Cytotoxicity of dental glass ionomers evaluated using dimethylthiazol diphenyltetrazolium and neutral red tests

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The purpose of this study was to assess the cytotoxicity of some commonly used glass ionomers. Three chemically cured glass ionomers (Fuji II, Lining cement, and Ketac Silver) and one light-cured (Fuji II LC) were tested. Extracts of mixed non-polymerized materials and polymerized specimens were prepared in accordance with ISO standard 10993-12. The polymerized specimens were cured and placed either directly in the medium (freshly cured), left for 24 h (aged), or aged plus ground before being placed in the medium. The cytotoxicity of extracts was evaluated on mouse fibroblasts (L 929), using dimethylthiazol diphenyltetrazolium (MTT) and neutral red (NR) assays. Further, the concentrations of aluminum, arsenic and lead were analyzed in aqueous extracts from freshly cured and aged samples, and the fluoride levels analyzed in aqueous extracts from freshly cured samples. All extracts except that of non-polymerized Ketac Silver were rated as severely cytotoxic in both assays. Extracts of polymerized material were significantly more cytotoxic than extracts of non-polymerized material. All freshly cured glass ionomers released aluminum and fluoride concentrations far above what is considered cytotoxic (aluminum >0.2 ppm and fluoride >20 ppm). Extracts from freshly cured Lining Cement contained the highest concentrations of aluminum and fluoride (215 ppm and 112 ppm). Extracts from freshly cured Ketac Silver had the lowest concentrations of aluminum and fluoride but the highest of lead (100 ppm). It can be concluded that all extracts from non-cured, freshly cured, and aged glass ionomers contained cytotoxic levels of substances. Curing did not reduce the toxicity significantly. ☐ *Cytotoxicity; glass ionomers; release of toxic substances*

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Glass ionomers are widely used for cementation of inlays, crowns, bridges, and orthodontic brackets, as cavity liners, crown and core build-ups, long-term temporary restorations, and as an alternative to amalgam and composite. The main constituents of a conventional glass ionomer are Ca-Al-F-silicate glass and poly (acrylic) carbonic acids. Light-cured glass ionomers additionally contain photo-curable monomers such as 2-hydroxyethyl dimethacrylate (HEMA) or a photo-curable side chain grafted onto the poly (acrylic) acid and ion-leaching glass (1). Polymerization is initiated chemically by mixing powder and liquid and additionally by energy from blue light for the light-cured types. Dental personnel are exposed when they handle the non-cured material but also when grinding cured material. Patients are exposed to non-cured materials during dental treatment and afterwards to the cured materials for a long time. Therefore an assessment of the biocompatibility of these materials is appropriate.

Cell culture systems provide convenient, controllable, and repeatable means to initially assess the biological response (2). Oliva et al. (3) tested the effects of 5 glass ionomer cements (Ketac-Fil, Ionocap, Fuji II, Fuji II LC, and Vitremer) on adhesion, morphology, and osteocalcin expression in human osteoblasts. Four of 5 glass ionomers tested were considered biocompatible. Only Vitremer showed high toxicity, probably due to the content of HEMA and unidentified acidic species. A concentration of 1–2 mM HEMA, which was half the amount identified released from Vitremer, was reported to cause total cell death. Leyhausen et al. (4) found the light-cured Vitrebond to be highly cytotoxic, whereas the other 3 glass ionomers, 2 light cured (Compoglass and Inoseal) and 1 chemically cured (Ketac Fil), showed excellent or good cellular compatibility when measuring DNA content and cell viability. In another study measuring the behavior of human pulp cells in the contact and adjacent zone, Fuji II was found to be cytotoxic (5).

The MTT test can been used to indicate the cytotoxic effects of substances. The viability and proliferation of the cells are assessed by means of the functional state of the cell mitochondria (6). Mitochondrial dehydrogenases in living cells reduce the yellow tetrazolium salt, MTT (3-(4,5-dimethyl) thiazol-2-yl) 2,5 diphenyl-tetrazolium bromide) to blue MTT formazan, which is then retained in the cell (6, 7–9). Formation of the formazan product has been found to correlate well with number of viable cells.

The neutral red assay is a so-called vital staining procedure (10, 11). The weakly cationic, supravital dye neutral red penetrates cell membranes readily by non-ionic passive diffusion and concentrates in the lysosomes. When cells preloaded with the vital dye are exposed to toxic substances damaging the plasma or lysosomal membranes, they are less able to retain neutral red. Determination of the amount of retained neutral red in

Table 1. Glass ionomers tested and their components*

Glass ionomer	Code	Lot no.	Manufacturer	Packaging	Components
Light-cured					
Fuji II LC	FULC	091077 and 131177	GC Corp., Japan	Capsule	Powder: Aluminium silicate glass 60–100 wt-% Liquid: TEGDMA 1–5 wt-% 2-HEMA 30–60 wt-% Polyacrylic acid 10–30 wt-%
Chemically cured					
Fuji II	FUCA	301075 and 180474	GC Corp., Japan	Capsule	Powder: Aluminium silicate glass 1–5%, Polyacrylic acid 60–100% Liquid: Polyacrylic acid 30–60 wt%, Tartar acid 10–30 wt-%
Lining cement	LC	010761 (powder) 280661 (liquid)	GC Corp., Japan	Powder, liquid	No information
Ketac Silver	KS	589/067 and 593/067	Espe GmbH, Germany	Capsule	Powder: Poly Maleinated Silver Glass Ionomer Liquid: Aqueous solution containing acid

* From manufacturer's product list.

cells exposed to test compounds, compared with controls, will indicate the degree of membrane damage, and thus the relative toxicity of test chemicals can be assessed. Uptake of neutral red depends on the number of viable cells in the culture and on lysosomal function in these cells, according to Repetto & Sanz (12). The method can distinguish between viable, damaged, and dead cells, on the basis of their specific lysosomal capacity for taking up the dye.

The aims of this study were to evaluate the cytotoxic potential of extracts made from non-cured, freshly cured, and aged glass ionomers specimens and to quantify the release of some potential toxic ionic species.

Materials and methods

Data on the tested glass ionomers are given in Table 1.

Preparation of extracts

Extracts of non-polymerized products were made by extruding the mixed materials into pre-weighed, foil-covered (on the outside) sterile glass vials, re-weighing, and calculating the volume of medium required, using 0.1 g/mL medium. To minimize the problem of serum protein denaturing, serum was added to the extracts immediately before the incubation with cells.

Preparation of freshly cured and aged samples was carried out in a tent with a nitrogen atmosphere, and the samples in the molds were stored at 37°C in airtight plastic bags for 12 h to prevent exposure to oxygen. Freshly cured and aged samples were prepared in cylindrical Teflon forms, 4 mm in diameter and 6 mm high. The material was covered with a 0.046-mm-thick polyester film and then light-cured for 40 sec at each end of the cylinder, using a 3M XL3000 blue light source (3M Dental Products, St. Paul, Minn., USA). The Vivadent Silamat amalgamator 2 (Vivadent, Amherst, Fla., USA) was used for high-speed mixing of encapsulated products. Aged samples were then removed from the molds, and the ends of half of the samples were ground with a diamond dental burr (Bego GmbH, Bremen, Germany), to remove the non-polymerized surface of the materials. Freshly cured samples were removed from the forms and placed in the medium for immediate extraction.

Extracts were prepared from non-cured, freshly cured, and aged samples of all the products and in accordance with ISO 10993-12 (13), using minimal essential medium (MEM) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 mg/mL of streptomycin, and 5% fetal bovine serum (all Gibco BRL, Paisley, UK). Extracts were prepared using water grade 2, ISO 3696:1987, for the quantification of aluminum, arsenic, lead, and fluoride.

Freshly cured and aged samples were placed in foil-covered (on the outside) sterile glass vials, using 1.25 cm^2 sample surface area/mL medium. The vials were shaken in a waterbath at 37°C, non-cured samples for 24 h, and freshly cured and aged for 6 days. Controls containing medium only were treated similarly. The samples were removed, and the extracts filtered using Millex-GS sterile filters (Millipore, Molsheim, France). The extracts were equilibrated for 30 min at 37°C in 5% CO_2 to stabilize the pH. The pH was measured in samples indicating low or high pH after equilibration and adjusted to 7 by titration of 1 M NaOH or 1 M HCl.

Cell cultures

L 929 mouse fibroblasts (American Type Culture Collection CCL 1) were maintained as continuous culture in cell culture medium at 37°C, in an air atmosphere of 5% CO_2. Cells were passaged when approximately 70% confluent by treating with 0.5 g/L trypsin/0.2 g/L ethylenediaminetetracetic acid in Earle's balanced salt solution (all GibcoBRL, Paisley, UK) for 8 min. Cell viability was measured with the trypan blue exclusion test (14) before being seeded. The cells were then seeded in 96-well cell culture clusters (Costar, Corning, N.Y., USA), at a density of 15,000 cells/well, in 100 µL, and incubated for 24 h to enable attachment. After incubation, medium was aspirated from all wells and replaced with 0.1 ml extract or control medium. The plates were further incubated for 24 h, and the cytotoxicity was assessed.

MTT test

The MTT assay was carried out in accordance with Edmondson et al. (6). Twenty microliters of a solution of 5 mg/mL MTT (Sigma, St. Louis, Mo., USA) in phosphate-buffered saline (PBS) was added to each well and incubated at 37°C for 4 h in the dark. After incubation, plates were aspirated and inverted to drain MTT, and 0.1 ml 0.04 mol/L HCl in isopropanol was added to each well, to solubilize the formazan product. The plates were shaken, and absorbance was read at 570 nm using a Multiskan EX spectrophotometer (Labsystems, Helsinki, Finland).

Neutral red test

The neutral red (NR) assay was carried out in accordance with a procedure described by Borenfreund & Puerner (11). All wells were aspirated, and 0.2 mL of a 50-µg/mL neutral red solution (Sigma) in phosphate-buffered saline was added to each well and incubated at 37°C for 3 h in the dark. After incubation, plates were inverted to drain, and the cells fixed for 2–3 min each with 100 µL formol-calcium (1% anhydrous $CaCl_2$ w/v in 0.4% formaldehyde). After aspiration, 0.1 mL 1% glacial acetic acid in 50% ethanol was added to each well to destain the cells, and the plates were incubated for 15–20 min. After incubation, the plates were shaken, and absorbance read at 540 nm with a Multiskan EX spectrophotometer.

Calculations/evaluation

Each extract was tested twice both with MTT and NR assays, using 6–8 replicates for each experiment. The results from the 2 experiments were compared, using a two-tailed *t* test. The significance level was $\alpha = 0.05$. When the MTT parallels and NR parallels differed significantly, both values were used in all calculations, and if not, only one value was used. Each result was further compared with controls. Mean value was calculated together with standard deviation (*s*) for each extract. Toxicity was compared between the four glass ionomers as non-cured, freshly cured, aged, and aged plus polished materials, using a *t* test. Further, within each material toxicity was compared between non-cured, freshly cured, aged, and aged plus polished stage, using a *t* test. Additionally, the mean values per experiment were calculated and expressed as percentage of control cells. Relative to controls, extracts were rated as severely (less then 30% viable cells), moderately (between 30% and 60% viable cells), and slightly cytotoxic (between 60% and 90% viable cells) and non-cytotoxic (>90% viable cells).

Quantification

The concentration of aluminum, arsenic, and lead in extracts from freshly cured and aged samples was analyzed by using an inductive coupled plasma optical emission spectrophotometer ARL 3580 (Termo-Optec, Ecublens, Switzerland). The concentration of fluoride in extracts from freshly cured samples was analyzed by potentiometric determinations with a fluoride ion selective electrode with a silver/silver chloride reference electrode. Calibration was performed using a sodium fluoride standard solution, prepared in total ionic strength-adjusting buffer (TISAB, pH 5.3), at concentrations ranging from 19.00 µg (as F-)/L to 190.0 mg/L. Samples were diluted in TISAB by a factor of at least 10 before analysis (15). Results are expressed as ppm because this was assumed to be equal to the concentration applied to the cells.

Results

Comparing extracts with controls by means of a *t* test showed that the cytotoxicity of all extracts except Ketac Silver (non-cured) differed significantly from that of controls ($P < 0.05$) in both tests. One non-cured extract from Ketac Silver had a mean value of 1.05 (*s*, 0.08) in the NR test, compared with 1.23 (*s*, 0.4) for the control.

Comparing the cytotoxicity of non-cured glass ionomers in the MTT test showed Fuji II LC to be most toxic, followed by Fuji II > Lining cement > Ketac Silver (least toxic). All differences were significant. In the NR test Fuji II LC and Lining cement were most cytotoxic (equal values), followed by Fuji II > Ketac Silver (least toxic), with significant differences between the last two materials.

Comparing the cytotoxicity of freshly cured glass ionomers in the MTT test showed Fuji II LC and Lining cement to be most cytotoxic (equal values), followed by Fuji II > KS. In the NR test, Fuji II LC was most cytotoxic, followed by Lining cement > Fuji II > Ketac Silver. All differences were significant.

Comparing the cytotoxicity of aged glass ionomers showed Ketac Silver to be most cytotoxic in the MTT test, followed by Fuji II LC > Lining cement > Fuji II, with significant differences in cytotoxicity between the first two

Table 2. Mean values of cytotoxicity with standard deviation (*s*) from the dimethylthiazole diphenyltetrazolium (MTT) and neutral red (NR) tests, based on at least 6 replicates. (Two values are given when there was a significant difference between the two MTT or NR tests.) Within each material, cytotoxicity is compared between non-cured and freshly cured, freshly cured and aged, aged and aged + polished material

	MTT				NR			
	Non-cured	Freshly cured	Aged	Aged + polished	Non-cured	Freshly cured	Aged	Aged + polished
1. FULC	0.03 (0.01)*>	0.02 (0.003)*>	0.01 (0.003)	0.01 (0.002)	0.28 (0.02)*>	0.1 (0.002)*>	0.07 (0.01)*>	0.06 (0.01)
2. FULC					0.22 (0.02)			
1. FUCA	0.05 (0.01)*>	0.03 (0.01)*<	0.05 (0.01)*<	0.15 (0.03)	0.44 (0.02)*>	0.11 (0.02)*>	0.04 (0.01)*<	0.08 (0.01)
2. FUCA	0.03 (0.01)				0.22 (0.05)			
1. LC	0.09 (0.01)*>	0.02 (0.01)	0.02 (0.003)*>	0.01 (0.001)	0.28 (0.03)*>	0.19 (0.01)*<	0.28 (0.02)	0.23 (0.02)
2. LC	0.17 (0.03)*>				0.67 (0.01)*>	0.22 (0.01)		
1. KS	0.25 (0.03)*>	0.05 (0.03)*>	0.01 (0.002)*<	0.02 (0.004)	0.32 (0.04)*>	0.18 (0.01)*>	0.06 (0.01)*>	0.04 (0.01)
2. KS					1.05 (0.08)*>		0.04 (0.01)	

* Significantly higher or lower mean value compared with the next curing stage.

materials. Fuji II was most cytotoxic in the NR test, followed by Ketac Silver > Fuji II LC > Lining cement, with significantly differences between the last two materials.

Comparing the cytotoxicity of aged plus polished materials showed Lining cement to be most cytotoxic in the MTT test, followed by Ketac Silver and Fuji II LC (equal) > Fuji II. Ketac Silver was most cytotoxic in the NR test, followed by Fuji II > LC Fuji II > Lining cement. The differences were significant between all aged plus polished material.

The effect of setting, aging, and polishing on the cytotoxicity is shown in Table 2 for the four glass ionomers, using the *t* test for analyzing. Significant differences are marked with an asterisk. Most materials were significantly more toxic as freshly cured than as non-cured, and in both the assays.

Fig. 1 shows the mean value of cytotoxicity calculated as a percentage of controls, and for different curing stages. All extracts except non-cured Ketac Silver were rated as severely cytotoxic, with less than 30% viable cells after exposure. Curing increased the cytotoxicity in both tests.

Analysis of extracts from freshly cured and aged glass ionomers showed high release of aluminum and fluoride (Table 3). Further, the level of aluminum dropped rapidly in extracts from glass ionomers that had been post-cured for 24 h before extraction.

Discussion

Leachables from glass ionomers may have effects on cell viability and function. Some chemicals may selectively affect the mitochondria of the cells and be detected in the MTT assay, and some may selectively affect the lysosomal function and be identified in the NR test. The results of the MTT and the NR assays were similar for most components tested in L929 cells, but the parallels in the NR test varied more widely. All glass ionomers except Ketac Silver were rated as severely cytotoxic even before setting, with less than 30% viable cells relative to controls in both MTT and NR assays. In both the MTT and NR tests, curing increased cytotoxicity in extracts from 3 of 4 glass ionomers, but in 1 extract from Fuji II curing reduced cytotoxicity. Sletten & Dahl (16) reported that curing reduced cytotoxicity in extracts from 7 of 9 compomers, assessed with the MTT test.

One factor contributing to the observed cytotoxicity might be the release of fluoride. A correlation between fluoride release and cytotoxicity has been observed by

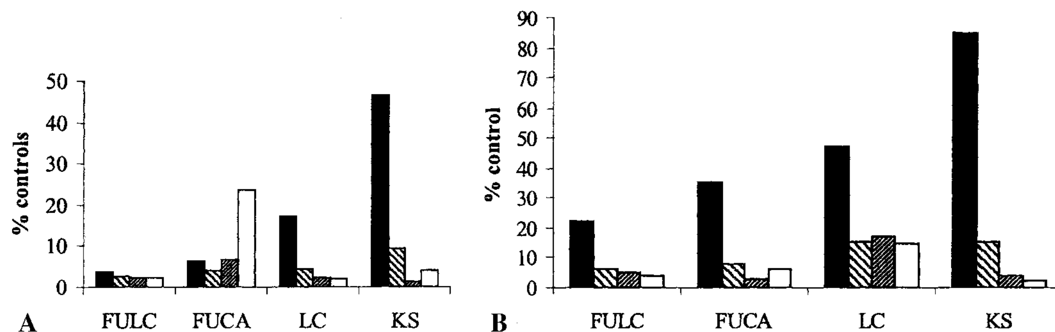

Fig. 1. Cytotoxicity expressed as mean value in percentage of control (based on at least 6 replicates). For each product the first bar represent non-cured material, the second freshly cured material, the third aged material, and the fourth aged plus polished material. A. Cytotoxicity assessed with the dimethylthiazol diphenyltetrazolium test. B. Cytotoxicity assessed with the neutral red test.

Table 3. The content (in ppm) of aluminum, arsenic, lead, and fluoride in aqueous extracts from four glass ionomers. Results based on one analysis per material

Glass ionomer	Aluminum		Arsenic		Lead		Fluoride
	Freshly cured	Aged	Freshly cured	Aged	Freshly cured	Aged	Freshly cured
Fuji II LC	29.5	2.0	*	*	0.03	*	47.5
Fuji II	39.2	0.04	*	*	*	*	44.8
Lining Cement	215.0	14.8	*	*	*	*	112.0
Ketac Silver	13.3	<0.05	*	*	100.0	*	35.5

* <0.01 ppm (detection limit).

Khalil & Da'dara (17), reporting that high doses of fluoride inhibited cell division and caused death of rat bone marrow cells. NaF induced dose-dependent cytotoxicity in cultured human proximal tubular cells (18). A concentration of about 1 mM fluoride (equivalent to 19 ppm) was cytotoxic to human epithelial cell at pH 7.3, and at lower pH, the cytotoxicity of fluoride increased (19). Glass ionomers provided a sustained fluoride release (20–23), and Hörsted-Bindslev & Larsen (24, 25) reported concentrations between 15 and 21 ppm after extraction in water for 24 h. The fluoride concentrations measured in the present study were much higher.

Glass ionomers also release aluminum, especially during early water contact (26), and chemically cured materials released more aluminum than light-cured ones (26), which to some extent also was confirmed in this study. The cytotoxicity of aluminum is suggested to occur through interaction with specific membrane lipids (27). Aluminum cytotoxicity has been observed at a concentration of about 0.2 ppm in cultured L929 mouse fibroblasts (28). In the present study the aluminum concentration in extracts of all freshly cured glass ionomers and of 2 of the aged glass ionomers exceeded the cytotoxic level observed for mouse fibroblasts. Aluminum release could be another factor contributing to the observed cytotoxicity.

Øilo (29) reported that glass ionomers might contain up to 2 mg/kg acid-soluble arsenic and 50 mg/kg lead. Analysis of the water extracts in this study showed no detectable levels of arsenic, but two materials released lead. Lead is considered more toxic than aluminum (30), and the extract of freshly cured Ketac Silver contained a lead concentration far above what is considered to be the toxic level for aluminum. Studies have reported that glass ionomers also release small amounts non-polymerized monomers (32, 33). The cytotoxicity of the released monomers varies; for example, TEGDMA was severely cytotoxic in 3T3 cells, whereas 2-HEMA was slightly cytotoxic (34). Fuji II LC contains both 2-HEMA and TEGDMA, which may be one reason for the high cytotoxicity.

In vitro studies are simple and inexpensive to perform, provide a significant amount of information, can be conducted under controlled conditions, and may elucidate the mechanisms of cellular toxicity, but it is difficult to predict the in vivo toxicity from such data.

It can be concluded that extracts of non-cured, freshly cured, and aged glass ionomers were cytotoxic and that the mechanisms of action in cultured cells include interference with both the cellular membranes and the mitochondrial action. A possible clinical implication of such findings must be given careful consideration, as it is difficult to predict the in vivo toxicity from such data. The clinical situation is different in that the leachables from a dental filling are diluted in the saliva and that the surface of the mucosa acts as a diffusion barrier against toxic substances. A thorough evaluation of the biocompatibility of dental material needs clinical or animal investigations. The increasing public concern about the use of animals in toxicity testing makes the use of in vitro testing in the evaluation of biocompatibility more practical, but it is necessary to be aware of the limitations.

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