Correlation of cytotoxicity, filler loading and curing time of dental composites

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Previous studies have shown that dental resin composites tested in cell culture produce cytotoxic effects on human gingival tissues. In this study, the cytotoxic potential of resin composites on primary human gingival fibroblast cultures was evaluated, based on inhibition of cellular protein synthesis measured by [35S] methionine incorporation. Both resin content and percentage of monomer conversion were considered as potential causes of cytotoxicity. Three resin composites were selected to provide a range of filler content from 45 to 86 wt%. Duplicate sample discs (1 mm thick × 10 mm diameter) of each composite were polymerized for 15, 30 and 60 s, followed by heat (110°C, 10 min), and the degree of monomer conversion for each sample group was measured using Fourier transform infrared spectrophotometry. Identically fabricated discs were placed into 35 mm culture dishes with gingival fibroblasts and incubated for 24 h at 37°C. The cell monolayers then were labelled at 24 h with [35S] methionine, washed and solubilized; then incorporated radioactivity was quantitated by liquid scintillation spectrometry. For each composite, as the percentage of monomer conversion increased, cellular toxicity decreased. In comparing different composites having similar monomer conversions, it was found that the filler/resin ratio was not the only factor determining the composite's relative toxicity.

Keywords: Cytotoxicity, dental materials, composites

Concerns over the toxicity of dental amalgam and an increased emphasis on aesthetics have popularized the commercial development and clinical use of dental composite restorative materials. However, it has long been recognized that such composite materials can result in localized inflammation. Composites placed in close proximity to pulpal or gingival tissues have been shown to induce toxicity in the form of inflammation and tissue damage. This inflammation may be produced by the presence of bacteria or bacterial products at the margins of restoration, by the toxic constituents of the resin, or a combination of both. Hanks et al. suggest that the toxicity of resins is a result of either new toxic products generated upon setting, or of residual toxic products following an incomplete setting reaction. There appear to be marked variations in composite formulation and purity of commercial resin systems, and such variations may affect the biological as well as the physical properties of the composites.

To ensure the biological safety of new materials, rapid and cost-effective standardized biocompatibility testing techniques have become a necessity. Various investigators have developed techniques based on mammalian cell culture assays to satisfy this need. Many of these assays are based on morphological criteria. Studies in our laboratory examined the effect of various resin cements on gingival cells utilizing a variety of viability, morphological and metabolic assays. The experiments resulted in a general correlation between the methods, but demonstrated that the dental materials can affect cell metabolism and produce injury to cells which is not immediately morphologically evident. The purpose of the current study was, using cell culture, to examine the differences in biological effects of three commercial composites having different proportions of filler and to correlate cellular toxicity with the degree of monomer conversion for each resin.

MATERIALS AND METHODS

Materials

Dental composite. The composites were selected on the basis of their formulations. They were P-50 (Shade U; 3M Corporation, St Paul, MN, USA), Prisma-Micro-Fine (Shade...
LYG; LD Caulk, Milford, DE, USA) and Multifil (Shade C-20; Kulzer, Irvine, CA, USA). All are Bis-GMA-based derivatives with a range of filler loading typical of a heavily-loaded hybrid, a microfill and a reinforced microfill, respectively.

**Methods**

**Sample fabrication.** Samples of composites 10 mm in diameter \( \times 1 \) mm in thickness were fabricated in polyethylene moulds. To determine the degree of toxicity of the composites and any relationship between the amount of polymerization and toxicity, three pairs of samples of each resin were fabricated. Pairs were polymerized for 15, 30 or 60 s, plus heating at 110°C for 10 min. All samples were polymerized from one side of the mould using a Visalux 2 light unit with an 11 mm tip (3M Corporation, St Paul, MN, USA).

**Sample burnout.** The percentage of filler weight of each brand of composite was determined by ashing, according to ISO Standard 404917. Empty crucibles were weighed on an analytical balance, heated, desiccated and reweighed to obtain their dry weight. Duplicate specimens of individual composites were weighed in the crucibles and heated at 575°C for 1 h, resulting in volatization of all organic content. The specimens were bench-cooled, desiccated for 3 min and weighed to the nearest 0.1 mg to obtain the ashed inorganic weight.

**Monomer conversion determination.** Disc-shaped specimens (10 mm diameter, 0.8 mm thick) of each of the three composite materials were fabricated and cured for the 15, 30 or 60 s with heating. A method was developed that allowed for determination of the monomer conversion of each face of this cured disc using Fourier transform infrared (FTIR) spectroscopy. To achieve this goal, small wafers of cured composite were obtained. Uncured resin was placed between thin sheets (0.05 mm) of Mylar (DuPont Company, Wilmington, DE, USA). The sheets were pressed until the height of aromatic \( \text{C} = \text{C} \) absorbance (1608 cm\(^{-1}\)) in the infrared spectrum was obtained using 16 scans at 2 cm\(^{-1}\) resolution. The mean monomer conversion of each cured wafer was calculated by determining the ratio of the height of the aliphatic carbon–carbon double bond (\( \text{C} = \text{C} \)) absorbance (1636 cm\(^{-1}\)) to the height of aromatic \( \text{C} = \text{C} \) absorbance (1600 cm\(^{-1}\)) in comparison to the ratio of the uncured material. The mean monomer conversion for each resin cured under each experimental condition was determined. The mean monomer conversion for the top and bottom of each brand of composite wafer was compared using an unpaired, one-tail \( t \)-test.

**Cell cultures.** Primary human gingival fibroblasts were obtained from explant cultures of pooled biopsy tissues taken from patients during their treatment in the Department of Periodontics at the Medical College of Georgia, GA, USA. The biopsy tissues were washed five times in Hank’s Balanced Salt Solution (BSS) supplemented with 200 units/ml penicillin and 200 \( \mu \)g/ml streptomycin. They were minced and placed in 35 mm plastic tissue culture dishes and incubated at 37°C in an atmosphere of 95% air-5% CO\(_2\). The medium used was Eagle’s Minimal Essential Medium (EMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 \( \mu \)g/ml streptomycin. These conditions provide maximum proliferative conditions for fibroblasts. The morphology of the cell layer which grew out from the explants was typical of fibroblasts. When the cells had formed a complete monolayer, they were serially passaged by trypsinization (0.25% trypsin, 0.02% EDTA in balanced salt solution) and allowed to grow to confluence in 75 cm\(^2\) flasks. Cells were used at passages 6–8 in all experiments. Viable cells (250,000), as determined by the trypsin blue dye exclusion method, were used per assay, as described below.

**Cytotoxicity assays.** Cytotoxicity was assessed by a method developed previously in our laboratory. After polymerization, duplicate samples of each specimen were immediately attached to the centre of 35 mm tissue culture dishes using dental sticky wax. Earlier studies had shown that dental sticky wax to be inert and not to interfere with cell growth and metabolism. Identically-sized polyethylene discs attached in the same manner served as inert controls. The prepared discs were then placed in an incubator at 37°C under an atmosphere of 95% air-5% CO\(_2\) for 2 h before cells were added. Human gingival fibroblasts (2.5 \( \times \) 10\(^{4}\) per dish) were seeded into the tissue culture dishes containing the sample composite discs, and incubated for 24 h at 37°C under a 95% air-5% CO\(_2\) atmosphere. Cell monolayers were observed by phase contrast microscopy for confluency; the culture media were then removed and 2 ml medium containing \( { }^{3} \text{H} \) methionine (20 \( \mu \)Ci/ml) was added to each dish to radio label proteins biosynthetically. The cultures were incubated for an additional 24 h at 37°C in 95% air-5% CO\(_2\). They were washed twice with isotonic phosphate-buffered saline, pH 7.4, and the cells were solubilized by the addition of sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate, 5% 2-mercaptoethanol, 10% glycerol) followed by boiling for 3 min. Triplicate aliquots of the solubilized cell extracts from duplicate plates were subjected to trichloroacetic acid...
precipitation. The acid-insoluble radioactivity representing \[^{35}S\]methionine incorporation into protein was quantitated in a Beckman 3801 liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA). To ensure that excess \[^{35}S\]methionine was present throughout the labelling period, additional experiments were performed to determine whether the composite materials absorbed significant amounts of the radiolabel. Plates containing \[^{35}S\]methionine media and composite discs but no cells, or containing cells and \[^{35}S\]methionine media but no composite discs were prepared and incubated as described above. Radioisotope incorporation by the cells and the amount of radioisotope remaining in the media were assayed by scintillation counting.

**RESULTS**

The percentage weight of filler of each composite was obtained from the sample ashing experiment. The average filler weight percentage for the composites ranged from 45% (Prisma-Fine) to 85% (P-50), as shown in Figure 2. The filler/resin ratios of the composites tested are representative of the range of commercially available products.

Figure 3 depicts the percentage of monomer conversion of the top and bottom surfaces of each set of specimens for each duration of cure, as determined by FTIR. Following 15 s exposure, the extent of polymerization of the top of the discs was nearly 40% for each resin, whilst the bottom cure ranged from 15 to 25%. The differences in extent of cure between the top and bottom values were significant for all three resins. Following 30 s exposure, the percentage of monomer conversion increased for each composite sample resulting from 15 second exposure to the curing light (15), 30 second exposure to the curing light (30), and 60 second exposure to the curing light and heat treatment at 120°C for 10 minutes (60+).

![Figure 2](image)

**Figure 2** Percentage of filler by weight of each composite calculated as the residual sample weight after ashing divided by the sample weight prior to ashing x 100.

![Figure 3](image)

**Figure 3** Percentage of monomer conversion at the top and bottom of each composite sample resulting from 15 second exposure to the curing light (15), 30 second exposure to the curing light (30), and 60 second exposure to the curing light and heat treatment at 120°C for 10 minutes (60+).

The cure of the top surface of each disc approached 45%, whilst the bottom surfaces of P-50 and Prisma-Fine approached only 25%. For this time of exposure, Multifil was about 30% cured on the bottom surface. Again, the three resins did not differ significantly from each other, but there were significant differences in cure between the top and bottom surfaces of each composite. Even after 60 s light cure followed by heating, none of the resins achieved >55% monomer conversion on the top surface. The discrepancy between top and bottom surfaces decreased with this latter exposure, with Multifil having nearly 50% conversion on the bottom disc surface while the others ranged from 40 to 45%. Significant differences between the top and bottom conversion values for P-50 and Prisma-Fine remained even after this treatment. There were no significant differences in conversion values between the three materials.

The composites tested produced a wide range of cytotoxic effects, related to both the specific resin and to the curing method. These results suggest that curing time directly influenced the degree of metabolic impairment as evidenced by reduction in \[^{35}S\]methionine incorporation. The observed decreases were not attributable to artifactual decreases in the \[^{35}S\]methionine pool, since experiments performed to determine whether the composite materials absorbed significant amounts of \[^{35}S\]methionine from the media indicated that the total medium-associated radioactivity remaining after incubation with the composite discs was >97.5% of the original concentration. Also, radio-labelling of cultures having cell densities ranging from \(1.5 \times 10^5\) to \(3.0 \times 10^5\), in the absence of composite discs, showed that the cells utilized a maximum of 9% of the available radioisotope. These data indicate that the observed decreases in radiolabel incorporation by cells in the presence of the composite materials was not due to limiting concentrations of \[^{35}S\]methionine.

As measured by inhibition of cellular protein synthesis, cellular toxicity decreased as the curing time and resultant monomer conversion increased for each composite sample (Figure 4). In comparing the samples cured for 15 and 30 s, monomer conversions were highest for the Multifil resin, but its cytotoxicity was the least. Prisma-Fine produced greater...
protein synthesis inhibition than the others at each curing time, even though its extent of cure was comparable to that of the other materials. The least cytotoxicity was observed in cultures exposed to P-50 and Multifil samples which had been light cured for 60 s and heated for 10 min at 110°C. Although Prisma-Fine was more inhibitory than P-50 and Multifil following the 60 s cure/heat treatment, the difference between them had narrowed.

**DISCUSSION**

Biocompatibility of dental materials has been evaluated in a variety of ways. The method chosen for the present study examined the influence of resin composite on protein production by primary human gingival fibroblasts. The choice of primary gingival fibroblasts for this study was based on several considerations. Since resin composites are often placed in close proximity to gingival tissue, any effects on the cell types of that tissue would be clinically important. Also, as suggested in our previous studies of resin composite luting agents, metabolic assays utilizing primary gingival fibroblasts have been shown to be more discriminating in cytotoxicity assessments than an epithelial cell line. Other studies have involved cells of the tooth pulp, but an extrapolation of the cytotoxic potential of a material to cells of the gingival tissues cannot be made from these results. Cytotoxic responses of pulpal tissues are likely to be different due to differences in cell type and the influences of a dentinal barrier. Primary gingival fibroblasts can be easily obtained in quantity as primary cell cultures from biopsy specimens. Because it has a finite lifetime without chromosomal aberrations, this cell type may more accurately correlate with the in vivo responses than would an established cell line. At the same time, the response of primary cell lines may change as the cultures approach senescence. Therefore, our studies were conducted using cells at similar culture ages to minimize this variable.

While morphology and simple viability are common methods of scoring cytotoxicity, these techniques tend to lack sensitivity and are difficult to quantify. For example, earlier studies examining toxicity of glass-ionomer cements showed that inhibition of RNA and protein synthesis occurred despite minimal morphologic effects. In contrast, methods which assess metabolic functions, such as the measurement of protein synthesis as determined by incorporation of radiolabelled precursors, are more sensitive, highly quantitative and easily reproducible.

In addition to duration of curing light exposure, the shade of the composite and the resin and filler formulation affect the degree of monomer conversion. Attempts were made to standardize each composite shade, but since different classes of composites from different manufacturers were tested, the only variable totally controllable by the experimenter was curing time. Our protocol produced a range of conversion values between the different composites at each curing time, and thus made it impossible to establish an absolute relationship between the filler/resin weight ratio and cytotoxicity. However, for all resins tested, as light curing time was increased, the extent of cytotoxicity decreased. The data also suggest that there may be differences in the amount or nature of cytotoxic substances that can leach out of resins of different compositions before achieving maximum cure. Whether this is due to different toxicities or the reaction rate is not clear at this time. Thus cytotoxicity is not solely determined by the proportion of filler and resin, but may also be influenced by formulation differences.

It should be noted that while 60 s light exposure plus post-cure heating was chosen to produce maximum curing of the samples, the results indicate that a uniform cure, even in these relatively thin mm samples, was not achieved. A differential still existed between conversion values of the tops and the bottoms of the discs. These findings emphasize that incremental polymerization and prolonged curing times are essential when placing resin composites, so that any potential for in vivo cytotoxic effects is minimized.

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**REFERENCES**