test. The results showed that the powder from Ketac Molar displayed genotoxicity only in the maximum concentration evaluated (100 μg/mL). In the same way, the liquid from Vitrebond at 0.1% dilution caused an increase of DNA injury. Significant differences ($P < 0.05$) in cytotoxicity provoked by all powders tested of glass ionomer cements were observed for exposure at 1000 μg/mL concentration. With respect to liquids of glass ionomer cements evaluated, the major toxic effect on cell viability was produced at 10%, beginning at the dilution of 0.5% for Vitrebond. Taken together, we conclude that some components of glass ionomer cements show both genotoxic and cytotoxic effects.

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1. Introduction

Since glass ionomer cements were first introduced in the early 70s by Wilson and Kent [1], they are extensively used in dentistry as restorative materials and adhesives for composite restorations. The usage also includes prosthetic and orthodontic devices. However, the data about the genotoxic potential of glass ionomer cements are still conflicting. A number of studies have demonstrated that glass ionomer cements are able to induce DNA breakage in various test systems like the bacterial UMU-test, the eukaryotic DNA synthesis inhibition test and the *in vivo* alkaline filter elution technique as well as the sister chromatid exchange test with human lymphocytes [2–4]. Nevertheless, negative results were detected *in vitro* [5, 6]. As a result and because these dental materials have the potential to be in contact with oral tissues over extended periods of time, further investigation is needed for complete risk of these compounds minimizing, therefore, potential risks to patients and clinicians.

Information regarding unfavorable reactions among biomaterials and living systems proceeds from retrospective studies in man and current knowledge about biomaterial tissue interactions has been gained through bioassays in vivo and *in vitro*. Taking into account biocompatibility tests available in general field, genotoxicity assays are of special concern since genotoxicity has gained widespread acceptance as an important and useful indicator of carcinogenicity [7]. To date, a variety of assays can assess genotoxicity, including those that assess metaphase chromosomal aberrations, micronuclei, sister chromatid exchanges and host cell reactivation. However, these methods are typically laborious and time-consuming or require highly trained technicians to accurately read and interpret slides. The single cell gel (comet) assay in alkaline version was developed as a rapid,

simple, and reliable biochemical technique for evaluating DNA damage in mammalian cells [8]. The basic principle of the single cell gel (comet) assay is the migration of DNA fragments in an agarose matrix under electrophoresis. When viewed under a microscope, cells have the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments or strands migrating towards the anode [9]. Our own recent studies have demonstrated that the single cell gel (comet) is a suitable tool to investigate genotoxicity of compounds used in dental practice [10–12].

Therefore, the purpose of this study was to investigate whether the components of glass ionomer cements can induce DNA breakage in Chinese hamster ovary (CHO) cells by the single cell gel (comet) assay. To monitor cytotoxic effects, the trypan blue exclusion test was applied. These results will contribute to a better understanding of the mechanism of dental materials upon cellular system.

2. Materials and methods

2.1. Cell culture

CHO K-1 cells were growth to confluence in 75 cm^2 culture flasks (Corning, NY, USA) using Ham's F-10 medium (Invitrogen Corporation, Grand Island, USA) supplemented with 10% fetal calf serum and 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen Corporation, Grand Island, USA) incubated in a 95% air 5% CO_2 atmosphere at 37°C. Cells were cultured for 5 days prior to treatment with test substances. Confluent cells were detached with 0.15% trypsin (Invitrogen Corporation, Grand Island, USA) for 5 min after that, 2 mL complete medium were added and cells were centrifuged at 1000 rpm (180 × *g*) during 5 min. Cell suspension was counted using a Neubauer® chamber and seeded in 96-well microtitre plated (Corning, NY, USA) at a density of 1×10^4 cells per well (at a concentration of 1×10^6/mL).

2.2. Treatment

The test materials, manufacturers and ingredients are listed in Table 1. Powder from all glass ionomer cements tested were prepared in increasing final concentrations ranging from 1 to 1000 μg/mL. Liquids from all glass ionomer cements tested were prepared with dilutions from 0.01 to 10%. The negative control group was treated with vehicle control (Phosphate buffer solution-PBS) and the positive control group was treated with methylmetasulfonate (MMS at 1 μg/mL, Sigma Aldrich, USA). After incubating for 1 h at 37°C, the cells were centrifuged at 1000 rpm (180 × *g*) during 5 min and washed twice with fresh medium and re-suspended with fresh medium. Each individual treatment was repeated three times consecutively to ensure reproducibility.

2.3. Cytotoxicity assay

Cell viability test for CHO cells was performed using Trypan blue staining after the treatment [13]. In brief, a freshly prepared solution of 10 μl trypan blue (0.05%) in distilled water was mixed to 10 μl of each cellular suspension during 5 min, spread onto a microscope slide and covered with a coverslip. Non-viable cells appear blue-stained. At least 200 cells were counted per treatment.

2.4. Genotoxicity assay

The protocol used for single cell gel (comet) assay followed the guidelines purposed by Tice et al. [8]. Briefly, a volume of 10 μl of cells ($\sim 1 \times 10^4$ cells) were added to 120 μl of 0.5% low-melting point agarose at 37°C, layered onto a pre-coated slide with 1.5% regular agarose, and covered with a coverslip. After brief agarose solidification in refrigerator, the coverslip was removed and slides immersed to lysis solution (2.5 M NaCI, 100 mM EDTA, 10 mM Tris-HCI buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO) for about 1h. Prior to electrophoresis, the slides were left in alkaline buffer (pH > 13) for 20 min and electrohoresed for another 20 min, at 25 V (0.86 V/cm) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCI (*p*H 7.5), fixed in absolute ethanol and stored at room temperature until analysis. In order to minimize extraneous DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination.

2.5. Comet capture and analysis

A total of 50 randomly captured comets per treatment (25 cells from each slide) [14] were examined blindly by one expertise observer at 400× magnification using a fluorescence microscope (Olympus) connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments, Sufolk, Haverhill, UK) calibrated previously with according to manufacturer's instructions. A computerized image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components and then evaluates the range of derived parameters. Undamaged cells have an intact nucleus without a tail and damaged cells have the appearance of a comet. To quantify the DNA damage, tail moment was evaluated. Tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail. The comet tail moment is positively correlated with the level of DNA breakage in a cell. The mean value of the tail moment in a

Table 1 Glass ionomer cements tested

Compound tested	Manufacturer	Lot no.	Country	Composition
Ketac Cem	3 M	161341	Germany	Powder: fluoroaluminosilicate glass; polycarboxylic acid; pigments Liquid: water; tartaric acid; benzoic acid
Ketac Molar	3 M	156133	Germany	Powder: fluoroaluminosilicate glass; ZnO; polycarboxylic acid; pigments Liquid: water; co-polymer acrylic calcium; maleic acid; tartaric acid; benzoic acid
Vitrebond	3 M	7510	Germany	Powder: fluoroaluminosilicate glass Liquid: HEMA (2-hydroxyethylmethacrylate), water and photo initiator

particular sample was taken as an index of DNA damage in this sample.

2.6. Statistical methods

Parameters from the comet assay and the cellular viability were assessed by the Kruskal-Wallis non-parametric test, using SigmaStat software, version 1.0 (Jadel Scientific, USA). The level of statistical significance was set at 5%.

3. Results

The main characteristics of glass ionomer cements tested are presented in Table 1. The materials were identified as commercial name, company, country of origin and ingredients.

The viability was greater than 90% for negative control group. In the positive control, MMS induced high DNA migration at high levels of viability (∼85%, data not shown). The dose-response relationship of powders from glass ionomer cements at concentrations ranging from 0–1000 μg/mL on cell viability assessed by trypan blue assay are shown in Fig. 1. Data indicate that cytotoxicity of components from glass ionomer cements was influenced by concentration of the agents. Cell viability after exposure to powders ranging from 1–100 μg/mL remained unchanged. Nevertheless, significant differences ($P < 0.05$) in cytotoxicity provoked by all powders tested of glass ionomer cements were observed for exposure at 1000 μg/mL concentration. The dose-response relationship between serial dilutions of liquids from glass ionomer cements evaluated are shown in Fig. 2, in which the major toxic effect on cell viability was produced at 10%, beginning at the dilution of 0.5% for Vitrebond (Fig. 2).

The single cell gel (comet) assay was used to measure DNA damage in CHO cells *in vitro*. DNA strand breaks were represented by the mean tail moment for 50 comets/sample. As seen in Table 2, powders from Ketac Cem and Vitrebond did not induce strand breaks in DNA in all concentrations tested. However, the power from Ketac Molar displayed genotoxicity only in the maximum concentrations evaluated

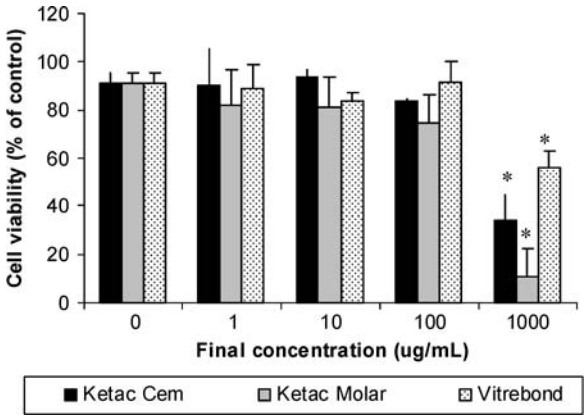

Fig. 1 Effects of serial concentrations of powders from glass ionomer cements on trypan blue exclusion test. Results are expressed as the mean percentage of control (mean and standard deviation.). $^{*}P < 0.05$ when compared to negative control (zero).

(100 μg/mL). Samples were further assayed for the comet assay in the presence of liquids from glass ionomer cements at dilutions that ranged from 1 to 0.01%. It was clearly observed that only Vitrebond at 0.1% dilution caused an increase of DNA injury. Fig. 3 shows undamaged CHO cell by negative control, Ketac-Cem treated-cell, and MMS-induced comet cell (positive control).

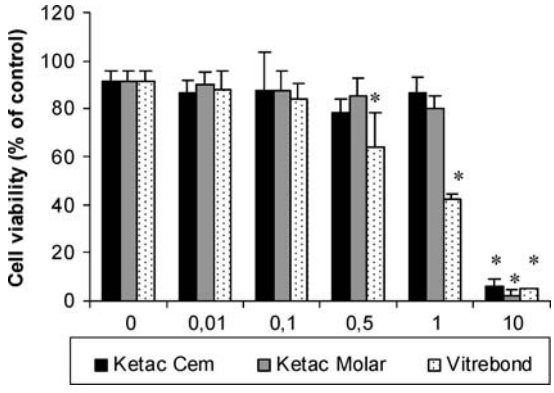

Fig. 2 Effects of serial dilutions (%) of liquids from glass ionomer cements on trypan blue exclusion test. Results are expressed as the mean percentage of control (mean and standard deviation.) $^{*}P < 0.05$ when compared to negative control (zero).

Table 2 Mean and Standard deviation of DNA damage (tail moment) in CHO cells exposed to powders and liquids from glass ionomer cements

Concentration	Ketac Cem[1]	Ketac Molar	Vitrebond
Powders (μg/mL)			
100	0.26 ± 0.21	$3.82 \pm 0.24^*$	0.48 ± 0.59
10	0.34 ± 0.15	0.63 ± 0.20	0.89 ± 0.42
1	0.59 ± 0.13	0.78 ± 0.56	0.45 ± 0.34
Liquids (%)			
1	0.78 ± 0.56	0.45 ± 0.10	–
0.5	1.18 ± 0.64	0.36 ± 0.23	–
0.1	0.64 ± 0.18	0.14 ± 0.10	$2.98 \pm 0.24^*$
0.01	0.54 ± 0.20	0.81 ± 0.48	0.29 ± 0.14
Negative control[1]	0.48 ± 0.11	0.48 ± 0.11	0.48 ± 0.11
Positive control[2]	$5.68 \pm 2.20^*$	$5.68 \pm 2.20^*$	$5.68 \pm 2.20^*$

[1]Phosphate buffer solution (ph 7.4).

[2]MMS at 1 μg/mL.

$^*P < 0.05$ when compared to negative control.

4. Discussion

The aim of this study was to evaluate the genotoxic damage in CHO cells *in vitro* using powders and liquids from different presentation forms of glass ionomer cements represented by second generation (Ketac Cem); high viscosity (Ketac Molar) and resin-modified glass ionomer cement (Vitrebond). The investigation was conducted utilizing the single cell gel (comet) assay. To our knowledge, this is the first study in which both genotoxic and cytotoxic effects from different categories of glass ionomer cements have been demonstrated *in vitro* by single cell gel (comet) assay and trypan blue assay, respectively. Single cell gel (comet) is a rapid and very sensitive fluorescent microscopic method to examine DNA damage at individual cell level. This assay has critically important applications in fields of toxicology ranging from aging and investigations to genetic toxicology and molecular epidemiology. Since the introduction of the alkaline comet assay in 1988 [15], a number of advancements have greatly increased the flexibility and utility of this technique for detecting various forms of DNA damage (e.g., single- and double-strand breaks, oxidative DNA base damage, and DNA-DNA/DNA-protein/DNA-drug cross-linking) in any eukaryotic cell.

In vitro studies are simple, inexpensive to perform, provide a significant amount of information, can be conducted under controlled conditions, and may elucidate the mechanisms of cellular toxicity [16]. Cell culture studies are commonly used in the evaluation of genotoxicity. The results obtained from these assays observed *in vitro* might be indicative of the effects observed in vivo. Our choice of cell line and use of cell in continuous culture permit an accurate evaluation of the changes, independently from factors such as age, metabolic and hormonal states of the donor that may influence the cell in primary culture. CHO cells have a small number of relatively large chromosomes, they grow fast; reproducible results can be obtained from the same cell source if cells are stocked in a frozen state.

The trypan blue exclusion test can be used to indicate cytotoxicity, where dead cells take up the blue stain of trypan blue, whereas the live cell have yellow nuclei. Recently, cytotoxicity was studied for powder and liquid components of chemically different matters dental in L929 fibroblasts using the integrity of mitochondria and lysosomes as end-points [17]. In our study, the trypan blue assay revealed that all powder components of glass ionomer cements were severely cytotoxic in the maximum concentration tested, and others as slightly to non-cytotoxic. These findings suggest that the cell membrane was the main target for the toxic agent, and that the damage occurred quickly. In the same way, the liquid dilutions were cytotoxic for all glass ionomers evaluated. All 10% dilutions had a strong effect on the cells with less than 10% viable cells after exposure. It seems that Vitrebond affects more selectively the cell membrane, because it was cytotoxic in intermediate dilutions. Furthermore, the high cytotoxicity of Vitrebond can be explained by the content of 2-hydroxyethyl-methacrylate (HEMA), a known cytotoxic agent [16, 18, 19]. Probably, HEMA was able to bind to CHO membranes, and it readily penetrates within the cells causing cellular death.

Cytotoxicity assessment is an integral part of the single cell gel (comet) assay. Since cytotoxicity produces strand breaks that show up as increased DNA migration, it is recommended that single cell gel (comet) assay should not be performed on samples showing more than 30% cytotoxicity [20]. Thus, the higher concentrations either to liquids or to powders from glass ionomer cements were not assayed for

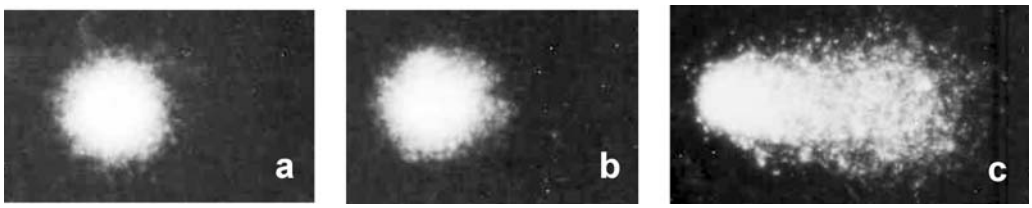

Fig. 3 Representative comet images from untreated control CHO cell (a), Ketac-Cem-treated-cell (b), and MMS-treated cell (positive control) (c). (DNA was stained with ethidium bromide; x40 magnification).

genotoxicity in this setting. Furthermore, in the present study, as well as in all of our previous investigations using the single cell gel (comet) assay, we have always excluded comets without clearly identifiable heads (i.e. comets with most of their DNA in the tails after the electrophoresis) during the image analysis. Although it should be emphasized that it is still not completely understood what these 'clouds' actually represent, this type of comet was excluded on the basis of the assumption that these cells represent dead cells, resulting from putative cytotoxic effects of glass ionomer cements rather than primary DNA-damage following a direct interaction between DNA and a genotoxic agent [21]. The approach of excluding comets with practically all DNA in the tail after the electrophoresis when evaluating potential genotoxicity in the single cell gel (comet) assay has also been used by others [22].

Regarding comet parameters, the tail moment represents a simple descriptor measured by the computerized image analysis system considering both the length of DNA migration in the comet tail and the tail intensity. This parameter is one of the best indices of induced DNA damage among the various parameters calculated by this method. The statistical analysis of tail moment data confirms that the powder from Ketac Molar at 100 μg/mL concentration can contribute to the induction of strand breaks in DNA. Herein, it is probable that some compounds of the powder of Ketac Molar are able to cause DNA damage, but the level damage depends on the concentrations used. It is important to stress that the single cell gel (comet) assay does not necessarily predict the mutagenic potential of the powder of glass ionomer cements; moreover, the genotoxicity of these compounds can be modulated in combination with other DNA-damaging agents. Powders from Ketac Cem and Vitrebond did not induce DNA strand breaks in all concentrations tested.

No measurable genotoxicity was found for liquids from Ketac Cem and Ketac Molar in all dilutions tested. On the other hand, the liquid from Vitrebond proved to be genotoxic at a final dilution of 0.1%. Our findings are in line with other genotoxicity study using UMU test [4]. However, other studies have addressed no genotoxic effect for Vitrebond [5, 6]. It is important to keep in mind that the strand break formation during excision repair processes may also cause DNA migration in the single cell gel (comet) assay [23]. Therefore, positive results obtained with the single cell gel (comet) assay reflect the presence of DNA damage and the activity of repair processes in a complex way [24]. Thus, we assumed that further studies are required to confirm these results.

It is generally accepted correlation between genotoxic and carcinogenic effects of a variety of chemicals. Whether the DNA damage is repaired or persists is important to the fate of cells targeted by chemical carcinogens. However, the development of tumors in target cells depends not only on the initial levels of induced DNA damage and its repair, but also on other contributing factors including the production of reactive metabolites, their distribution, and their effect on cell proliferation [25]. For this reason, genotoxicity tests do not always reflect carcinogenicity. Moreover, *in vitro* and in vivo genotoxicity tests detect compounds that induce genetic damage directly or indirectly by various mechanisms. Therefore, no single test is capable of detecting all genotoxic agents. For a more detailed judgment on the genotoxic potential of glass ionomer cements, battery of tests is feasible.

Concluding, some tested substances induced significant DNA migration detected by single cell gel (comet) assay as a sign for limited genotoxic effects in higher concentrations. However, with the highest levels of DNA migration being combined with elevated cytotoxic effects, a low in vivo genotoxic effect could be observed. Since DNA damage and cellular death are important steps in events leading from carcinogen exposure to cancer, our study represents a relevant contribution to the correct evaluation of the potential health risk associated with the exposure to dental materials.

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