Saudi Dental Journal (2018) **30**, 38–42

King Saud University

Saudi Dental Journal

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ORIGINAL ARTICLE

Genotoxicity and cytotoxicity induced by eluates from orthodontic glass ionomer cements in vitro

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Received 5 January 2016; revised 4 September 2017; accepted 3 October 2017
Available online 24 October 2017

KEYWORDS

Genotoxicity;
Cytotoxicity;
Glass ionomer cements;
Murine fibroblasts

Abstract The aim of this study was to investigate genotoxicity and cytotoxicity of some orthodontic glass ionomer cements commercially available by means of the single cell gel (comet) assay. For this purpose, five commercial orthodontic glass ionomer cements (Vidrion C®, Meron®, Optiband®, Multicure® and Ultra Band Lok®) were tested in murine fibroblasts in vitro. For this purpose, eluates from each cement were prepared according manufactures instructions at 0, 2, 4, 8, 18, 32 and 64 days of immersion in artificial saliva at 37 °C. All orthodontic glass ionomer cements failed to induce cytotoxicity to murine fibroblasts for all periods evaluated in this study. However, Vidrion C® was able to induce genotoxicity after 64 days of exposure to eluates. Meron® also demonstrated genotoxicity as depicted by increasing DNA damage on 2nd day. Multicure® demonstrated genotoxicity on 32nd day and Ultra band Lok on 18th, 32nd days of exposure. Taken together, our results demonstrated that orthodontic cements derived from resin-modified glass ionomer composite (Multicure®) and compomer (Ultra Band Lok®) cause genetic damage in mammalian cells in vitro.

1. Introduction

Nowadays, orthodontic therapy is increasing around the world as a result of establishment masticatory function and/or esthetic purposes. Herein, orthodontists have routinely used glass ionomer cements with success for bonding brackets. In order to facilitate its manipulation as far as to increase its immediate tensile strength, resin-modified glass ionomer cements and compomers have been incorporated to clinical usage, by incorporation of 2-hydroxyethyl methacrylate (HEMA), triethylene glycol dimethacrylate (TEGMA), urethane dimethacrylate (UDMA) and bisphenol A glicidyl

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sdentj.2017.10.001

methacrylate (BisGMA) (Reichl et al., 2006a,b). Taking into consideration that these dental materials are in close contact with oral tissues over long periods of time, it is mandatory to evaluate the behavior on biological test systems to ensure protection either to patients or to clinicians (Ribeiro et al., 2006a,b).

Biocompatibility is the ability of some material to perform with an appropriate host response when applied to biological tissues. To date, there are many experimental models able to evaluate the biocompatibility of materials used in dental and/or medical practices using different methodologies and end-points so far. To evaluate genotoxicity and cytotoxicity are particularly relevant biologically, because they are closely related to the initiation phase of carcinogenesis process, due to cell cycle proliferation stimulus, or an error of mitotic phase secondary to cell deoxyribonucleic acid (DNA) damage with subsequent repair capacity impaired (Bull et al., 2006). For this reason, it would important to investigate if, and to what extent, some glass ionomer cements used in orthodontics induce genetic damage and cellular death in mammalian cells.

The single-cell gel (comet) assay in alkaline version was designed as a rapid, simple and reliable biochemical technique for evaluating DNA strand breaks in mammalian cells (McKelvey-Martin et al., 1993). Some advantages of the single cell gel (comet) assay have been elected when compared to other genotoxicity assays because it is cheap and with reproducible results (Brendler-Schwaab et al., 2006). The basic principle of the single-cell gel (comet) assay resides on the migration of DNA fragments as a result of double strand breaks, single strand breaks, adducts and incomplete repair sites through agarose matrix under electrophoresis conditions. Nucleoids look like comets with a head (the nuclear region) and a tail containing DNA fragments by conventional light microscopy (Tice et al., 2000). Previous studies conducted by our research group have performed the single cell gel (comet) assay under different protocols and paradigms (Angelieri et al., 2011; Da Silva et al., 2007; Braz et al., 2006; Guilheiro et al., 2014). Therefore, the methodology is useful for assessing DNA damage in mammalian cells.

Accumulating evidence suggests that cell membrane integrity is a suitable biological phenomenon for distinguishing dead from live cells (Kroemer et al., 2009). The trypan blue method is a suitable assay for evaluating cytotoxicity in experimental investigations (Zanatta et al., 2012) including biocompatibility tests. The rationale of this methodology resides on the fact that dead cells incorporates trypan blue dye into the cytoplasm as a result of loss of membrane selectivity, whereas live cells remain unstained (Tennant, 1964).

The aim of this study was to evaluate genotoxicity and cytotoxicity induced by orthodontic glass ionomer cements by single cell gel (comet) assay and trypan blue exclusion test in vitro. Certainly, such data will contribute for a better understanding the biological behavior of these compounds on eukaryotic cells.

2. Materials and methods

2.1. Cell Culture

Murine fibroblast cells (lineage 3 T3-L1) were obtained from the American Type Culture Collection and cultured at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were maintained in a growth medium containing the following constituents: Dulbecco's modified Eagle's medium (Invitrogen Corp., Grand Island, NY, USA) with 25 mmol/L glucose, 1 mmol/L pyruvate, 4.02 mmol/L L-alanyl-glutamine and 10% fetal calf serum (Sigma Aldrich, St. Louis, MO, USA). Confluent cells were detached with 0.15% trypsin (Invitrogen Corp.) for 5 min. After that, 2 mL of complete medium was added and the 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 Glass, Corning, NY, USA) at a density of 1×10^4 cells per well (at a concentration of 1×106/mL).

2.2. Cell treatment

Two specimens (6 mm × 2 mm) of each cement were made according to manufactures' instructions. The following orthodontic cements were used in this study: two conventional glass ionomer cements (Vidrion C®, SS White, Brazil and Meron®, VOCO, Germany), two resin-modified glass ionomer cements (Optiband®, Ormco, USA and Multicure®, 3 M Unitek, USA) and a compomer (Ultra Band Lok®, Reliance Orthodontics, USA). The resin-modified glass ionomer cement and cement were light cured for 40 s.

Each specimen was individually immersed in 7 ml of artificial saliva at 37 °C for a period up to 64 days. One milliliter of each eluate was extracted at 0, 2, 4, 8, 18, 32 and 64 days. After that, each eluate obtained from experimental periods established in this setting were exposed to murine fibroblasts for 1 h at 37 °C in triplicate as described elsewhere (Angelieri et al., 2011).

The negative control group was treated with vehicle control (artificial saliva), during 1 h at 37 °C. For the positive control group, both cells were exposed to methyl methanesulfonate (MMS, Sigma Aldrich) at 1 μmol/mL during 3 min at 37 °C. Each treatment was performed consecutively three times to ensure reproducibility. The protocol was established in a previous study conducted by our research group (Angelieri et al., 2011; Matsumoto et al., 2014).

2.3. Trypan blue exclusion test

After completing the experimental periods, murine fibroblasts cells from all groups (1×105 mL cells) were incubated to 90 μL 0.4% trypan blue solution diluted in PBS for 5 min. Neubauer chamber was used to measure the total number of death cells.

2.4. Single cell gel (comet) assay

For genotoxicity assay (Tice et al., 2000), a volume of 10 μl of murine fibroblasts cells ($\sim 1 \times 10^4$ cells) for all experimental groups was added to 120 mL 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 (slides were prepared in duplicate per treatment). After brief agarose solidification in a refrigerator, the coverslip was removed and the slides were immersed in the lysis solution (2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid [EDTA], 10 mM Tris-HCl buffer pH = 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO) for 1 h. Prior to electrophoresis, the slides were

left in an alkaline buffer (0.3 mM NaOH and 1 mM EDTA, pH > 13) for 20 min and electrophoresed 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-HCl (pH 5 7.5) for 15 min, fixed in absolute ethanol, and stored at room temperature. All of the steps described above were conducted in the darkened room to prevent additional DNA damage.

A total of 50 randomly captured comets per treatment (25 cells from each slide) were blindly examined by one expertise observer (D.A.R.) at 400× magnification using a fluorescence microscope (Olympus, Center Valley, Pa) connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments, UK). 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 "migrated tail". Damaged cells have a broken and spread nucleus with the appearance of a comet. To quantify the DNA damage, tail moment was evaluated. The comet tail moment is positively correlated with the level of DNA breakage in a cell and is calculated as the product of the tail length (DNA migration) and tail intensity (% of present DNA in the tail estimated by fraction of DNA in the comet tail). The mean value of the tail moment in a particular sample was taken as an index of DNA damage in this sample.

2.5. Statistical analysis

All data were checked to the Shapiro–Wilk test for evaluating normality ($p < .05$). Genotoxicity and cytotoxicity data were assessed by repeated measure ANOVA followed by multiple comparisons of Tukey test using SigmaStat software, version 1.0 (Jandel Scientific, Rafael, CA, USA). A *P* value less than .05 was considered statistically significant.

3. Results

Cytotoxicity assay was conducted by trypan blue exclusion test. The results are presented in Table 1. All eluates from orthodontic cements did not induce cellular death after 2, 4, 8 18, 32, and 64 days of exposure. No significant statistically differences were detected among groups ($p > .05$).

The results from single cell gel (comet) assay are demonstrated in Table 2. Such findings demonstrated that Vidrion C® induced genetic damage from 64 days of eluates exposure only. The same picture occurred to Optiband®, i.e. eluates obtained from 64 days were able to induce genetic damage. Meron® provoked genetic damage in murine fibroblasts after 2 days of eluates exposure. Other periods did not induce DNA strand breaks in this setting.

Multicure® induced DNA breakage after 32 days and 64 days of eluates exposure eluates. Tail moment values were significant statistically ($p < .05$) when compared to negative control.

Ultrabandlok® exerted moderate genotoxicity since eluates obtained from 18, 32 and 64 days of exposure were able to induce genetic damage in murine fibroblasts in vitro.

4. Discussion

The aim of this study was to evaluate if orthodontic glass ionomer cements are able to induce genotoxicity and cytotoxicity in vitro. In vitro tests investigating genotoxicity and cytotoxicity have gained large acceptance among biocompatibility assays because the methodology is able to assess subtle metabolism changes of cells (Tice et al., 2000) and isolate potential biases from environment, allowing to evaluate data with more accuracy (Matsumoto et al., 2014).

The results revealed that all eluates derived from orthodontic cements did not induced cellular death in vitro. By comparison, a previous study conducted by our research group has detected positive findings in cytotoxicity for orthodontic cements. For example, high cytotoxicity was detected to powder and liquid from Vidrion C® (Angelieri et al., 2012). In a similar manner, paste A from OptiBand® induced cellular death at the highest tested concentration (Angelieri et al., 2012). Band-Lok® paste B was also able to induce cytotoxicity at the highest tested concentration (Angelieri et al. 2012). Probably, these discrepancies are explained by differing in experimental design, since this study was able to evaluate the effects of eluates obtained from orthodontic cements and not the powder, liquid or pastes of these ones. Anyway, further studies are necessary to elucidate the issue.

Regarding genotoxicity, the majority of orthodontic cements tested did cause genetic damage in the highest concentration used, while Meron®, showed early genotoxicity in this setting (2 day-eluate of exposure). Ultrabandlok® was able to induce genotoxicity after 18, 32 and 64 days of exposure, whereas Multircure® induced genotoxicity after 32 and 64 days of exposure. Therefore, it is assumed that genotoxicity to murine fibroblasts induced by eluates was in a dose-dependent fashion. This occurred characteristically for resin-modified and compomer glass ionomer cements. Such findings

Table 1 Cellular death (% of viable cells) in murine fibroblasts exposed to eluates from glass ionomer cements.

Days of exposure	Vidrion	Meron	Optiband	Multicure 3 M	Ultraband Lok
0	90 ± 5	90 ± 5	90 ± 5	90 ± 5	90 ± 5
2	87 ± 4	88 ± 3	90 ± 5	93 ± 3	78 ± 8
4	84 ± 5	93 ± 5	78 ± 10	79 ± 8	90 ± 8
8	88 ± 6	92 ± 5	77 ± 14	90 ± 5	90 ± 2
18	77 ± 13	84 ± 7	90 ± 3	86 ± 2	77 ± 9
32	89 ± 9	78 ± 6	78 ± 14	84 ± 6	87 ± 9
64	90 ± 10	83 ± 7	90 ± 6	88 ± 10	81 ± 7

$p > .05$.

Table 2 DNA damage (tail moment) in murine fibroblasts exposed to eluates from glass ionomer cements.

Days of exposure	Vidrion	Meron	Optiband	Multicure 3 M	Ultraband Lok
0	0.6 ± 0.3	1.2 ± 0.4	0.4 ± 0.3	1.2 ± 0.6	0.7 ± 0.4
2	1.2 ± 0.8	2.4 ± 0.7*	0.7 ± 0.3	0.8 ± 0.4	0.9 ± 0.6
4	0.5 ± 0.3	1.2 ± 0.6	0.8 ± 0.6	0.9 ± 0.5	0.7 ± 0.6
8	1 ± 0.7	0.6 ± 0.2	1.1 ± 0.8	1.3 ± 0.5	1.2 ± 1
18	1.2 ± 1	1.4 ± 1.2	0.5 ± 0.4	0.9 ± 0.4	4.3 ± 1.8*
32	1.5 ± 0.9	1.2 ± 0.8	1.7 ± 0.5	3.5 ± 1.8*	3.5 ± 1*
64	2.1 ± 0.7*	1.7 ± 1.5	3.5 ± 1.2*	3.2 ± 1.1*	3.7 ± 1.2*
Positive control[a]	4.5 ± 1.4*	4.5 ± 1.4*	4.5 ± 1.4*	4.5 ± 1.4*	4.5 ± 1.4*

* $p < .05$ when compared to negative control (zero).
[a] Methylmetanesulfonate (MMS) at 10 μg/mL.

are important to better understand that some orthodontic cements from resin-modified and compomers are able to release some compounds that in turn cause genetic damage in mammalian cells. In fact, it has been demonstrated that some compounds are released from glass ionomer cements (Muller et al., 2003; Forss, 1993). For example, resin-modified and compomers contain HEMA, a known genotoxic agent present low performance with regard to biocompatibility (Falconi et al., 2007). Muller et al. (2003) used UMU-test to demonstrate in vitro biocompatibility of light-cured glass ionomer cements to Ham's F12 cells. The authors have postulated that HEMA is able to induce genetic damage at concentrations from 27 mM to 66.7 mM. The powder of Vitrebond® (resin-modified glass ionomer cement) induced genetic damage. The authors concluded that genotoxicity should not be attributed to this compound solely. However, Ribeiro et al. did not demonstrate in vitro genotoxicity to mouse lymphoma cells exposed to powder of Vitrebond® in concentrations ranging from 1 $\mu g/mL^{-1}$ to 100 $\mu g/mL^{-1}$ by single cell gel (comet) assay (Ribeiro et al., 2006b). On the other hand, they demonstrated that the liquid from Vitrebond® was genotoxic in a final volume of 0.1% (Ribeiro et al., 2006b). Therefore, both studies concluded that powders and liquids from glass ionomer cements cause genetic damage in mammalian cells in a dose-dependent manner.

In present study, in vitro genotoxicity to murine fibroblasts was mainly attributed to commercial Ultraband Lok®, a compomer very similar to composite resins. In general, composite resins contain TEGMA, UDMA e BisGMA in their composition, which is able to induce DNA injury in several concentrations (Reichl et al., 2006a). It is important to stress that genotoxicity is intimately related to the multistep carcinogenesis process, since the injured cells remain in proliferative cycle rather than undergo apoptosis (exclusion from proliferative cycle).

In summary, our results demonstrated that orthodontic cements derived from resin-modified glass ionomer composite (Multicure®) and the compomer (Ultra Band Lok®) cause genetic damage in mammalian cells in vitro. Since in vitro studies do not taking into account the complex homeostatic scenario that occurs in vivo, experimental models and clinical studies are welcomed to better understanding the biological behavior of orthodontic cements on mammalian cells.

Acknowledgments

YSS and DAR are recipients of CNPq (National Council for Scientific and Technological Development) fellowship.

Conflict of interest

None declared.

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