Cytotoxic effects of extracts of compomers

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We have studied the cytotoxicity of 10 commercially available compomers. Extracts were taken in cell culture medium of non-cured, freshly-cured, and aged samples. Murine L-929 fibroblasts were exposed to the extracts for 24 h and the cytotoxicity was evaluated using dimethylthiazol diphenyltetrazolium (MTT) assay and neutral red uptake (NRU). Extracts were rated as severely, moderately, or slightly cytotoxic when the activity relative to controls was less than 30%, between 30% and 60%, or greater than 60%, respectively. Extracts of non-cured materials were rated severely toxic with both methods, with one exception. All but one freshly-cured material exhibited moderate to severe toxicity in both assays. Aged test specimens were rated moderately to severely toxic. Non-cured materials were generally more toxic than cured, with two exceptions. Aging and polishing the samples to remove non-polymerized surface film affected cytotoxicity to a varying degree. Curing reduced cytotoxicity in the MTT test from severe to moderate in 7 of 9 materials, but had relatively little effect in the NRU assay. Aging and polishing, however, had little effect on cytotoxicity evaluated by the MTT test, but markedly reduced cytotoxicity in NRU in 6 of 8 extracts. To conclude, extracts made from compomers used for dental fillings were found to be cytotoxic both before and after setting. □ *MTT test; neutral red uptake*

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It is suggested that compomers, or polyacid-modified composite resins, combine beneficial adhesion properties, fluoride release, and biocompatibility of glass-ionomers with the convenience of light-curing composites (1, 2). A limited number of reports on compomers have been published, the majority addressing the physical properties. They have been compared with composites, conventional glass-ionomers and resin-modified glass-ionomers with respect to setting and mechanical properties, water sorption, and fluoride release. Compomers resemble composites, but their performance is inferior (3, 4). However, they have superior mechanical properties compared to glass-ionomers (4). They show similar levels of long-term fluoride release to glass-ionomers (5, 6) and there is evidence of the release of residual monomers and additives (7, 8).

Data from biocompatibility studies of compomers are scarce. Dyract® exhibited only minor inflammatory reactions when applied as an intraosseous implant (9). Compoglass® and Dyract were rated as non-irritants to primary odontoblasts and pulp tissues when placed in class 5 cavity preparations (10). Extracts in medium from freshly-cured and aged samples of Compoglass did not affect proliferation of HGF and 3T3 cells measured using a DNA- intercalating stain, and the growth of HGF was unaffected by direct contact with Compoglass (11). However, in another study, Compoglass and Dyract extracts in serum-free medium had a moderately toxic effect on 3T3 cell proliferation measured using a DNA-intercalating stain (7). Luxat® caused a reduction in cell number but no lysis in L-929 cells using the agar overlay test, and both Ionosit® and Luxat caused mild local inflammation when implanted into muscle, but no inflammation in oral mucosa (product documentation, DMG Chemisch-Pharmazeutische Fabrik GmbH).

Cell culture systems provide convenient, controllable, and repeatable means for an initial assessment of biological responses (12). There are a number of cytotoxicity tests available measuring a variety of parameters. The toxicity parameters should ideally be appropriate to the chemical nature of the test material (13). Hydrophilic substances are more likely to affect intracellular enzyme and energy-consuming functions, and their effects should be assessed by using a functional assay like the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test (14). The ability of cells to reduce the tetrazolium salt, MTT, to the formazan product indicates mitochondrial activity, which is seen only in living cells (15). The formazan product accumulates within the cell, being unable to cross the plasma membrane. Determination of formazan production in cells exposed to test compounds, compared with controls, enables the relative toxicity of test chemicals to be assessed. Lipophilic substances are more likely to disrupt membrane integrity, therefore a permeability assay such as neutral red uptake (NRU) is more useful. The NRU assay is a so-called vital staining procedure (16, 17). Viable cells incubated in the presence of NR take up and retain the dye compound. On exposure to substances damaging the plasma or lysosomal membranes, the cell no longer retains NR. Determination of the amount of retained NR in cells exposed to test compounds, compared with controls, enables the relative toxicity of test chemicals to be assessed. NRU has been applied to ranking cytotoxicity of chemicals according to

Table 1. Investigated compomers and their components*

Compomer	Code	Lot no.	Manufacturer	Components
ANA-compomer	AC	Batch no. 97300033	Nordiska Dental, Sweden	Methacrylates (UEDMA) (10–20%) Methacrylated phosphoric acid (1–5%) Glass ionomer (81%)
Compoglass	CO	Lot no. 909415	Vivadent, Liechtenstein	UDMA (5–10%) [70 mg/g] BisGMA (1–5%) [30 mg/g] TEGDMA [40 mg/g] HEMA CADCADM-cycloaliphatic dicarboxylic acid dimethacrylate [60 mg/g] Silanized barium fluorosilicate [630 mg/g] Silanized spheroidal mixed oxides [60 mg/g] YbF_3 [100 mg/g]
Dyract	DY	Lot no. 9712000297	Dentsply De Trey, Germany	Methacrylates: dicarboxylic acid dimethacrylate (UDMA, TEGDMA) (25%) Strontium fluorosilicate glass PENTA: dipentaerythritol pentaacrylate monophosphate
Dyract-AP	DP	Lot no. 9803001061	Dentsply De Trey, Germany	Methacrylate M (10–30%) glass particles (60–100%)
F-2000	FT	Lot no. 19970926	3M Dental Products, MN, USA	Fluoro-aluminium silicate glass (65–85%) 2-hydroxy-1,3-dimethacryloxy- propane CDMA oligomer (7–10%) 2-(2-hydroxy-5-methylphenyl)-benzotriazol (0.05–0.15%) BisGMA (0.005–0.01%)
Freedom	FR	Lot no. 70932	Southern Dental Industries, Australia	Dimethacrylate resin Camphorquinone Tetramethylaniline Butylated hydroxytoluene Fluoride glass
Hytac Aplitip	HY	Lot no. FW0042872	Espe, Germany	UDMA (60–100%) Aliphatic dimethacrylate Aromatic dimethacrylate Polycarboxyl polymethacrylate Glass, silica
Ionosit Seal	IO	Lot no. 97110296	DMG, Germany	
Luxat	LU	Lot no. 97310013	DMG, Germany	Ionomer glass (81%) UDMA (12%) Methacrylated phosphoric acid (2.5%) Maleic acid ester (2.5%) Silica (1%)
Primaflow	PR	Lot no. 98150170	DMG, Germany	Ionomer glass and silica silan. 64,28% Resins (UDMA, dodecanediol dimethacrylate, mono-methacryloyloxyethyl maleate, methacrylated phosphoric acid) (35, 11%)

* Product information provided by the manufacturers ( ) or adapted from [11].

potency, and to elucidate their structure-toxicity relationships (18). These studies have demonstrated that different cell types may vary in sensitivity to test agents, but that the overall ranking remains equivalent. In a study where a number of materials are to be compared and where identities and amounts of leachables are uncertain, it is an advantage to use more than one test (19). In this study, the MTT test and NRU have been employed to assay the cytotoxic effects of extracts made from compomers on L-929 cells, in order to elucidate the cytotoxic potential of these materials.

Materials and methods

Preparation of extracts

The products tested in this study (VITA shade A2, VITA Zahnfabrik, Germany) are listed in Table 1. Extracts were prepared from non-cured, freshly-cured, and aged samples of all the products. Freshly-cured and aged samples were prepared in cylindrical metal molds 4 mm in diameter and 6 mm high. The material was covered with 0.046-mm thick polyester film to exclude air, then light-cured for 40 sec at each end of the cylinder,

using a 3M XL3000 blue light source (3M Dental Products, MN, USA). Freshly-cured samples were removed from the molds and transferred immediately to the extraction solution. The preparation and curing of aged samples were carried out in a tent with a nitrogen atmosphere and the samples, in the molds, were stored at 37°C in air-tight plastic bags for 8 h to prevent exposure to oxygen. The samples were then removed from the molds and the ends ground using a sintered diamond dental burr (Bego GmbH, Germany) in order to remove any non-polymerized material.

Extracts were prepared in accordance with ISO 10993-12 (20) using minimal essential medium (MEM) supplemented with 100 units/mL of penicillin, 100 mg/mL of streptomycin, 2 mM L-glutamine, and 5% fetal bovine serum (all GibcoBRL, Paisley, UK) as the extraction medium. Extracts of non-cured materials were made by extruding the materials into pre-weighed, foil-covered sterile glass vials, re-weighing and calculating the volume of medium required using 0.1 g material per mL medium. Freshly-cured and aged samples were washed in distilled water and placed in foil-covered sterile glass vials using 1.25 cm^2 sample surface area/mL medium. The vials were shaken in a water-bath at 37°C, non-cured samples for 24 h and freshly-cured and aged for 5 days. Control samples containing medium only were treated similarly. The specimens were removed and the extracts filtered using Millex-GS sterile filters (Millipore, France) to ensure sterile conditions.

Cell culture

L929 mouse fibroblasts (American Type Culture Collection CCL 1) were maintained in continuous culture in MEM supplemented with 100 units/mL of penicillin, 100 mg/mL of streptomycin, 2 mM L-glutamine, and 5% fetal bovine serum, at 37°C in an air atmosphere containing 5% CO_2 and 95% relative humidity. 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, UK) for 8 min. Cell viability was measured using the trypan blue exclusion test (21). The cells were then plated in 96-well cell culture clusters (Costar, USA) at a density of 15,000 cells/well, in 100 µL, and incubated for 24 h to allow attachment. The medium was replaced with 100 µL test extract or control medium, which had been equilibrated in air/5% CO_2 at 37°C for 30 min. After 24 h incubation, the cytotoxicity was assessed.

MTT test

The MTT assay was carried out according to Edmondson et al. (14); 20 µL of a solution of 5 mg/mL MTT (Sigma, MO, USA) in warm phosphate-buffered saline (PBS) was added to each well. The cells were incubated in darkness for 4 h at 37°C and the plates inverted and blotted on paper to remove excess MTT medium; 100 µL 0.04 M HCl in isopropanol was added to solubilize the formazan product, the plates were shaken, and absorption at 570 nm read using a Multiskan EX spectrophotometer (Labsystems, Finland).

Neutral red uptake

The NRU assay was carried out according to a procedure described by Borenfreund and Puerner (16). All wells were aspirated, and 200 µL of 50 µg/mL NR (Sigma, MO, USA) in phosphate-buffered saline was added to each well. After 3 h incubation at 37°C in darkness, all wells were aspirated and the cells fixed for 2–3 min each with 100 µL formol-calcium (1% anhydrous $CaCl_2$w/v in 0.4% formaldehyde). All wells were aspirated again and incubated with 100 µL 1% glacial acetic acid in 50% ethanol for 15–20 min, to destain the cells. The plates were shaken and absorption at 540 nm read, using a Multiskan EX spectrophotometer (Labsystems, Finland).

Data processing and evaluation of cytotoxicity

Mean test absorptions were calculated and expressed as a percentage of control cells. Each value represents the mean of 3 experiments for non-cured and aged materials, and 4 experiments for freshly-cured materials, using at least 6 replicates of each extract per experiment. Extracts were rated as severely, moderately, or slightly cytotoxic, where activity relative to controls was less than 30%, between 30% and 60%, or greater than 60%, respectively. Statistical analysis using a one-way variance (ANOVA) was carried out. Differences were considered to be significant at a *P*-value of less than 0.05.

Results

Fig. 1 shows the cytotoxicity of extracts of non-cured, freshly-cured, and aged samples of 10 commercially available compomers, using NRU and the MTT tests. The cytotoxicity of all extracts of both non-cured and freshly-cured materials differed significantly from controls ($P < 0.05$). Extracts of all non-cured materials were rated severely toxic, with activities of less than 10% of controls as assessed using both methods, with the exception of Freedom, which was slightly toxic. Extracts of freshly-cured materials exhibited moderate to severe toxicity in both assays, except Freedom, which was slightly toxic in the MTT test. Aged Ionosit and F2000 were rated severely toxic in both tests, and Hytac in the MTT test. All the other extracts were rated moderately toxic, except that of Primaflow, which was rated slightly toxic.

Aging the samples and removal of non-polymerized surface film affected the cytotoxicity of the extracts to a varying degree, and the results in Fig. 1 are grouped in order to reflect this observation. The cytotoxicity rating was changed from severely cytotoxic in the non-cured state to moderately or slightly cytotoxic, in both tests, for ANA-

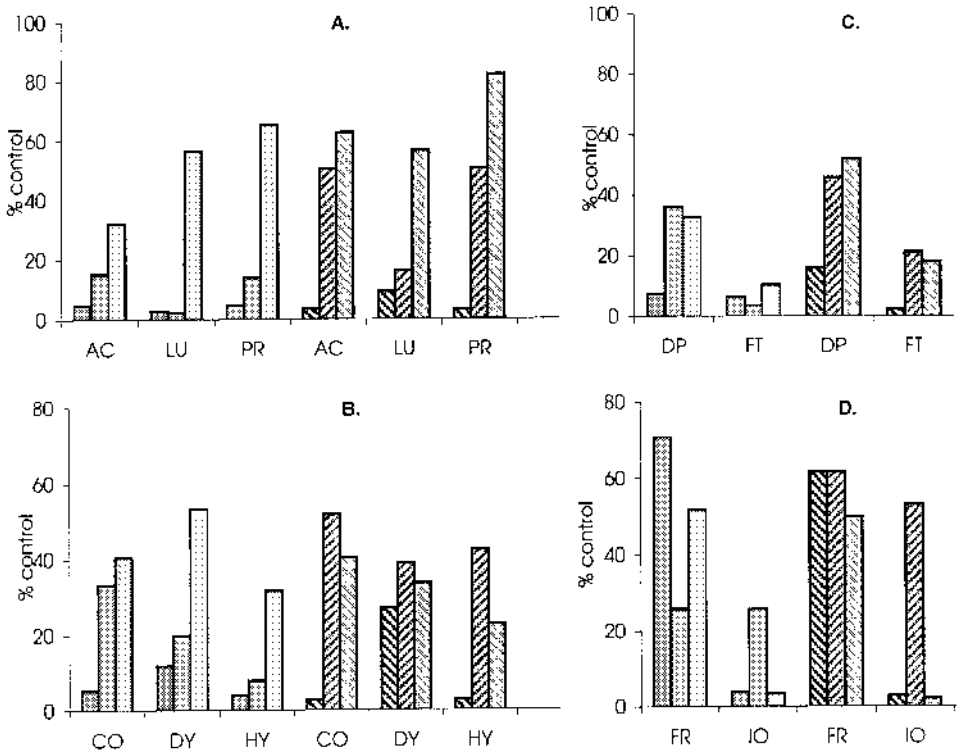

Fig. 1. Cytotoxic effects of 10 compomers, demonstrated using neutral red uptake (solid bars) and MTT test (hatched bars). For each product, the first bar represents non-cured material, the second freshly-cured material, and the third aged and polished material. The materials are grouped according to the effects of aging and polishing on the cytotoxicity. A. Reduced cytotoxicity in both tests. B. Reduced cytotoxicity in NRU, increased or unaltered in MTT test. C. No significant alteration in either test. D. Materials not conforming to any of the above categories.

Compomer, Luxat, and Primaflow, which are shown in Fig. 1A. Three products showed reduced cytotoxicity in NRU, but increased or unaltered cytotoxicity in the MTT test, as can be seen in Fig. 1B. No alteration in either test was seen in two products (Fig. 1C), and two of the materials, Freedom and Ionosit, did not conform to any of the above categories (Fig. 1D). Extracts of Freedom showed little variation in toxicity following curing and aging in the MTT test, but in NRU in contrast to non-cured or aged Freedom, freshly-cured Freedom was severely cytotoxic.

Fig. 2 shows the changes in cytotoxicity rating following curing and aging. Curing reduced cytotoxicity in the MTT test from severe to moderate in 7 of 9 materials, but had relatively little effect in the NRU assay. Aging and polishing, however, had little effect on cytotoxicity evaluated by the MTT test, but markedly reduced cytotoxicity in NRU in 6 of 8 extracts.

Discussion

Severely cytotoxic reactions to nearly all the non-cured compomer extracts were observed. Compomers contain modified methacrylate monomers, such as the urethane dimethacrylates (UDMA) and bisphenol-A-glycidylmethacrylate (BisGMA), and bifunctional monomers, such as dicarboxylic acid dimethacrylates (DCDMA) (3). Information available from the manufacturers of the products used in this study is summarized in Table 1. The dimethacrylate components of all these materials are cytotoxic to varying degrees. The cytotoxic effect of some resin components on DNA and protein synthesis has been demonstrated in 3T3 fibroblasts, ethoxylated bisphenol-A-dimethacrylate (BisEMA) being the most toxic, then UDMA and BisGMA; triethyleneglycol dimethacrylate (TEGDMA) is slightly less toxic (22). Cytotoxicity of resin components was ranked using the MTT test in 3T3 fibroblasts: BisGMA > UDMA > TEGDMA ≫ hydroxyethyl methacrylate (HEMA) (23). Thirty-nine acrylates and methacrylates were tested in HeLa S3 cells using the MTT test. The cytotoxicity ranking of the most widely used monomers was BisGMA > UDMA > TEGDMA > HEMA > MMA. It was further shown that the cytotoxicity of these substances was related to their lipophilicity (24). The effects of 35 monomers/additives on DNA content in primary cultures and 3T3 cells demonstrated a high cytotoxic potential for BisGMA, bisphenol-A-dimethacrylate (Bis-

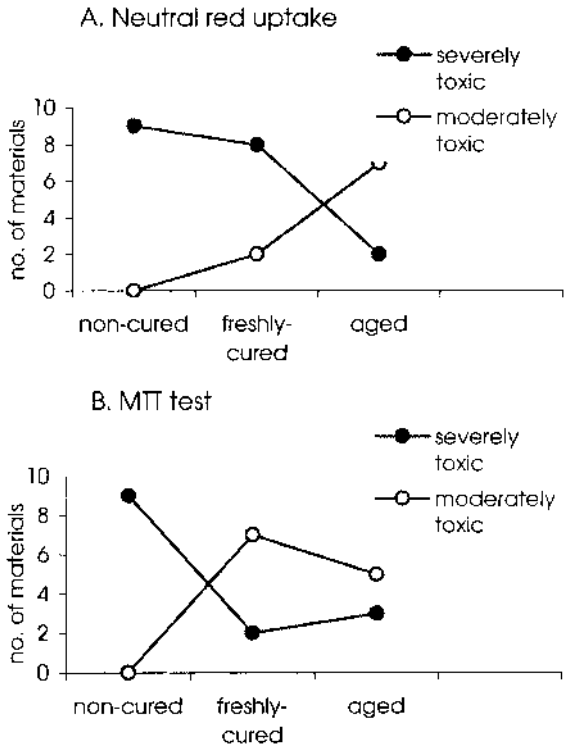

Fig. 2. The effects of curing and aging on the cytotoxicity rating of severely toxic extracts of non-cured compomers, seen in A. neutral red uptake and B. MTT test.

MA), UDMA and TEGDMA, significantly less for HEMA, and moderate for the photoinitiator camphorquinone (25). Methacrylates have been shown to interfere with cellular cholesterol and phospholipids, which could result in an alteration in membrane-associated functions (26). Methyl methacrylate alters inner mitochondrial membrane structure, uncouples oxidative phosphorylation, and disrupts electron transport, resulting in inhibition of ATP synthesis (27). Constituents may interact and alter the toxic response. Low concentrations of BisGMA combined with any toxic amount of HEMA or TEGDMA have resulted in antagonism, whereas higher concentrations of BisGMA gave synergistic reactions with HEMA or TEGDMA (23).

The moderate to severe cytotoxicity of the freshly-cured extracts tested in this study indicates the presence of cytotoxic residual monomers or newly-formed compounds in the extracts. Light-induced polymerization is normally the major factor contributing to the setting of compomers, the acid-base reaction contributing to a lesser degree as the materials contain no water, but the aqueous environment during extraction may influence degree or rate of polymerization. Post-curing in compomers continues for approximately 24 h after initial curing, suggesting availability of unconverted monomers within this period of time (28). Aqueous extracts of freshly-cured Compoglass and Dyract have been analyzed. Compoglass was shown to release the monomers DEGDMA, TEGDMA, TEEGDMA, PEGDMA, and HEGDMA (di-, tri-, tetra-, penta- and hexaethylene glycol methacrylates) and the base monomer BisMA, whereas Dyract released photostabilizer and co-initiator substances. Both compomers released HEMA and BisGMA (7). Cured composites have previously been shown to contain identifiable leachable components with cytotoxic activity, and subsequent extraction of the leached samples gave 90% reduction in cytotoxicity (29). Nearly all of the leachable species in composites are eluted within 24 h (30, 31), although glass-ionomers have been shown to release cytotoxic substances up to 48 h after curing (32). Cytotoxicity of glass-ionomers was not accounted for by fluoride release (33), and since compomers released significantly less fluoride than glass-ionomers (4), it seems unlikely that any cytotoxicity is related to fluoride.

The in vitro release of substances from polymerized materials may be due to elution of monomers or additives, and depends on the degree of monomer conversion. Possible degradatory effects of enzymes in extraction media and serum should also be taken into account. Enzymatic hydrolysis in vitro using non-specific esterase and salivary hydrolases has demonstrated that monomers such as TEGDMA, BisGMA, and HEMA were degraded to methacrylic acid (34, 35).

In general, non-cured materials were more cytotoxic than cured. Curing altered the degree of toxicity to a variable extent; for example F2000, whose cytotoxicity decreased with curing in the MTT test, but remained unaltered in NRU. Earlier studies using composites and cements have indicated that cytotoxicity is strongly dependent on setting time, and that unmixed components and freshly-mixed materials exhibit greater cytotoxicity than the set materials (36, 37). Samples cured for the same lengths of time, but aged (post-cured) for varying periods of time, showed a decrease in cytotoxicity, particularly in the first 24 h of aging (38).

The effects of aging and polishing on the cytotoxicity of extracts varied, but there was little decrease in cytotoxicity in most materials. Increasing the post-curing time increases, in theory, the degree of monomer conversion. However, a surface film of incompletely polymerized material has been demonstrated for dental polymers, due to oxygen inhibition of free-radical polymerization (39). The marked decrease in cytotoxicity observed in aged Luxat and Primaflow, and also, to a lesser extent, ANA Compomer (Fig. 1A), made under nitrogen and ground, compared to both non- and freshly-cured, suggests the presence of such an incompletely polymerized, cytotoxic film in the freshly-cured samples. The same pattern is seen in Compoglass, Dyract and Hytac in the NRU assay (Fig. 1B). Resins cured in an argon atmosphere show a higher degree of monomer conversion than resins cured in air (40). Cured composites released formaldehyde, unground, air-polymerized

specimens releasing higher levels than ground specimens polymerized in the absence of air (41). The absence of this decrease in cytotoxicity in the MTT test could indicate other toxic effects from the exposed inorganic, ion-leachable glass in the polymer matrix. The presence of the surface film may impede the release of these cytotoxic substances, such as barium, but polishing subsequently allows their extraction. The severe cytotoxicity observed in freshly-cured composites, irrespective of curing time, was ameliorated by polishing (38).

Freedom (Fig. 1D) exhibits a constant level of cytotoxicity in the MTT test, regardless of curing state, but the freshly-cured material appears to be more toxic to NRU. The incompletely polymerized layer found in freshly-cured samples of Freedom may contain intermediate compounds, which are more toxic to NRU than non-cured monomer, but have little effect on the MTT test.

Ionosit (Fig. 1D) exhibits a different pattern of cytotoxicity, where the aged samples appear to have similar cytotoxicity to the non-cured material, in both tests, but freshly-cured Ionosit was much less toxic. Curing greatly reduced cytotoxicity, but polishing this material increased it to the same levels found in non-cured, suggesting that in this case the non-polymerized film inhibits release of toxic substances from freshly-cured material. Formation of the non-polymerized film is dependent on the presence of oxygen, which is able to diffuse further into a material with a low viscosity (39), and Ionosit is the most fluid material in this study. It is difficult to explain why aging and polishing gave no significant alteration in cytotoxicity in the remaining two materials (Fig. 1C).

Fig. 2 summarizes the overall effects of curing and aging on the cytotoxicity ratings of extracts of non-cured compomers. Curing appears to reduce the cytotoxicity caused by substances which primarily affect the MTT test but not NRU, whereas aging and polishing reduces cytotoxicity caused by substances affecting NRU rather than the MTT test. As previously discussed, hydrophilic substances such as methyl methacrylate affect oxidative processes, resulting in inhibition of ATP synthesis (27). Lipophilic substances are more likely to disrupt membrane integrity, and methacrylates alter cellular cholesterol and phospholipids, which could alter membrane-associated functions (26). Non-cured materials may tend to release both hydrophilic and hydrophobic substances, explaining their severely toxic effects in both tests. Curing may reduce the availability of the more hydrophilic substances, thus reducing cytotoxicity in the MTT test. The more hydrophobic substances, such as UDMA and TEGDMA, may be less readily available in freshly-cured materials, and may first become available to extraction after aging and polishing.

To conclude, extracts from 10 commercially available compomers were found to be cytotoxic both before and after setting. Non-cured materials were generally more cytotoxic than cured, and the effect of aging varied considerably between the different materials.

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