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Orthodontic cements induce genotoxicity and cytotoxicity in mammalian cells *in vitro*

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ABSTRACT

Background: This study assessed the genotoxic and cytotoxic potential of three different glass ionomer cements used in Orthodontics (Vidrion C, OptiBand, and Band-Lok).

Materials and Methods: The tested cements were exposed *in vitro* to mouse fibroblast cells for 1 h at 37°C. The genotoxicity and cytotoxicity were evaluated by means of the single cell gel (Comet Assay) and the trypan blue exclusion test, respectively. All data were assessed by the Kruskal–Wallis non-parametric test, followed by Dunn's test. $P < 0.05$ was considered for statistical significance.

Results: Significant statistically differences ($P < 0.05$) in cytotoxicity were observed for both Vidrion C powder and liquid at the tested concentrations, with exception to the group presenting the lowest powder concentration. OptiBand similarly presented induced cellular death at the highest tested concentration for paste A ($P < 0.05$). Band-Lok paste B was also able to induce cytotoxicity at the highest tested concentration. Regarding the comet assay, Band-Lok paste B and OptiBand paste A resulted in increased DNA injury ($P < 0.05$).

Conclusion: The obtained results support the thought that some glass ionomer cement components present both genotoxic and cytotoxic effects when in high concentrations. Since DNA damage and cellular death are important events during oncogenesis, this study represents relevant contribution to estimate the real risks induced by these materials upon cellular systems.

Key Words: Cytotoxicity, genotoxicity, glass ionomer cements, mouse fibroblast cells

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INTRODUCTION

Several dental materials have been recently used as orthodontic cement, but interest is now focusing on the use of glass ionomer cements. The advantages of glass ionomer cements are: low solubility, ability to release fluoride, and good adhesive properties.[1] Glass ionomer cements also uptake fluoride from topical fluoride applications, allowing them to act as long-term fluoride-releasing agents.[2] The glass ionomer cements' mixing procedure can be however unhandy and technique-sensitive.

Due to the variation in chemical composition and setting reaction among glass ionomer cements, they have been categorized as conventional, resin-modified glass ionomers or modified composites.[3] Conventional and resin-modified glass ionomer cements (RMGICs) are able to adhere to base metal alloys, as well as to unetched enamel, making them attractive for orthodontic banding. RMGICs are a hybrid of composite resin and glass ionomer groups.[4] These cements are often marketed in capsules and their setting includes an acid–base reaction.

Biocompatibility is the capacity of a material to do in a formal manner in a specific application,[5] meaning patient's tissues contacting this material do not undergo any toxic, inflammatory, allergic,

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genotoxic, or carcinogenic action.[6] To the best of our knowledge, no information is yet available on the biocompatibility of glass ionomer cements used in orthodontic practice.

Genotoxicity assays, within the biocompatibility tests available in the general field, are of particular importance because genotoxicity is an important and useful indicator of carcinogenicity.[7] This is due to these assays' ability to measure an initiating tumorigenesis event. To date, a variety of assays are able to assess genotoxicity, including those assessing genetic damage, DNA repair capacity, metaphase chromosomal aberrations, micronuclei, and sister chromatid exchanges.[8] 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.[9,10] The single cell gel [comet] assay was revealed as a promising tool for the detection of DNA damage induced by compounds used in dental practice.[11]

Cytotoxicity is a rapid and standardized test able to determine if biomaterials contain significant quantities of harmful extractables and their effect on cellular components. The trypan blue exclusion test can be used to indicate cytotoxicity, where dead cells stain blue from the trypan blue, and living cells present yellow nuclei.[12]

The purpose of this study was to investigate whether three glass ionomer cements used for orthodontic cementation can induce DNA breakage in mouse fibroblasts by the single cell gel (Comet Assay). To monitor cytotoxic effects, the trypan blue exclusion test was employed.

MATERIALS AND METHODS

Cell culture

Murine fibroblast cells (lineage 3T3-L1) were obtained from American Type Culture Collection and cultured at 37°C in an incubator supplied with humidified air containing 5% CO_2/95% O_2. The cells were maintained in a growth medium containing the following constituents: Dulbecco's modified Eagle's medium (Invitrogen, USA) with 25 mmol/L glucose, 1 mmol/L pyruvate, 4.02 mmol/L l-alanyl-glutamine, and 10% fetal calf serum (Sigma, USA).

Confluent cells from third passage were detached with 0.15% trypsin (Invitrogen Corporation) for 5 min. Then, 2 mL of complete medium was added and cells were centrifuged at 1000 rpm (180 g) for 5 min. Cell suspension was counted using a Neubauer chamber and seeded in 96-well microtitre plates (Corning) at 1×10^4 cells density per well (at a concentration of 1×10^6/ mL).

All procedures in this study followed the ethical conducts described by Ethics Committee of Federal University of Sao Paulo, UNIFESP, SP, Brazil.

Treatment

The following glass ionomer cements were tested: Vidrion C (SS White, USA – Lot 070709); Band-Lok (Reliance Orthodontic Products Inc., Itasca, IL, USA – Lot Paste A 0802185/ Paste B 0802202) and OptiBand (Ormco Co, Orange, CA, USA – Lot 507180/507190). The cements composition is: (a) Vidrion C powder: sodium fluorosilicate, calcium, and aluminum, polyacrylic acid; and its liquid, tartaric acid, and distilled water; (b) OptiBand: uncured methacrylate ester monomers, inert mineral fillers, fumed silica, activators, preservatives, and colorant; and (c) Band-Lok: hidroxietilmetacrilate (HEMA), silica, inert mineral fillers, and activators.

Powders or pastes of the abovementioned glass ionomer cements were prepared in increasing final concentration solutions ranging from 10 to 1000 mg/ mL, while liquids were prepared with dilutions from 0.1 to 10%. The negative control group was treated with vehicle control (Dimethylsulfoxide, DMSO), while the positive control group was treated with methylmetasulfonate (MMS at 10 mg/mL, Sigma Aldrich, USA). After 1 h incubation at 37°C, cells were centrifuged at 1000 rpm (180 *g*) for 5 min, washed twice with fresh medium and re-suspended with fresh medium. Each individual treatment was consecutively repeated three times to ensure reproducibility.

Cytotoxicity assay

The cell viability test for mouse fibroblast cells was performed using Trypan blue staining after the treatment.[12] 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. Nonviable cells appeared blue-stained. At least 200 cells were counted per treatment.

Genotoxicity assay

The protocol used for the cell gel (Comet Assay) followed the guidelines proposed by Tice *et al.*[8] Briefly, 10 µl of cells (~l × 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 in duplicate, and covered with a coverslip. After brief agarose solidification in a refrigerator, the coverslip was removed and slides immersed into 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 1 h. The slides were left in alkaline buffer (pH > 13) for 20 min prior to electrophoresis and then electrophoresed for 20 min, at 25 V (0.86 V/cm) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris–HCI (pH 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.

Fifty randomly captured comets per treatment (25 cells from each slide)[13] were blindly examined by one experienced observer. The observations were performed at 400× magnification, using a fluorescence microscope (Olympus, Optical Co. Ltd, Tokyo, Japan) connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments, Haverhill, UK), previously calibrated in accordance to the manufacturer's instructions. A computerized image analysis system acquired images, computed the integrated intensity profiles for each cell, estimated the comet cell components, and then evaluated the range of derived parameters. Undamaged cells have an intact nucleus without a tail and damaged cells presented a comet-like appearance. Tail moment was evaluated to quantify the DNA damage and was calculated as the product of tail length and DNA fraction 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 particular sample was taken as an indicator of DNA damage for this sample.

Statistical methods

Parameters from the comet assay and the cellular viability were assessed by Kruskal–Wallis followed by Dunn's test using SigmaStat software, version 1.0 (Jadel Scientific, USA). $P < 0.05$ was considered for statistical significance.

RESULTS

The viability was greater than 90% for the negative control group. In the positive control, MMS induced high DNA migration at high levels of viability (~87%, data not shown). The dose–response relationship on cell viability of the glass ionomer cement powders or pastes at concentrations ranging from 0 to 1000 mg/ mL, assessed by trypan blue assay, is shown on Table 1. Data indicate that cytotoxicity of components from glass ionomer cements was influenced by their concentration. Significant statistically differences ($P < 0.05$) in cytotoxicity were observed for both Vidrion C powder and liquid at the tested concentrations, with exception to the group presenting the lowest powder concentration. OptiBand similarly presented induced cellular death at the highest tested concentration for paste A [Table 1]. Band-Lok paste B was also able to induce cytotoxicity at the highest tested concentrations.

The single cell gel (Comet Assay) was used to measure the *in vitro* DNA damage in mouse fibroblast cells. DNA strand breaks were represented by the mean tail moment at 50 comets per sample. As seen in Table 2, Vidrion C powder was not able to induce genetic damage at the highest tested concentration. Samples were further assessed for the comet assay with pastes A and B from Band-Lok and OptiBand. The results revealed that Band-Lok paste B and OptiBand paste A resulted in increased DNA injury. A representative comet image is shown in Figure 1.

DISCUSSION

The aim of this study was to evaluate *in vitro* the genotoxic damage and cytotoxicity induced by orthodontic cements using murine fibroblasts. The investigation was conducted using the single cell gel

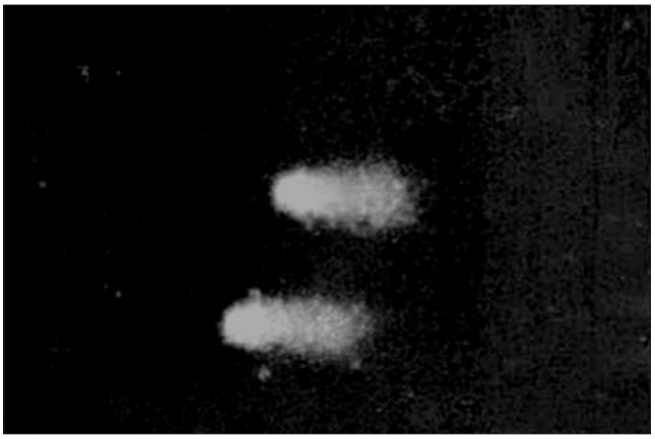

Figure 1: Representative comet image in murine fibroblasts cells treated with orthodontic cements. Syber green stain, ×40 magnification

(Comet Assay) and trypan blue exclusion test. The glass ionomer cements were chosen due to their wide use in the orthodontic clinic, Vidrion C being as a conventional glass ionomer cement and Band-Lok and OptiBand as resin-modified glass ionomer cements. To the best of our knowledge, this assessment has not been demonstrated so far.

In vitro studies are simple and inexpensive to perform; they provide a significant amount of information, can be conducted under controlled conditions,[14] and may elucidate the mechanisms of cellular toxicity. Cell culture studies are commonly used to assess genotoxicity. Our choice for this cell line, i.e., fibroblast cells, allows an accurate evaluation of alterations, excluding factors such as age and donor metabolic and hormonal states, which might influence cells in primary culture. In particular, previous studies conducted by our group have demonstrated that murine fibroblasts cells are suitable for investigating genotoxic potential of dental materials.[15,16]

It is important to stress that the approach for studying powders and liquids separately was adopted in this study because it is imperative to know if, and to what extent, such compounds are able to induce genetic damage and/or cellular death as described elsewhere.[17,18] Such increasing concentrations represent a critical evaluation for toxicological purposes in genotoxicity and cytotoxicity.[8]

In this study, we chose the trypan blue assay because the test is recommended by the comet assay expert group as an indicative of cytotoxic potential of chemicals in studies involving gentoxicity. The trypan blue assay revealed that all glass ionomer cement powders or pastes were severely cytotoxic at the highest tested concentrations, while other concentrations presented slight to non-cytotoxic. Vidrion C liquid dilutions showed strong cytotoxicity to mouse fibroblasts. Because the cytotoxicity assay used in the study is able to evaluate the membrane integrity as an indicator of cellular death, these findings support the idea that cell membrane was

Table 1: Mean and standard deviation of cellular death (% of viable cells) in murine fibroblast cells exposed to powders and liquids from orthodontic cements

Concentration	Vidrion C	OptiBand		Band-Lok	
		Paste A	Paste B	Paste A	Paste B
Powders or pastes (mg/mL)					
10	78 ± 5.3[2]	76 ± 6.2	84 ± 5.5	90 ± 5.2	75 ± 10.3
100	69 ± 7.4*	75 ± 8.2	78 ± 4.3	84 ± 5.2	62 ± 8.4*
1000	52 ± 6.4*	67 ± 3.2*	80 ± 7.2	77 ± 5	51 ± 3.4*
Liquids (vol. %)					
0.1	62 ± 4.5*	–	–	–	–
1	47 ± 7.2*	–	–	–	–
10	38 ± 6.5*	–	–	–	–
Negative control[1]	90 ± 8.4	90 ± 8.4	90 ± 8.4	90 ± 8.4	90 ± 8.4

[1]Dimethylsulfoxide (DMSO). [2]Standard deviation of three independent experiments. *$P < 0.05$ when compared to negative control

Table 2: Mean and standard deviation of DNA damage (tail moment) in murine fibroblast cells exposed to powders, pastes, and liquids from orthodontic cements

Concentration	Vidrion C	OptiBand		Band-Lok	
		Paste A	Paste B	Paste A	Paste B
Powders or pastes (mg/mL)					
10	0.6 ± 0.3[2]	1.2 ± 0.4	0.4 ± 0.3	1.2 ± 0.6	1.7 ± 0.4*
100	–	2.4 ± 0.7*	0.7 ± 0.3	0.8 ± 0.4	–
1000	–	–	0.8 ± 0.6	0.9 ± 0.5	–
Liquids (vol. %)					
0.1	–	–	–	–	–
1	–	–	–	–	–
10	–	–	–	–	–
Negative control[1]	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.3
Positive control[3]	4.3 ± 1.2	4.3 ± 1.2	4.3 ± 1.2	4.3 ± 1.2	4.3 ± 1.2

[1]Dimethylsulfoxide (DMSO). [2]Standard deviation of three independent experiments. [3]Methylmetasulfonate (MMS) at 10 mg/mL. *$P < 0.05$ when compared to negative control

the main target for toxic agents, and that damage occurred quickly. By comparison, previous studies conducted by our group have demonstrated *in vitro* strong cytotoxicity, induced by resin-modified glass ionomer cements.[17,18] Furthermore, the resin-modified glass-ionomer has exhibited inhibition on growth and differentiation of osteoblasts surface.[19] *In vivo* studies have postulated low biocompatibility properties of resin-modified glass ionomer cements, depicted by the presence of coagulation necrosis and intense inflammatory infiltrate induced by Vitrebond.[20] Taken as whole, it seems that glass ionomer cements exert cellular death. Further studies using other cytotoxicity parameters such as MTT or protein synthesis would be useful at discovering early cytotoxic effects of glass ionomer cements, mainly in lower concentrations.

Cytotoxicity assessment is an integral part of the single cell gel (Comet Assay). Cytotoxicity produces strand breaks representing increased DNA migration. Therefore, it is recommended that the single cell gel (Comet Assay) should not be performed on samples showing more than 30% cytotoxicity.[21] In this regard, no cytotoxicity signals were assessed through genotoxic damage in this study. Moreover, comets without clearly identifiable heads, i.e. comets with most of their DNA in the tails after the electrophoresis, were excluded during the image analysis. Although the representation of these "clouds" is still not completely understood, this type of comet was excluded based on 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.[22] The approach of excluding comets with practically all DNA in the tail when evaluating potential genotoxicity in the single cell gel (Comet Assay) has also been used by others.[23]

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 several parameters calculated by this method.[13] The statistical analysis of tail moment data confirms that Band-Lok and OptiBand components induce genetic damage. No measurable genotoxicity was also found in all tested dilutions of Vidrion C powder or liquids. Such findings are in agreement with previous studies.[24] On the other hand, studies have observed positive genotoxicity for resin-modified glass ionomer cements by the UMU-test.[25] It is important to discuss that strand break formation during excision repair processes may cause DNA migration in the single cell gel (Comet Assay).[26] Nevertheless, DNA lesions detected with the single cell gel (Comet Assay) do not represent premutagenic lesions. Thus, this assay does not necessarily predict the mutagenic potential of the tested compound.[27]

Taken together, the obtained results support the thought that some glass ionomer cement components present both genotoxic and cytotoxic effects when in high concentrations.

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REFERENCES

1. Maijer R, Smith DC. A comparison between zinc phosphate and glass ionomer cement in orthodontics. Am J Orthod Dentofacial Orthop 1988;93:273-9.
2. Diaz-Arnold AM, Holmes DC, Wistrom DW, Swift EJ Jr. Short-term fluoride release/uptake of glass ionomer restoratives. Dent Mater 1995;11:96-101.
3. McCabe JF. Resin-modified glass-ionomers. Biomaterials 1988;19:521-7.
4. Yoshida Y, van Meerbeek B, Nakayama Y, Snauwaert J, Hellemans L, Lambrechts P, *et al.* Evidence of chemical bonding at biomaterial-hard tissue interfaces. J Dent Res 2000;79:709-14.
5. Willians DF. Definitions in biomaterials. Proceedings of a Consensus Conference of the European Society for Biomaterials. England co. 4, New York: Elsevier; 1986.
6. Vahey JW, Simonian PT, Conrad EU 3rd. Carcinogenicity and metallic implants. Am J Orthop (Belle Mead NJ) 1995;24:319-24.
7. Ribeiro DA. Do endodontic compounds induce genetic damage? A comprehensive review. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;105:251-6.
8. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, *et al.* Single cell gel/comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutagen 2000;35:206-21.
9. Martins RA, Gomes GA, Aguiar O Jr, Ribeiro DA. Biomonitoring of oral epithelial cells in petrol station attendants: Comparison between buccal mucosa and lateral border of the tongue. Environ Int 2009;35:1062-5.
10. Olive PL, Banáth JP, Durand RE. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the comet assay. Radiat Res 1990;112:86-94.
11. Ribeiro DA. Single-cell gel (Comet Assay) as a promising tool for the detection of DNA damage induced by compounds used in

dental practice: The oral cancer risk assessment. Crit Rev Oncog 2008;14:165-75.
12. Mckelvey-Martin VJ, Green MH, Schmezer P, Pool-Zobel BL, De Méo MP, Collins A. The single cell gel electrophoresis assay (comet assay): A European review. Mutat Res 1993;288:47-63.
13. Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, *et al.* Recommendations for conducting the *in vivo* alkaline comet assay. 4th International Comet Assay Workshop. Mutagenesis 2003;18:45-51.
14. Geurtsen W. Substances released from dental resin composites and glass ionomer cements. Eur J Oral Sci 1998;106:687-95.
15. Marins JS, Sassone LM, Ribeiro DA. Biopure MTAD Induces DNA Damage but Not Cellular Death: An *in vitro* Study. Eur J Dent 2009;3:285-9.
16. Zeferino EG, Bueno CE, Oyama LM, Ribeiro DA. Ex vivo assessment of genotoxicity and cytotoxicity in murine fibroblasts exposed to white MTA or white Portland cement with 15% bismuth oxide. Int Endod J 2010;43:843-8.
17. 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.
18. Ribeiro DA, Marques ME, Salvadori DM. Biocompatibility of glass-ionomer cements using mouse lymphoma cells *in vitro*. J Oral Rehabil 2006;33:912-7.
19. Imazato S, Horikawa D, Nishida M, Ebisu S. Effects of monomers eluted from dental resin restoratives on osteoblast-like cells. J Biomed Mater Res B Appl Biomater 2009;88:378-86.
20. 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.
21. Eisenbrand G, Pool-Zobel B, Baker V, Balls M, Blaauboer BJ, Boobis A, *et al.* Methods *in vitro* toxicology. Food Chem Toxicol 2002;40:193-236.
22. Ribeiro DA, Pereira PC, Machado JM, Silva SB, Pessoa AW, Salvadori DM. Does toxoplasmosis cause DNA damage? An evaluation in isogenic mice under normal diet or dietary restriction. Mutat Res 2004;559:169-76.
23. Hartmann A, Speit G. The contribution of cytotoxicity to DNA-effects in the single cell gel test [comet assay]. Toxicol Lett 1997;90:183-8.
24. Bakopoulou AA, Triviai IN, Tsiftsoglou AS, Garefis PD. *In vitro* assessment of cytotoxicity of resin-based dental restorative materials on WEHI 13 var fibroblasts. Int J Prosthodont 2006;19:13-6.
25. Müller BP, Eisenträger A, Jahnen-Dechent W, Dott W, Hollender J. Effect of sample preparation on the *in vitro* genotoxicity of a light curable glass ionomer cement. Biomaterials 2003;24:611-7.
26. Gedik CM, Ewen SW, Collins AR. Single-cell gel electrophoresis applied to the analysis of UV-C-damage and its repair in human cells. Int J Radiat Biol 1992;62:313-20.
27. Hartmann A, Speit G. Genotoxic effects of chemicals in the single cell gel (SCG) test with human blood cells in relation to the induction of sister chromatid exchanges (SCE). Mutat Res 1995;346:49-56.

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