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Effects Cytotoxic of Orthodontic Cementation Materials: An in vitro Study about Cell Viability. Should this be a Concern?

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Abstract

Introduction: The biocompatibility of cementation materials in orthodontics is still questionable despite these materials have been used in contact to gingival tissues.

Objective: The objective of this study was to test the null hypothesis that cementation materials are not toxic to mice fibroblasts (lineage NIH/3T3), comparing different brands and types of cements, especially the composers.

Methods: Ninety specimens were divided into six groups (n=3 each) according to trademark (Meron™, Vitro Cem™, Vidrion C™,

Glass Ionomer Band Cement Multicure™, Fuji Ortho LC™, and Ultra Band Lok™) and cell viability assessment time point (1 h, 24 h, 48 h, 7 days, and 28 days). Cell viability was assessed using the MTT assay. Cellular growth was used as negative control (-) and sodium hypochlorite 1% as positive control (+). The data were analyzed using ANOVA and Tukey's HSD test, with $p<0.05$.

Results: All cementation materials evaluated showed low values of cell viability and similar to positive control at all times assessed, except Meron™, at 28 days. Toxicity peaks were observed at 1 h for Fuji Ortho LC™; at 48 h for Vitro Cem™, Vidrion C™, and Multi Cure Ionomer Band Cement™; and at 7 days for Meron™ and Ultra Band Lok™. After 28 days, the cell viability had increased considerably, but the materials (except Meron™) still showed toxicity values similar to those of the positive control, suggesting even cell toxicity.

Conclusions: All the cementation materials investigated showed low values of cell viability suggesting toxicity to mice fibroblasts at all times assessed, regardless of their composition, representing a concern about toxicity to clinical application, since they are materials that are in intimate contact with the gingival tissues.

Keywords: Orthodontics, Toxicity, Materials, Cimentation, Biocompatibility.

Introduction

The biocompatibility of some materials used in orthodontic clinical practice, especially cements, have been the subject of great concern. [1-4] In particular, glass ionomer cements (GICs) and composers, widely used in orthodontic practice as cementation materials [5, 6] have attracted the attention of investigators worldwide ever since their development because of their physical and chemical properties, e.g., adherence to tooth structure, and anticariogenic action due to the release of fluoride [6-8] however pouco se sabe sobre a biocompatibilidade.

According to Kao et al. (2007), a material is considered to be biocompatible when there are no manifestations of any toxic, irritating, inflammatory, or allergic events after its placement in the oral cavity [9]. Among the GICs commercially available, the release of ions, e.g., triethylene glycol dimethacrylate (TEGDMA), urethane dimethacrylate (UDMA), 2-hydroxyethyl methacrylate (HEMA), bisphenol A-glycidyl dimethacrylate (Bis-GMA), and methyl methacrylate (MMA), and their diffusion in the oral tissues, have been associated with cytotoxic and genotoxic effects, compromising their biocompatibility. [10,11] Conversely, studies investigating compomers are not available in the literature.

The adverse effects potentially caused by GICs in patients include inflammatory processes and allergic reactions. In the dental professional and team, problems may also occur as a result of long-term exposure to the monomer (the substance may cause sensitization after a single contact, even at low concentrations). Furthermore, GICs may induce dermatitis of different types by skin contact, and their vapour, once inhaled, may cause respiratory problems [12].

Based on these findings, in the concern about toxicity to clinical application, in the scarcity of studies on the cytotoxicity of compomers and evaluation of cementation materials in the long term, the objective of this study was to test the null hypothesis that cementation materials are not toxic to mice fibroblasts.

Materials and Methods

The sample comprised 90 specimens fabricated from the following materials: three conventional GICs, namely, Meron™ (Voco, Cuxhaven, Germany), Vitro Cem™ (DFL, Jacarepaguá, Rio de Janeiro, Brazil), and Vidrion C™ (SS White, Rio de Janeiro, Brazil); two resin-reinforced GICs, namely, Multi Cure Glass Ionomer Band Cement™ (3M Unitek, Monrovia, USA) and Fuji Ortho LC™ (GC Corp., Tokyo, Japan); and a composer, Ultra Band Lok™ (Reliance Orthodontic, Itasca, USA). Three specimens were fabricated from each material for each of the five assessment time points, at a total of 15 specimens per material. Cellular growth (no specimen) was used

as negative control (-), and 1% sodium hypochlorite as positive control (+). Details and characteristics of each material are described in Table 1.

Material	Composition
Meron™	Mixture of silicate, polyacrylic acid, tartaric acid, paraben fluoride, and initiator particles
Vitro Cem™	Strontium and aluminum silicate, dehydrated polyacrylic acid, and iron oxide
Vidrion C™	Sodium, calcium, and aluminum fluorosilicate, polyacrylic acid, tartaric acid, and distilled water
Multi Cure Ionomer™	Fluoro-alumino-silicate glass, strontium, potassium persulfate, ascorbic acid, blue dye, cherry essence, distilled water, HEMA, butylated hydroxytoluene, diphenyl hexafluorophosphate, camphorquinone, and iodine
Fuji Ortho LC™	Fluoro-alumino-silicate glass, polyacrylic acid copolymers, maleic acid, HEMA, di-2-methacryloxyethyl-2,2,4-trimethyl, hexamethylene dicarbamate, water, camphorquinone
Ultra Band Lok™	Glass, amorphous silica, Bis-GMA, and sodium fluoride particles

Bis-GMA = bisphenol A-glycidyl dimethacrylate; HEMA = 2-hydroxyethyl methacrylate.

Table 1: Basic composition of the cementation materials assessed.

Specimen Fabrication

Specimens were fabricated according to the manufacturers' instructions. All specimens were fabricated by one single operator. They were prepared and handled under aseptic conditions to avoid the influence of any microbiological contamination on cell culture tests.

In order to ensure standardization, a steel matrix was used for specimen fabrication; it measured 112 x 40 x 3 mm and contained 10 holes with 3-mm diameter, 3-mm high. Holes were assigned to the different experimental groups (n=3 each), according to the commercial brands assessed. Following fabrication, specimens were removed from the matrix and stored in a sterile environment until the toxicity test. Specimens were assessed after 1 h, 24 h, 48 h, 7 days, and 28 days.

Cytotoxicity Test

Mice fibroblasts (lineage NIH/3T3) (ATCC™-American Type Culture Collection-TCC, Old Town, USA) were thawed and cultured in 25 cc culture flasks (TPP™, Zollstrasse, Switzerland) in Dulbecco's modified eagle medium (DMEM; Invitrogen™, Carlsbad, USA) supplemented with 10% fetal bovine serum, 100 U/mL gentamycin, and 100 U/mL

penicillin/streptomycin (Gibco™) in a humidified incubator (5% CO2 at 37 °C) (Sanyo®, Kadoma, Osaka, Japan) until reaching 80% confluence.

Subsequently, cells were harvested by trypsinization with trypsin 0.1% EDTA (Gibco™, Invitrogen Corporation, Grand Island, USA), stained and counted in a Neubauer chamber (Optik Labor, USA) with the aid of a microscope. Indirect analysis was used for this test. A total of 900 µL of DMEM with no supplementation was added to the specimens, which were then cultured for 1 h, 24 h, 48 h, 7 days, and 28 days for the extraction of residual monomer or cytotoxic substances, according to the methodology preconized by Malkoc et al.11 The extraction method here employed is in accordance with ISO 10993 standard, part 5, on in vitro toxicity tests.

Twenty-four hours before reaching the desired exposure time, 96-well plates were prepared with a cell density of 4.5 x 105 cells per well. Each well was filled with 200 µL supplemented DMEM plus the predefined number of cells. DMEM was aspirated and the wells rinsed with 200 µL of Dulbeco's phosphate buffer saline (DPBS). Subsequently, 200 µL of the extract in contact with the specimens was aspirated, always in triplicate.

Wells containing only cell cultures (no specimens) were used as negative controls, free of toxicity; for positive control, 1% sodium hypochlorite was added to the wells. Both negative and positive controls were cultured for 24 h in an incubator.

Subsequently, the extracts were removed, and 200 µL of MTT solution was added to each well, at a concentration of 5 mg/mL. Cells were then incubated for 4 h in a humidified incubator (5% CO2 at 37 °C), and then removed. The intracellularly stored MTT was solubilized in 100 µL of dimethyl sulfoxide (DMSO) for 30 minutes at room temperature.

Optical densities were measured at a 570 nm wavelength in an ELISA™ reader (Bio-Rad Benchmark, Hercules, USA). Cell viability was calculated according to the following equation:

$$\% \text{ cell viability } (\%) = \frac{\text{Optical density in test group}}{\text{Optical density in control group}} \times 100$$

The Kolmogorov-Smirnov and Levine tests were used to assess data normality and homogeneity, respectively, with significance set at 5%. Based on the normal, homogeneous distribution obtained, analysis of variance (ANOVA) and Tukey's HSD test were used to assess differences between the groups, again at a significance level of 5%.

Results

The negative (-) and positive (+) control groups showed the lowest and highest means for cell viability results, respectively, confirming their validity as reference groups for comparison.

In intragroup analysis, no statistical differences were found in cell viability results across the different time points assessed ($p>0.05$) (Table 2). Notwithstanding, it was possible to observe reduction of cell viability with toxicity peaks: after 1 h for Fuji Ortho LC™; after 48 h for Vitro Cem™, Vidrion C™, and Multi Cure Ionomer Band Cement™; and after 7 days for Meron™ and Ultra Band Lok™. In all groups, cell viability at 28 days was higher than at all the preceding time points, except for the Fuji Ortho LC™ group.

Intergroup analysis revealed presence of toxicity in all groups ($p<0.05$), with results significantly different from the negative control group ($p<0.05$) and statistically similar to those of the positive control group, except the Meron™ at 28 days. The Meron™, Vitro Cem™, and Vidrion C™ groups (conventional GICs) showed higher cell viability results than the Multi Cure Ionomer Band Cement™, Ultra Band Lok®™, and Fuji Ortho LC™ groups (resin-reinforced GICs and composer), but this difference was not significant ($p>0.05$).

After 24 h, there was an evident reduction in cell viability among conventional GICs, differently from the findings observed for resin-reinforced GICs and for the composer, which showed a mild increase in cell viability results.

After 48 h, all groups continued to be statistically similar to the positive control group ($p>0.05$), suggesting the presence of toxicity. At this point, the Vitro Cem™, Vidrion C™, and Multi Cure Ionomer Band Cement™ groups showed peaks of cell viability reduction.

After 7 days, the Meron™ group showed the highest (peak) cytotoxicity values, based on low cell viability results. The Vitro Cem™, Vidrion C™, and Multi Cure Ionomer Band Cement™ groups, in turn, showed signs of increased cell viability, differently from Ultra Band Lok™ and Fuji Ortho LC™, which showed reduced values, suggestive of increased toxicity. Despite these differences, all groups remained statistically similar with the positive control group.

Finally, at 28 days, cell viability was evidently increased in virtually all the groups assessed (no significant differences among the groups). However, they all continued to be significantly different from the negative control group ($p<0.05$), suggesting the presence of toxicity. Of all the materials assessed, Meron™ was the only one to show signs of improvement in relation to toxicity, with significant differences also in relation to the positive control group.

Groups	1 hour	24 hours	48 h	7 days	28 days
Control (-)	0.387 ± 0.110^{Aa}	0.387 ± 0.110^{Aa}	0.387 ± 0.110^{Aa}	0.387 ± 0.110^{Aa}	0.387 ± 0.110^{Aa}
Meron™	0.111 ± 0.004^{Ba}	0.092 ± 0.001^{Ba}	0.089 ± 0.006^{Ba}	0.085 ± 0.011^{Ba}	0.229 ± 0.106^{Ba}
Vitro Cem™	0.118 ± 0.026^{Ba}	0.092 ± 0.004^{Ba}	0.056 ± 0.002^{Ba}	0.079 ± 0.010^{Ba}	0.161 ± 0.070^{BCa}
Vidrion C™	0.139 ± 0.006^{Ba}	0.090 ± 0.018^{Ba}	0.057 ± 0.001^{Ba}	0.070 ± 0.008^{Ba}	0.150 ± 0.043^{BCa}
Multi Cure Ionomer™	0.061 ± 0.008^{Ba}	0.085 ± 0.011^{Ba}	0.058 ± 0.001^{Ba}	0.081 ± 0.002^{Ba}	0.136 ± 0.040^{BCa}
Ultra Band Lok™	0.087 ± 0.006^{Ba}	0.101 ± 0.012^{Ba}	0.126 ± 0.005^{Ba}	0.079 ± 0.007^{Ba}	0.134 ± 0.022^{BCa}
Fuji Ortho LC™	0.083 ± 0.006^{Ba}	0.105 ± 0.016^{Ba}	0.126 ± 0.011^{Ba}	0.085 ± 0.022^{Ba}	0.117 ± 0.011^{BCa}
Control (+)	0.078 ± 0.003^{Ba}	0.078 ± 0.003^{Ba}	0.078 ± 0.003^{Ba}	0.078 ± 0.003^{Ba}	0.078 ± 0.003^{Ca}

Different superscript letters indicate statistical differences ($p\leq0.05$): A, $p<0.000$; B, $p<0.001$; C, $p<0.01$.

Uppercase letters refer to statistical results within columns; lowercase letters refer to statistical results within rows.

Table 2: Cell viability results (mean ± standard deviation) obtained in the different groups assessed

Discussion

In this study all cementation materials evaluated showed low values of cell viability at all times assessed being similar to positive control, except Meron™ at 28 days, suggesting that the use of these materials can be a concern to clinical application. It's important to point that this is a in vitro test representing the first step to analyse the biocompatibility.

Cell cultures have been used for over 30 years to determine the cytotoxicity of different materials.13 According to Santos et al., [3] cell culture cytotoxicity tests may also be used to assess the aggressiveness of materials used in orthodontics. In the present study, we chose to assess cytotoxicity based on the viability of cells exposed to extracts in contact with specimens fabricated from different cementation materials for different lengths of time. For that purpose, cultures of mice fibroblast (lineage NIH/3T3) were employed were employed. This cell lineage was chosen due to its similarity with cells from the lamina propria of the oral mucosa. Other studies have used other lineages, namely L9294 or human cells [14-15].

The biocompatibility of conventional GICs was described in 1991 by Nicholson & Czarnecka (2008). In that study, after 1 h and 24 h of contact between the cells and material extracts, all

groups showed toxicity when compared with the negative control group ($p<0.05$). After 1 h, conventional GICs showed higher cell viability results than resin-reinforced GICs and compomers, despite the absence of statistical differences ($p>0.05$). The presence of resin in orthodontic materials therefore seems to be associated with increased toxicity12.In agreement with those findings, the study by Sasanaluckit et al. (1993) assessed the effects of constituents released by conventional GICs on the metabolism of cells from the oral mucosa of hamsters. The authors found that the substances released by the materials were indeed able to affect the macromolecular synthesis and replication of cells. For those authors, these reactions could be associated with the release of fluoride [16].

In the same vein, Kan et al. (1997) demonstrated a relationship between toxicity and fluoride release in GICs, despite the apparent absence of a causal relationship. Notwithstanding, according to those authors, cytotoxicity cannot be explained by the release of this ion only14. Savarino et al. (2000), comparing the release of fluoride and aluminium in GICs and composers, found that GICs showed the highest release rates after 1 h and after 1 week, but especially after 1 h [17].

In the study conducted by Santos et al. [3] to assess the toxicity of resin-reinforced GICs used in the cementation of orthodontic bands, the authors found that after 1 h, 24 h, and 48 h, the materials were cytotoxic to L929 fibroblasts, except for Multi Cure Ionomer Band Cement™. The latter material showed absence of toxicity after 48 h at a statistically similar result to that of the cell growth control.

Costa et al. (2003) assessed the toxicity of restorative GICs in odontoblast lineage cells (MDPC-23) and also found that resin-reinforced GICs were more toxic than conventional GICs18. Malkoc et al. investigating resin-reinforced GICs used for the cementation of orthodontic bands in L929 cells, found that, after 24 h, all cements showed high toxicity levels when compared with the control group [11]. In contrast, Kan et al. (1997) [14] and Coimbra et al., [19] analyzing cell viability via the MTT assay in both conventional and resin-reinforced restorative GICs and in composite resin, reported minimum toxicity of these materials.

In 2010, Aranha et al. found that correct light-curing wavelength is directly related with cytotoxicity. In our study, when using light-cured cements, wavelength was constantly monitored using a radiometer and kept at 600 mw/cm2, so as to avoid any negative influence on the results. In clinical practice, insufficient light-curing is probably frequent, as the irradiation of light to cure the cement found between the band and the tooth does not always reach the whole area in contact with oral tissues. [10]

Another factor that may increase the toxicity of materials is the release of ions such as TEGDMA, UDMA, HEMA, Bis-GMA, and MMA, present in orthodontic resin adhesive systems. The release of these ions and their diffusion in oral tissues have both a cytotoxic and a genotoxic effect.10,11 In particular, HEMA is associated with increased toxicity, allergic reactions, and undesirable effects on cells. When not light-cured, HEMA solubilizes in the oral environment, affecting cell membranes and possibly inducing cell death. [13,19-26]

In the present study, after 48 h, all groups remained statistically similar to the positive control group ($p>0.05$), suggesting the presence of toxicity. The Vitro Cem™, Vidrion C™, and Multi Cure Ionomer™ groups showed their toxicity peaks at this time point. These findings are similar to those described by Sasanaluckit et al. (1993), who observed that substances released by GICs were able to affect the macromolecular synthesis and replication of cells, especially in the first 48 hours, in part due to the release of fluoride [16].

In the present study, we chose to assess the cytotoxicity of materials used for the cementation of orthodontic bands for longer periods of time, e.g., 7 and 28 days. Most of the published studies, however, report results on shorter times only, a difference that limits, at least in part, the comparison between our results and those available in the literature. Meron™ showed peak cytotoxicity values after 7 days of culture. Vitro Cem™, Vidrion C™, and Multi Cure Ionomer™, in turn, showed signs of increased cell viability at this time point, whereas the Ultra Band Lok™ and Fuji Ortho LC™ groups showed reduced viability results, suggestive of higher toxicity. Despite these differences, all groups remained statistically similar to the positive control group.

At 28 days, it became evident that cell viability was increased in all groups assessed, with no differences between them. However, the materials continued to be different from the negative control group ($p<0.05$), suggesting toxicity. Of all groups, only Meron™ showed signs of improvement with regard to toxicity, with a significant difference also in relation to the positive control group.

Sletten & Dahl (1999) assessed the cytotoxicity of 10 compomers commonly used in orthodontic practice by analyzing their extracts combined with MTT solution and found high toxicity results for all of them [24].

In 2010, Malkoc et al.[11] also tested the biocompatibility of two commercially available compomers and one resin-reinforced GIC used for the cementation of orthodontic bands. All materials were cytotoxic when compared to the negative control group, but the resin-reinforced GIC was statistically more toxic to the cells than the compomers (the latter being statistically similar between themselves).

Özturk et al. (2012) assessed genotoxicity and cytotoxicity of some cements used with orthodontic bands in cells from the human oral mucosa over 1 week and 1 month. The authors found that all materials had both genotoxic and cytotoxic effects on the cells. In the present study, Meron™ was the cement showing the least genotoxic effects among all materials assessed [25].

Finally, Its important to cite that despite this results show the toxicity of these materials, clinical studies must be made to confirm them. We would like to emphasize the importance of the in vitro tests because they are the beginning tests to analyze the biocompatibility.

Conclusions

Our findings suggest that all the cementation materials investigated were toxic to mice fibroblasts at all times assessed, regardless of their composition, representing a concern about toxicity to clinical application. Notwithstanding, a conventional GIC (Meron™) showed the best cell viability results after 28 days.

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