Cytotoxicity of liquids and powders of chemically different dental materials evaluated using dimethylthiazol diphenyltetrazolium and neutral red tests

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The purpose of this study was to assess and compare the cytotoxicity of liquid and powder components of chemically different dental materials using 2 basic unspecific cell culture methods. Three chemically cured glass ionomers (Fuji II, Lining cement, and Ketac Silver), 1 light-cured glass ionomer (Fuji II LC), and 2 chemically cured acrylates (Swedon and Super Bond) were tested. The liquids were diluted 1:10 in cell culture medium. The liquids from chemically cured acrylates were further diluted 1:100, 1:1000, and 1:10000. Extracts were made by incubating the powders in cell culture medium for 24 h at 37°C according to the ISO standard 10993-12. The cytotoxicity was assessed in transformed mouse fibroblasts (L-929) using two viability assays, dimethylthiazol diphenyltetrazolium (MTT) and neutral red (NR). Severe cytotoxicity was observed when testing powder extracts of Swedon, Fuji II, and Lining cement, whereas powder extracts of Ketac Silver, Fuji LC, and Super Bond induced slight to non-cytotoxicity. All of the 1:10 liquid dilutions were severely cytotoxic in the MTT assay. In the NR assay, however, four 10% dilutions were severely cytotoxic and 4 moderately cytotoxic. Further dilution of the liquids of the chemically cured acrylates reduced the toxicity, while the Super Bond catalyst was severely cytotoxic even as the 1:100 dilutions. □ *Chemical cured acrylates; cytotoxicity; liquids; powders; glass ionomers*

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Dental personnel consider chemically cured acrylates to be the most harmful materials to handle and glass ionomers to be less risky, according to a Swedish study (1). Both chemically cured acrylates and glass ionomers are commonly used and their application varies from cementing prosthetic constructions and orthodontic brackets to core build-ups and temporary restorations.

Dental personnel handle the different components prior to mixing, and materials with characteristic and strong smell are probably handled with more care than non-smelling products, as they are regarded as more hazardous (1). This might not reflect the biological hazard of the products and an assessment of the biocompatibility of these chemically different materials is therefore appropriate.

Cell culture systems provide convenient, controllable, and repeatable means to initially assess the biological response of a given chemical (2). The dimethylthiazol diphenyltetrazolium (MTT) test can been used to indicate cytotoxic effects by assessing the functional state of the cell mitochondria after exposure to chemicals (3). 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 (3, 4–6). Formation of the formazan product has been found to correlate well with number of viable cells.

The neutral red (NR) test is also a viability test, a so-called vital staining procedure (7, 8). 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 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 the lysosomal function in these cells, according to Repetto & Sanz (9). The aim of this study was to assess and compare the cytotoxicity of liquid and powder components of some chemically different dental materials.

Materials and methods

Chemically different materials were selected based on what was considered very hazardous/less hazardous to handle in dental clinics (1). Data on the tested materials are given in Table 1.

Preparation of extracts

Extracts were prepared from the powder components of all products according to ISO 10993-12 (10) using minimal essential medium (MEM) supplemented with

Table 1. Dental materials tested and their components*

Material	Code	Lot no.	Manufacturer	Packaging	Components
Chemically cured acrylates					
Swedon	SW	219609 (powder), 049510 (liquid)	Svedia Dental Industri AB, Sweden	Powder, liquid	Powder: Polymethylmethacrylate (PMMA) 80–100% Benzoic acid 1–5% Benzoyl peroxide 1–5% Monomer liquid: Methylmethacrylate (MMA) 30–10% Etandiol-1, 2-dimethacrylate 5–10% Dimethyl-p-toluidin 0–1% p-Metoxifenol 0–1%
Super Bond	SB	70901 (powder), 70903 (liquid)	Sun Medical Co. Ltd, Japan	Powder, liquids	Powder: Polymethylmethacrylate (PMMA) 60–100 wt% Monomer liquid: Methylmethacrylate (MMA), 4-Methacryloxyethyl trimellitate anhydride
Super Bond	SBc	711011 (catalyst)	Sun Medical Co. Ltd, Japan	Liquid	Catalyst liquid: Partial oxidized tri-n-butyl-borane (TBB) >50%
Chemically cured glass ionomers					
Fuji II	FU	301075 and 180474	GC Corporation, 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 Corporation, 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
Light-cured glass ionomer					
Fuji II LC	FULC	091077 and 131177	GC Corporation, Japan	Capsule	Powder: Aluminium silicate glass 60–100 wt% Liquid: TEGDMA 1–5 wt% 2-HEMA 30–60 wt% Polyacrylic acid 10–30 wt%

* From manufacturer's product list.

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 shaken for 24 h in water-bath at 37°C, then filtered using Millex-GS sterile filter (Millipore, Molsheim, France). The extracts were equilibrated for 30 min at 37°C in 5% CO_2 to stabilize the pH. Extracts indicating low or high pH after equilibration were adjusted to pH 7 by titration of 1M NaOH or 1M HCL. A 30% liquid solution was prepared by mixing polyacrylic acid in distilled water. The liquids were diluted to 1:10 in 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). The liquids of chemically cured acrylates were further diluted to 1:100, 1:1000, and 1:10,000 because it was observed that the 10% dilutions corroded the vials used. Data were discarded and the tests repeated using glass vials for all experiments. It was also observed that the 1%, 0.1%, and 0.01% liquid dilutions had to be tested separately because the 10% dilutions affected the nearby rows resulting in higher toxic response than expected. Each extract and dilution was tested at least twice both with MTT and NR assays, using 8 replicates for each experiment.

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 ethylene diamine tetracetic acid in Earle's balanced salt solution (all GibcoBRL, Paisley, UK) for 8 min. Cell viability was measured using the trypan blue exclusion test (11) before seeding. The cells were then seeded in 96-well cell culture clusters Costar® (Corning, New York, USA) at a density of 15,000 cells/well, in 100 µL, and incubated for 24 h to allow attachment. After incubation, medium was aspirated from all wells and replaced with 0.1 mL extraction or control medium. The plates were further incubated for 24 h and the cytotoxicity was assessed.

MTT test

The MTT assay was carried out according to Edmondson et al. (3). A 20 µL solution of 5 mg/mL MTT (Sigma, 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, the medium was aspirated and the plates 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 assay was carried out according to a procedure described by Borenfreund & Puerner (8). The

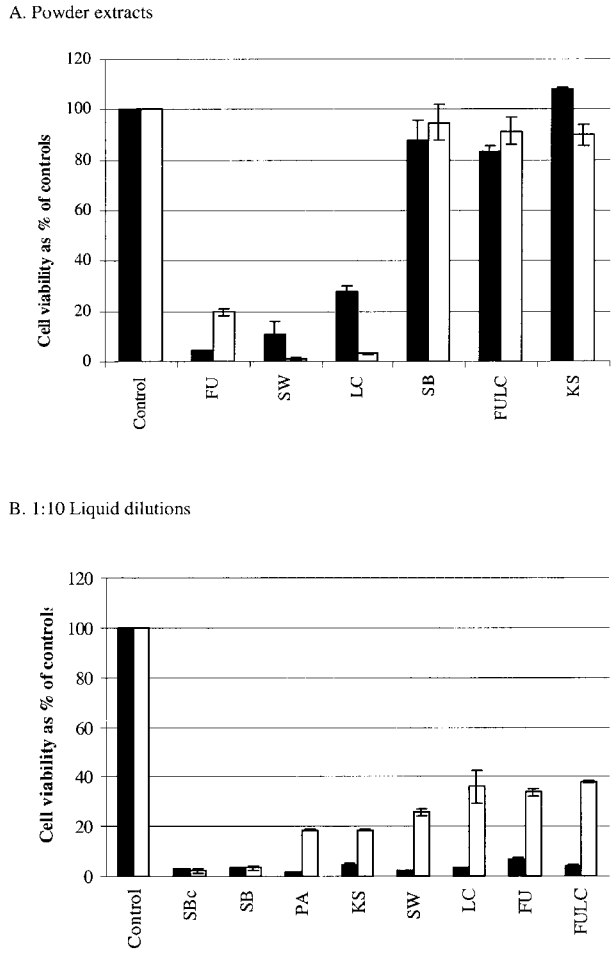

Fig. 1. Cytotoxic effects of chemically different dental materials demonstrated using MTT assay (black bars) and NR assay (white bars). Material tested: Fuji II (FU), Swedon (SW), Lining Cement (LC), Super Bond liquid, and powder (SB), Super Bond catalyst (SBc), Fuji II LC, (FU LC), Ketac Silver (KS), and Polyacrylic acid (PA). A. Cytotoxicity of powder extracts based on 2×8 replicates. B. Cytotoxicity of 1:10 liquid dilutions based on 2×8 replicates. Results presented as cell viability in percent of controls with mean values and standard deviations.

test solutions were aspirated and 0.2 mL of a 50 μg/mL neutral red solution (Sigma, Mo., USA) in phosphate-buffered saline was added to each well and incubated at 37°C for 3 h in 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 using a Multiskan EX spectrophotometer.

Calculations/evaluation

Mean test absorptions were calculated and expressed as a percentage of control cells together with standard deviation (*s*). Each value represents the mean of 2× at least 8 replicates. The results from the 2 experiments were compared using a *t* test. Each result was further compared with controls. The significance level was $\alpha = 0.05$. Extracts were rated as severely, moderately, slightly, or not cytotoxic where activity relative to controls was less than 30%, between 30% and 60%, between 60% and 90%, or greater than 90%, respectively. Further, the materials were ranked according to toxicity.

Results

Three of the powder extracts were severely cytotoxic (Fuji II, Swedon and Lining cement), while 3 powder extracts (Ketac Silver, Fuji LC, and Super Bond) were slightly to non-cytotoxic assessed by the MTT and NR assays. Ranking of the powder extracts, Fuji II was most cytotoxic, followed by Swedon and Lining cement in the MTT assay, while Swedon was most cytotoxic in the NR test, followed by Lining cement and Fuji II (see Fig. 1A).

The 10% dilutions of chemically cured acrylates corroded the vials used. Thus, data were discarded and new experiments carried out using glass vials. It was also observed that testing different dilutions in the same well, 10% dilutions of chemically cured acrylates influenced the results of nearby materials resulting in higher cytotoxic response, compared to testing the material separately. Thus, additional tests were carried out without 10% dilutions in the same well. All 1:10 dilutions were severely cytotoxic in the MTT assay. The 10% liquid dilutions were ranked according to cytotoxicity as: Polyacrylic acid > Swedon > Super Bond catalyst > Lining Cement > Super Bond monomer > Fuji LC > Ketac Silver > Fuji II in the MTT assay. In the NR assay. however, 10% dilutions of Super Bond catalyst, Super Bond monomer, Polyacrylic acid, and Ketac Silver were rated as severely cytotoxic and the others as moderately cytotoxic. The 10% dilutions were ranked as: Super Bond catalyst > Super Bond monomer > Polyacrylic acid, and Ketac Silver > Swedon > Fuji II > Lining cement in the NR assay (see Fig. 1B).

Cell viability was calculated and expressed as a percent of control cells for 1:10, 1:100, 1:1000, and 1:10,000 liquid dilutions of chemically cured acrylate. Super Bond catalyst was severely cytotoxic as 10% and 1% dilutions, while monomer liquids of Swedon and Super Bond were severely cytotoxic only as 10% dilutions in both the MTT and NR assays. Some dilutions, particularly 1%, 0.1%, and 0.01% of Super Bond monomer, enhanced the cell activity (see Table 2).

Discussion

Cytotoxicity of powder and liquid components of chemically different dental materials were studied in

Table 2. Mean values of cytotoxicity with standard deviation (*s*) from the dimethylthiazol diphenyltetrazolium (MTT) and neutral red (NR) tests, based on 2 × 8 replicates. Results presented as cell viability as percent of controls

		MTT		NR	
	Dilutions	Test I	Test II	Test I	Test II
SW monomer liquid	10%	2.3 (*0.01*)	2.7 (*0.01*)	23.2 (*0.04*)	27.9 (*0.06*)
	1%	97.3 (*0.06*)	98.4 (*0.05*)	41.5 (*0.05*)	50.4 (*0.01*)
	0.1%	101.3 (*0.05*)	103.9 (*0.05*)	92.6 (*0.05*)	99.9 (*0.1*)
	0.01%	114.1 (*0.03*)	116.5 (*0.07*)	98.1 (*0.04*)	98.9 (*0.1*)
SB monomer liquid	10%	3.4 (*0.004*)	3.6 (*0.005*)	3.1 (*0.004*)	3.2 (*0.02*)
	1%	110.5 (*0.07*)	111.6 (*0.05*)	112.6 (*0.06*)	142.4 (*0.05*)
	0.1%	102.1 (*0.07*)	105.3 (*0.03*)	112.1 (*0.06*)	115.0 (*0.05*)
	0.01%	106.9 (*0.03*)	115.9 (*0.04*)	90.7 (*0.2*)	115.0 (*0.05*)
SB catalyst liquid	10%	2.6 (*0.01*)	2.9 (*0.01*)	2.5 (*0.01*)	1.6 (*0.01*)
	1%	1.2 (*0.01*)	3.3 (*0.01*)	1.1 (*0.01*)	1.3 (*0.01*)
	0.1%	80.0 (*0.1*)	83.0 (*0.06*)	89.6 (*0.1*)	91.5 (*0.1*)
	0.01%	90.2 (*0.06*)	94.4 (*0.05*)	86.6 (*0.1*)	94.8 (*0.1*)

L929 mouse fibroblasts using the integrity of mitochondria and lysosomes as the end-points. The MTT assay detects living cells based on the amount of formazan formation in the mitochondria, and the NR assay detects living cells based on the amount of neutral red uptake in the lysosomes. The MTT and NR assays exhibited almost identical reaction patterns for all extracts of powder components in L929 cells by identifying the same three powder extracts as severely cytotoxic, and the other as slightly to non-cytotoxic.

The liquid dilutions, however, seemed more to selectively affect the mitochondria of the cells, because the response was in general more pronounced in the MTT than in the NR assay. All 10% dilutions had a strong effect on the cells with less than 10% viable cells after exposure, according to the MTT tests, but in the NR test, three liquids were identified as moderately toxic (Lining cement, Fuji LC, and Fuji II), the other was severely cytotoxic. Lining cement 10% dilutions differed in the NR test, but both tests were classified as moderately cytotoxic. The 10% dilutions of chemically cured acrylates not only corroded the vials used, they also affected the nearby rows, probably due to release of vapor. When testing the 1%, 01%, and 0.01% dilutions separately, lower toxic responses were observed.

Dilution of the 10% liquids resulted in lower toxic response, except for Super Bond catalyst. The high cytotoxicity of Super Bond catalyst can be explained by the content of tri-n-butyl-borane, classified as hazardous, toxic, and with the potential to cause burns, according to the Material Safety Data Sheet, and the content of 4-META. Polyacrylic acid was even more toxic than Super Bond catalyst to the mitochondria function of the cell. Results also indicated that dilutions, e.g. some monomer dilutions, might stimulate cell activity.

The differences in toxicity between powder extracts might be explained by the composition and the ability of the constituents to leach out into an aqueous solution. Fuji II powder contains polyacrylic acid, shown in this study to be seriously cytotoxic. Swedon powder contains Benzoyl peroxide and Benzoic acid, both with well-known irritation and sensitization potential.

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 exposure to 10% liquid dilutions based on polyacrylic acid (no odor) and exposure to monomer liquids or catalyst of chemically cured acrylates (with strong characteristic odor) produced a similar severe response to the mitochondria function of the cells. The cell membranes were severely affected by exposure to half of the dilutions, and moderately affected by exposure to the others. Results indicate that more detailed cell culture studies are needed to clarify the biocompatibility of dental material.

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