

# Evaluation of cytotoxicity and degree of conversion of glass ionomer cements reinforced with resin

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**SUMMARY** The objective of the present study was to evaluate the cytotoxicity and degree of monomer conversion of resin-reinforced glass ionomer cements (RGIC) over different time periods. Four RGICs: Fuji Ortho LC (FOLC), Fuji Ortho Band (FOB), Orthoglass (OGL), and Multicure Glass Ionomer (MCI) were evaluated for cytotoxicity in fibroblastic L929 cells and for their degree of monomer conversion over different time periods. Three control groups were also analysed: positive control (C+), consisting of Tween 80 cell detergent; negative control (C–), consisting of phosphate-buffered saline; and cell control (CC), consisting of cells exposed to any material. To evaluate the cytotoxicity, the dye-uptake technique was used and the degree of conversion was evaluated using infrared spectroscopy. The data obtained were analysed by analysis of variance and the Tukey's test.

The results showed cytotoxicity of the RGICs at 1 and 24 hours; the viability values of these materials were statistically different from the C– and CC groups ( $P < 0.05$ ). After 48 hours, the FOLC group was statistically similar to the CC and C– groups but different from the others. At 1 hour, there was no difference in the degree of conversion between the FOLC and OGL groups ( $P > 0.05$ ) or between the FOB and MCI ( $P < 0.05$ ) groups. However, at 48 hours, the FOLC group had greater conversion values than the other groups ( $P < 0.05$ ).

There is a direct relationship between the degree of conversion and RGIC cytotoxicity. Following initial polymerization, cytotoxicity decreases and, consequently, the degree of conversion of the material increases.

## Introduction

White spot lesions and marginal gingivitis are of concern to clinicians who are alert to new materials that minimize and prevent such damage (Romano *et al.*, 2005; Pithon *et al.*, 2006). Among these, are glass ionomer cements (GIC) developed by Wilson and Kent (1971). They bond chemically to enamel, dentine, and other surfaces as well as release fluoride (Pascotto *et al.*, 2004; Pithon *et al.*, 2006). The physical properties of GICs have contributed to a reduction of dental caries (Wilson and Prosser, 1982; McCarthy and Hondrum, 1994). Despite the favourable characteristics of these materials, their retention to dental enamel is still not adequate to resist chewing forces and orthodontic mechanotherapy (Silverman *et al.*, 1995; Varlik and Ulusoy, 2009).

However, GICs incorporating a resin matrix to combine the retention capacity with ionomer properties creates resin-reinforced glass ionomer cements (RGIC). Despite improvement in the mechanical properties, the cytotoxic effects of RGIC are more evident in comparison with those of GIC (Lan *et al.*, 2003).

Hydrophilic monomers, such as 2-hydroxyethyl-methacrylate (HEMA) and polymerization initiator (Wilson

and Prosser, 1982), were incorporated into the RGICs. Thus, the retention of the RGICs is a result of an acid–base reaction, the characteristic of the GICs, and resin monomer polymerization, that is light activated (Wilson and Prosser, 1982). The photopolymerization reaction forms the polymeric matrix, which protects the acid–base reaction from possible initial humidity contamination (Wilson and Prosser, 1982). However, sub-polymerization results in inadequate conversion of monomers into polymers and these residual monomers, on becoming liberated from the material, can cause a significant cytotoxic effect (Stanislawski *et al.*, 1999; Costa *et al.*, 2003; Siqueira Gonçalves *et al.*, 2008). The presence of non-polymerized monomers can be considered as an aetiological factor in increasing adverse immunological reactions (Macedo de Menezes *et al.*, 2009).

The degree of monomer conversion is a measure of the percentage of double bonds between the carbons that convert into single bonds during polymerization. The conversion levels reached during polymerization directly influence the physical, mechanical, and biological properties of the material (Vande Vannet and Hanssens, 2007; Jonke *et al.*, 2008; Shin and Rawls, 2009; Shinya *et al.*, 2009).

The aim of this research was to evaluate the cytotoxicity and degree of conversion of RGIC at different time points.

## Materials and methods

### Cytotoxicity

**Cell culture.** The cell line used for this study was mouse L929 (cell line mouse) fibroblasts (American Type Culture Collection-TCC, Old Town, Maryland, USA) and cultivated in Eagle's minimum essential medium (MEM; Cultilab, Campinas, São Paulo, Brazil). The cell culture was supplemented with 2-mM L-glutamine (Sigma, St. Louis, Missouri, USA), 50 g ml<sup>-1</sup> gentamicin (Schering Plough, Kenilworth, New Jersey, USA), 2.5 g ml<sup>-1</sup> fungizone (Bristol-Myers-Squibb, New York, USA), 0.25-mM sodium bicarbonate solution (Merck, Darmstadt, Germany), 10-mM HEPES (Sigma), and 10 per cent foetal bovine serum (Cultilab) and kept at 37°C in a 5 per cent CO<sub>2</sub> environment.

**RGIC evaluated.** Details of the manufactures, composition, and presentation of the four RGICs evaluated are shown in Table 1.

**Test sample preparation.** To prepare and standardize the test samples that were 5 mm in diameter and 2 mm thick, stainless steel bipartite matrices were used.

The experimental materials Fuji Ortho LC (FOLC), Orthoglass (OGL), and Multicure Glass Ionomer (MCI) were handled according to the manufacturers' instructions, that is, respecting the ratio of powder/liquid recommended and using paper blocks and a sterile plastic spatula. This procedure was not necessary for Fuji Orthobond since the material was pre-measured and dispensed.

After sample preparation, the metallic matrix was placed on a glass blade and the RGIC was injected with a syringe (Centrix, Shelton, Connecticut, USA). Once the matrix was full, a new glass blade was placed on top so the materials could subsequently be photoactivated. The photopolymerization apparatus were fixed on a rod to ensure that the distance from the specimens remained constant. An Ultralux curing light (Dabi Atlante, Ribeirão Preto, Brazil) with a lamp intensity of 550 mw cm<sup>-2</sup>, calibrated regularly with a radiometer (Demetron, Danbury Connecticut, USA), was used for 40 seconds for photopolymerization. After photopolymerization, the samples were removed from the matrices. Thirty samples of each material were immersed in the culture for post-cytotoxic evaluation and 15 were evaluated for the degree of monomer conversion.

**Controls.** To verify the cell response, three other groups were included in the study: group CC (cell control), consisting of cells not exposed to any material; group C+ (positive control), consisting of Tween 80 (polyoxyethylene 20 sorbitan); and group C- (negative control), consisting of phosphate-buffered saline (PBS) in contact with the cells.

### Assessment of cytotoxicity

The materials were previously sterilized by exposing them to ultraviolet light (Labconco, Kansas, Missouri, USA) for 1 hour. Thirty samples of each material were then placed in 24-well plates containing Eagles MEM. The culture medium was replaced every 24 hours, and the supernatants were collected after 1, 24, and 48 hours for toxicity analysis to L929 cells. The supernatants were placed in 96-well plates containing a single layer of L929 cells and then incubated at 37°C for 24 hours in a 5 per cent CO<sub>2</sub> environment. After incubation, cell viability was determined using a modification of the dye-uptake technique (Neyndorff *et al.*,

**Table 1** Materials tested with their respective manufacturers, presentation, composition, and manufacturing. FOLC, Fuji Ortho LC; FOB, Fuji Ortho Band; OGL, Orthoglass; MCI, Multicure Glass Ionomer; HEMA, 2-hydroxyethyl-methacrylate; DPICL, diphenyliodoniumchloride.

Materials tested	Manufacture	Presentation	Composition	Lot
FOLC	GC Corp., Tokyo, Japan	Pre-dosed capsules	Glass particles of fluoroaluminosilicate, co-polymers of polyacrylic acid, maleic acid, HEMA, di-2-metacriloxietil-2, 2,4-trimetyl hexamethylene dicarbamate, water, and camphoroquinone as a photo-initiator DPICL	509011
FOB	GC Corp.	Two pastes, blue and white, in pre-dosing device	The blue paste contains fluoroaluminosilicate glass, dimethacrylate, and HEMA; and the white paste polyacrylic acid, distilled water, silicon dioxide, and an initiator	304141 39460
OGL	DFL, Rio de Janeiro, Brazil	One flask of liquid and another of powder	Powder: strontium aluminium silicate, fumed silica, activators, and iron oxide; liquid: HEMA, polyacrylic acid solution, activators, co-activators, and camphoroquinone	6091426
MCI	3M Unitek, Monrovia, California USA	One flask of liquid and another of powder	Powder: glass fluoroaluminosilicate strontium, potassium persulphate, ascorbic acid, blue dye, and cherry essence; liquid: distilled water, HEMA co-polymer Vitrebond, butylate hydroxy toluene, diphenyl hexafluorophosphate camphoroquinone, and Iode	8HA/8EU

1990). After 24 hours, 100  $\mu$ l of 0.01 per cent neutral-red staining solution (Sigma) was added to each well, and these were incubated for 3 hours at 37°C to allow the dye to penetrate into the living cells. The cells were fixed using 100  $\mu$ l of 4 per cent formaldehyde solution (Reagen, Rio de Janeiro, Brazil) in PBS (130 mM NaCl, 2 mM KCl, 6 mM Na<sub>2</sub>HPO<sub>4</sub> 2H<sub>2</sub>O, 1 mM K<sub>2</sub>HPO<sub>4</sub>, pH = 7.2) for 5 minutes. Finally, 100  $\mu$ l of 1 per cent acetic acid solution (Vetec, Rio de Janeiro, Brazil) with 50 per cent methanol (Reagen) was added to the medium to remove the dye. Absorption was measured after 20 minutes using a spectrophotometer (BioTek, Winooski, Vermont, USA) at a wave length of 492 nm.

#### Analysis of degree of conversion

After polymerization, the test samples ( $n = 15$ ) were ground to obtain the ionomer powder. This was subsequently mixed with potassium bromide (KBr), at a ratio of 1/20. This powder was placed in a tablet maker under an approximate pressure of 8 tons. A spectrophotometer (Bomen—MB-102, Dawson, Yukon, Canada) was used to assay out the infrared spectrum measurements, using the Fourier transformation method, to determine the percentage degree of monomer conversion.

The following equation was used to determine the degree of conversion taking account of the fact that in the double bond of aliphatic carbon-carbon (C=C aliphatic), the infrared absorption characteristics are around 1638 cm<sup>-1</sup>, while the double bond carbon-oxygen (C=O) has an absorption value at 1720 cm<sup>-1</sup>.

$$CD = 1 - \frac{\text{Area of band C=C (polymer)/area of band C=O (polymer)}}{\text{Area of band C=C (monomer)/area of band C=O (monomer)}} \times 100.$$

#### Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences version 13.0 (SPSS Inc., Chicago, Illinois, USA). Means and standard deviations were calculated. The number of viable cells and degree of conversion were submitted to analysis of variance to determine whether statistical differences existed between the groups, and Tukey's test was applied.

## Results

#### Cytotoxicity

All RGICs were shown to be cytotoxic at 1 and 24 hours. After 48 hours, FOLC showed an absence of cytotoxicity and was not statistically different from groups C- and CC. The average values of viable cells are shown in Table 2.

#### Degree of conversion

At all time periods, FOLC and OGL showed most monomer conversion. Conversion occurred progressively up to 48

hours. The material that showed the least monomer conversion was Fuji Ortho Band (FOB), followed by MCI. The degree of conversion values of the RGICs at the different evaluation periods are shown in Table 3.

## Discussion

The use of resin components in GIC has resulted in some improvements such as a reduction in sensitivity to humidity, low mechanical resistance, and improved handling and working characteristics (Xie *et al.*, 2004; Aranha *et al.*, 2006). However the addition of components such as HEMA could give rise to cytotoxic effects (Vermeersch *et al.*, 2005; Souza *et al.*, 2006). This toxicity could be provoked by the fact that HEMA is a monomer of low molecular weight (130) and can quickly spread into the buccal fluids (Xie *et al.*, 2004).

Based on this premise, the present research evaluated the cytotoxicity of different RGICs for orthodontic use. In addition, the degree of monomer conversion of these materials was determined, as the presence of residual monomers could cause cellular and metabolic alterations (Schuster *et al.*, 2000; Xie *et al.*, 2004).

**Table 2** Statistical analysis with means and standard deviation (SD) of the number of viable cells. FOLC, Fuji Ortho LC, FOB, Fuji Ortho Band; OGL, Orthoglass; MCI, Multicure Glass Ionomer; C+, positive control; C-, negative control; CC, cell control; mean, mean values for the amount of viable cells.

Groups	1 hour		24 hours		48 hours	
	Mean (SD)	St.*	Mean (SD)	St.*	Mean (SD)	St.*
FOLC	127.4 (31.72)	A	850.3 (28.0)	A	529.8 (21.2)	A
FOB	66.4 (19.62)	B	391.1 (41.6)	B	255.1 (29.9)	B
OGL	105.2 (27.6)	A	626.2 (94.0)	C	402.4 (34.6)	C
MCI	81.66 (11.62)	B	407(34.4)	B	397.1 (60.3)	C
C+	72.66 (7.36)	B	358 (70.7)	B	215.6 (41.2)	B
C-	726.88 (7.36)	C	1086.33 (110.7)	D	555 (67.2)	A
CC	763.11 (30.09)	C	1190 (119.147)	D	561.1 (49.8)	A

St\*, same letters → no statistical difference ( $P > 0.05$ ).

**Table 3** Average values, standard deviation (SD), and degree of conversion of the resin-reinforced glass ionomer cements. FOLC, Fuji Ortho LC; FOB, Fuji Ortho Band; OGL, Orthoglass; MCI, Multicure Glass Ionomer; mean, mean values for the amount of viable cells.

Groups	1 hour		24 hours		48 hours	
	Mean (SD)	St.*	Mean (SD)	St.*	Mean (SD)	St.*
FOLC	53 (5)	A	55 (3)	A	67 (8)	A
FOB	32 (2)	B	34 (1)	B	38 (4)	B
OGL	48 (7)	A	50 (2)	A	54 (9)	C
MCI	34 ± (1)	B	36 (5)	B	52 (7)	C

St\*, same letters → no statistical difference ( $P > 0.05$ ).

Cell cultures are recommended for evaluation of the biological behaviour of materials to be placed in contact with human tissues (Jorge *et al.*, 2004; Estrela, 2005; Santos *et al.*, 2008). In this study, cytotoxicity testing was carried out using the L929 cell line (mouse fibroblasts) that is frequently used for cytotoxicity evaluation of orthodontic materials (Alcaide *et al.*, 2008; Donadio *et al.*, 2008; Feizzadeh *et al.*, 2008; Jin *et al.*, 2008; Franz *et al.*, 2009).

The neutral red analysis is a test of cell viability/survival based on the capacity of viable cells to incorporate and combine neutral red in the lysosomes. The test is carried out in adhering cells. Neutral red is a weak cationic dye but quickly penetrates into the cell membrane and accumulates intracellularly in the lysosomes (pH lysosomal < pH cytoplasmatic), where it combines with the anionic part of the matrix (Griffon *et al.*, 1995). Changes on the cell surface or on the sensitive lysosomal membrane result in lysosomal weakening. These alterations are due to xenobiotics that cause a reduction of neutral red absorption. Thus, it is possible to distinguish viable, damaged, or apoptotic cells. The quantity of dye incorporated in the cells is measured by spectrometry and is directly proportional to the number of cells with intact membranes. This method demonstrated that after 1 hour of polymerization all the materials were cytotoxic; FOB showed the greatest cytotoxicity and FOLC the least. The average viability of cells increased after 24 hours of photopolymerization; however, it was not sufficient for these materials to be considered biocompatible. After 48 hours, FOLC showed less cytotoxicity, with no statistical difference from the CC and C- groups. The OGL and MCL groups showed intermediate values of cell viability but not sufficient to be considered biocompatible, although with cell viability values greater than group C+. The FOB group demonstrated continued cytotoxicity at 48 hours. Such results could be explained by a deficiency in the polymerization process of these materials.

The degree of monomer conversion of the RGICs was evaluated together with the acquisition of the cytotoxicity results. Different methods, such as differential thermal analysis, magnetic resonance imaging, determination of magnetic resistance, and infrared spectroscopy, can also be used to determine the degree of conversion of polymer materials. Infrared spectroscopy is the most commonly used method as it directly quantifies the unreacted groups of methacrylates in the material (Franz *et al.*, 2009). Consequently, this method was chosen for the present study.

As described by Wan *et al.* (1999), the cement discs were pulverized and transformed into discs, with KBr. The RGIC/KBr mixture used was 1:20 by weight. KBr is a pure salt and is inert (transparent) to infrared spectrometry and thus when mixed with the test material no spectral line appears.

The majority of the degree of conversion studies in the literature refer to composite resins that have Bis-GMA as their main monomeric ingredient. The chemical formula of Bis-GMA has two aromatic rings and, therefore, a double

aliphatic carbon-carbon bond (open chain) and an aromatic bond. These studies refer to the use of an analytical peak of 1638  $\text{cm}^{-1}$  in relation to the double aliphatic bond and an internal standard peak of 1608  $\text{cm}^{-1}$  for the double bonds present in the aromatic ring (Cunha *et al.*, 2009; Shin and Rawls, 2009). However, when studying RGIC, Bis-GMA is not present and HEMA becomes the main monomeric component. As HEMA does not have an aromatic ring in its formula, this permits the use of an internal standard peak value of 1608  $\text{cm}^{-1}$  (Li *et al.*, 1995). Therefore, as in similar studies (Eliades and Palaghias, 1993; Li *et al.*, 1995), the internal standard wavelength used was the double bond carbon-oxygen (C=O) which is an ester at 1712  $\text{cm}^{-1}$  with a peak of 1636  $\text{cm}^{-1}$ . This was defined as the analytical reference corresponding to the methacrylate group. This technique has been used to determine the curing efficiency of non-aromatic resins (Eliades and Palaghias, 1993).

The monomer conversion values observed corroborated those found in the cytotoxicity test, showing the importance of residual monomer in cell viability, at all time periods evaluated. The FOLC group presented the greatest degree of conversion and the FOB group the least. These results suggest that as monomers convert into polymers, cytotoxicity reduces.

## Conclusions

1. There is a direct relationship between the degree of conversion with RGIC cytotoxicity;
2. After initial polymerization, cytotoxicity decreases over time and, consequently, the degree of conversion of the materials increases;
3. Over a 48-hour period, only FOLC demonstrated biocompatibility.

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