Cytotoxicity of composite resins polymerized with different curing methods

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Abstract

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Aim To compare the relative cytotoxicity of resin-based composite materials polymerized with three different curing methods on L 929 cells over a period of 1 week.

Methodology Ten discs of each material (Flowline®, P 60® and Z 250®) were cured from one side with either standard cure (Optilux 401®), soft-start cure (Elipar Free Light®) or fast cure (Hilux Ultra Plus®). Then the samples were aged for 1, 2, 3, 5 and 7 days in Dulbecco's Modified Eagle Medium/Ham's F12 (DMEM/F12). After each ageing interval, cytotoxicity of the extracts to cultured fibroblasts (L 929) was measured by MTT assay. The degree of cytotoxicity for each sample was determined according to the reference value represented by the cells with a pure culture medium. Statistical significance was determined by one-way analysis of variance (ANOVA), followed by the Student's Newman–Keuls test.

Results Exposure of L 929 cells to the test materials resulted in a high survival fraction at 1 and 7 days. Flowline® specimens, either cured with Optilux 401® or Elipar Free Light®, had no toxic effect on the cells, whereas the other groups were moderately toxic on the 2-day interval. All experimental groups presented lower cell viability than the control at the 3- and 5-day intervals.

Conclusions The composite resins used in this study were cytotoxic after 48 h pre-incubation, but this toxicity disappeared after pre-incubation in a biological medium for 7 days. Curing did not have a significant effect on the cytotoxicity of the composite materials tested.

Keywords: composite resin, cytotoxicity, light curing.

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Introduction

Resin composites have been used with increasing frequency as posterior restorative materials as a result of the demand for both aesthetic restorations and concerns over the adverse effects of mercury from amalgam (Sweeney *et al.* 2002). However, it has long been recognized that such composite materials can result in localized pulp inflammation. Although the physical properties of resin composites are constantly being improved, *in vivo* studies have shown that their use is occasionally associated with necrosis and irritation of the pulp (Caughman *et al.* 1991), as well as the periodontium (Nasjleti *et al.* 1983).

Adequate polymerization is a crucial factor in maximizing the physical properties and clinical performance of composite resin restorative materials. The cytotoxicity is a result of residual uncured monomer or oligomer (Caughman *et al.* 1991). Even in fully set restorative materials, substantial amounts of short-chain polymers remain unbound (Ferracane & Condon 1990), with the result that there is possible elution of leachable toxic components towards the pulp (Ferracane 1994). There is also a correlation between the amount of uncured leachable resin in the composite and the magnitude of the cytotoxic effect (Inoue *et al.* 1988). To overcome the problem of inadequate polymerization, new curing methods have been introduced such as soft-start and fast cure methods.

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Table 1 Manufacturers and monomer contents of materials

Product	Manufacturer	Composite type	Matrix
Flowline®	Heraeus Kulzer, Hanau, Germany	Flowable	TEGDMA, (meth-) acrylate
P 60®	3M Dental Products, St Paul, MN, USA	Condensable	BIS-GMA, UDMA, BIS-EMA
Z 250®	3M Dental Products, St Paul, MN, USA	Hybrid	BIS-GMA, UDMA, BIS-EMA

Whereas the cytotoxicity of dental composites and their components have been studied (Hanks *et al.* 1988, Caughman *et al.* 1991, Hanks *et al.* 1991, Rathbun *et al.* 1991, Wataha *et al.* 1994, Ratanasathien *et al.* 1995, Schedle *et al.* 1998, Bouillaguet *et al.* 2002, Quinlan *et al.* 2002), little is known about the effect of polymerization techniques on the cytotoxicity of composites. For this reason, the aim of this study was to compare the relative cytotoxicity of resin-based composite materials polymerized with three different curing methods on L 929 cells over a period of 1 week.

Materials and methods

Cells

The cells used for the experiments were L 929 mouse skin fibroblasts (L 929 An2 Hükük 95030802; Şap Enstitüsü, Ankara, Turkey). The cells were grown as monolayer cultures in 25 T-flasks (Costar, Cambridge, MA, USA), subcultured three times a week at 37 °C, in an atmosphere of 5% CO_2 in air and 100% relative humidity, and maintained at third passage. The culture medium was Dulbecco's Modified Eagle Medium/Ham's F12 (DMEM/F12) nutrient mixture (1 : 1; Sigma, St Louis, MO, USA) supplemented with 10% (v/v) foetal bovine serum (FBS; Biochrom, Berlin, Germany) without antibiotics. Adherent cells at a logarithmic phase were detached with a mixture of 0.025% trypsin (Sigma) and 0.02% EDTA (Sigma), incubated for 2–5 min at 37 °C and used for cell inoculation.

Sample preparation

Three resin composite materials and three curing methods were tested. These materials were: (i) flowable composite (Flowline®; Heraeus Kulzer, Hanau, Germany), (ii) condensable composite (P 60®; 3M Dental Products, St Paul, MN, USA) and (iii) hybrid composite (Z 250®; 3M Dental Products, St Paul, MN, USA). The composite resins were placed into round Teflon molds (6 mm diameter and 1 mm depth), covered with mylar strips and pressed with a glass plate. Three different curing methods were applied for each composite material. The samples were cured from one side with either standard cure unit (Optilux 401®; Demetron, Kerr, Danbury, USA) of 600 mW cm^{-2} intensity (continuous energy output for 40 s) with halogen light, soft-start cure unit (Elipar Free Light®; 3M ESPE, Germany) of 400 mW cm^{-2} intensity (during the first 12 s, the light intensity increased exponentially, and in the final 28 s, the light intensity was stable at 400 mW cm^{-2}) with light-emitting diode (LED) or a fast cure unit (Hilux Ultra Plus®; Benlioğlu Dental, Ankara, Turkey) of 1400 mW cm^{-2} intensity (continuous energy output for 10 s) with halogen light. All specimens were prepared by the same operator. The contents of each material and the details of each curing unit are given in Tables 1 and 2.

According to the composites used and the different cure conditions, the samples were divided into 9 groups of 10 samples each as shown in Table 3.

The samples were kept for 45 min under ultraviolet light to prevent bacterial contamination, and then the

Table 2 The light curing units used, modes and source

Curing light units	Light and cure type	Light intensity output	Curing modes
Optilux 401[a] (Demetron, Kerr, Danbury, CT 06810, USA) Serial #4223926	Halogen standard cure	600 mW cm^{-2}	Continuous energy output for 40 s
Hilux Ultra Plus[b] (Benlioğlu Dental, Ankara, Turkey) Serial #P2080878	Halogen fast cure	1400 mW cm^{-2}	Continuous energy output for 10 s
Elipar Free Light[c] (3M ESPE, Germany) Serial #939800001010	LED soft-start cure	0–400 mW cm^{-2} + 400 mW cm^{-2}	Exponential energy output automatically increase to full energy within 12 s + 28 s full energy

[a]Intensity of halogen light was measured using curing radiometer (Curing Radiometer, Model 100; Demetron, Kerr, Danbury, CT 06810, USA).
[b]Intensity of halogen light was measured using 950-700-3 radiometer placed on the curing unit itself.
[c]Stated output intensity was confirmed manufacturers' suggestions.

Table 3 Composites and curing methods for experimental groups

Composite	Curing method	Group
Flowline®	Standard cure	A1
	Soft-start cure	A2
	Fast cure	A3
P60®	Standard cure	B1
	Soft-start cure	B2
	Fast cure	B3
Z250®	Standard cure	C1
	Soft-start cure	C2
	Fast cure	C3

samples were aged for 1, 2, 3, 5 and 7 days in DMEM/F12. Two samples from each group were immersed in 5 mL of sterile DMEM/F12 for each test in order to obtain enough ratio of the surface area to volume, which is recommended to be within the range 0.5–6.0 cm^2 mL^{-1} by the International Standards Organization (1997; ISO 10993-5). The ratio of the surface area of the discs to the volume of medium was 0.75 cm^2 mL^{-1} in this study. The samples were stored at 37 °C in 5% CO_2 and 95% air. After each ageing interval, the extracts were used to assess cytotoxicity.

Cell viability assays (MTT assay)

The L 929 cell suspension was prepared at a concentration of 3×10^4 cell mL^{-1} and dispensed onto 96-well cluster cell culture plates (100 μL $well^{-1}$). The multiwell plates were incubated at 37 °C, 5% CO_2 in air for 24 h. After 24 h, the culture medium was removed from the wells and equal volumes (100 μL) of the experimental material were added into each well. In control wells, 100 μL DMEM/F12 was added. Following removal of the test extracts, 100 μL $well^{-1}$ growth medium and 10 μL MTT (tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) were added to each well and kept in a dark environment for 4 h at 37 °C. Then, MTT was aspirated and 100 μL $well^{-1}$ of dimethyl sulfoxide (DMSO) was added to each well followed by 12.5 glycine buffer. Subsequently, the absorbance at 570 nm was measured using a UV-visible spectrophotometer (LPB Pharmacia, Bromma, Sweden). MTT assays were repeated in three separate experiments.

Statistics

Statistical significance was determined by one-way analysis of variance (ANOVA), followed by Student's Newman–Keuls test with Bonferroni correction for multiple comparisons. Statistical significance was defined at $P < 0.05$.

Results

In the MTT assay, exposure of L 929 cells to the test materials resulted in a high survival fraction at 1 and 7 days (Figs 1 and 2). There were no statistically significant differences at 1 and 7 days, whereas the differences were found to be statistically significant at 2, 3 and 5 days among the groups. Student's Newman–Keuls test was

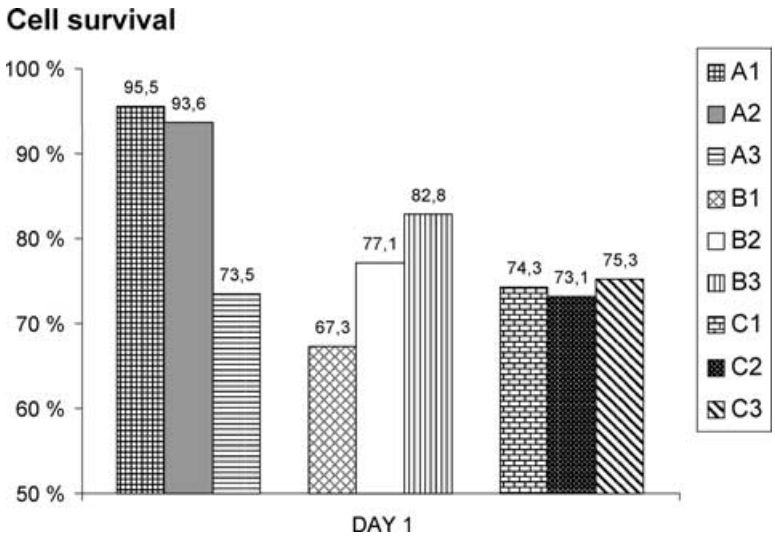

Figure 1 Ratio (%) of OD values of extract-treated cells to the control cells by MTT assay at 1 day.

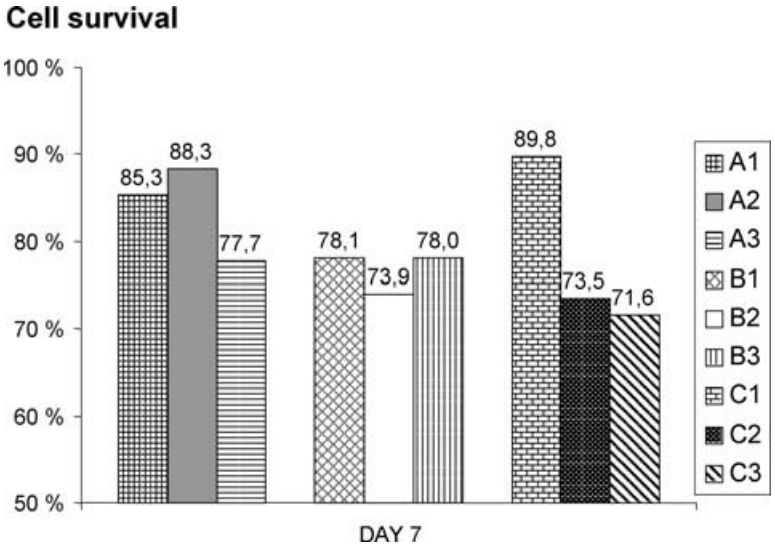

Figure 2 Ratio (%) of OD values of extract-treated cells to the control cells by MTT assay at 7 days.

used to indicate the groups which differed from one another.

Exposure of L 929 cells to the materials in Group A1 and A2 resulted in a survival fraction (defined as the percentage of optical density (OD) values compared to the OD value of control) of 94.9 and 89.9%, respectively (Fig. 3) at 2 days. There were no statistically significant differences between Group A1, A2 and the control ($P > 0.05$); on the other hand, comparison of these groups with the others revealed that the differences were significant ($P < 0.05$).

All experimental groups presented lower cell viability than the control at 3 and 5 days (Figs 4 and 5). The differences between the experimental groups and the control were found to be statistically significant ($P < 0.05$), whereas there were no statistically significant differences between the experimental groups ($P > 0.05$).

Discussion

Biocompatibility of dental materials has been evaluated in a variety of ways. In the present study, the effects of three composite resins on L 929 fibroblasts were investigated with the MTT assay. The MTT assay is a good indicator of cell viability. This assay is based on the reduction of the MTT by those cells that remain viable after exposure, and incubation with a test chemical or device. Mitochondrial dehydrogenases at the

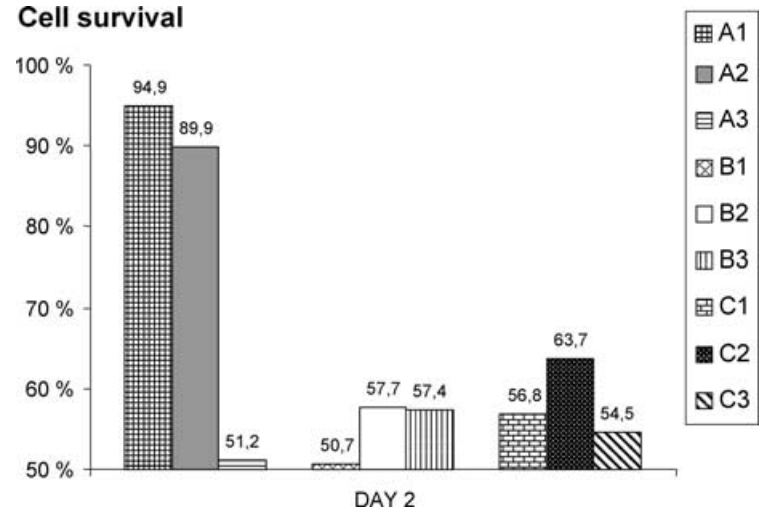

Figure 3 Ratio (%) of OD values of extract-treated cells to the control cells by MTT assay at 2 days.

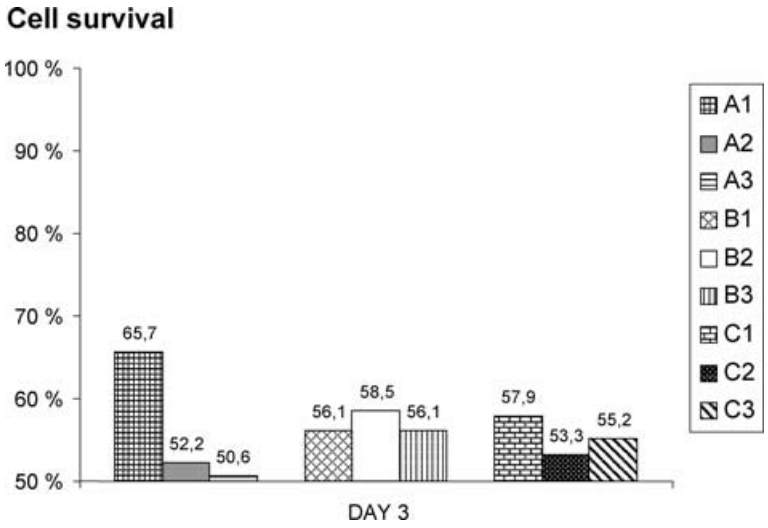

Figure 4 Ratio (%) of OD values of extract-treated cells to the control cells by MTT assay at 3 days.

cytochrome *b* and cytochrome *c* sites of viable cells convert the yellow water-soluble form of the salt to an insoluble, intracellular purple formazon metabolite. Formazon solubilized by extraction with alcohol or DMSO can be quantified spectrophotometrically with results related to the proportion of viable cells (Bean *et al.* 1995).

Composite resins have been shown to be cytotoxic in several tissue culture systems (Hanks *et al.* 1988, Caughman *et al.* 1991, Hanks *et al.* 1991, Rathbun *et al.* 1991, Wataha *et al.* 1994, Ratanasathien *et al.* 1995, Schedle *et al.* 1998, Bouillaguet *et al.* 2002, Quinlan *et al.* 2002). However, it is difficult or even impossible to compare the results from different cell culture experiments because of the many variations in experimental conditions, such as the cell type, the cell material contact method and the exposure time (Spangberg 1981). The results of the current study revealed that the tested materials had no toxic effect on the cells until the second day of incubation, whereas the toxic effects of materials were evident up to 7 days incubation. Our data suggest that the cytotoxic effects elicited by composite materials disappeared after 7 days. Only Flowline® samples, whether cured with standard (94.9% cell viability) or soft-start cure (89.9% cell viability) method, did not cause toxicity on the cells at 2 days incubation, whereas the Flowline® samples cured with fast cure method showed mild cytotoxicity (51.2% cell viability). Hofmann *et al.* 2002) compared the release of leachable components from resin composites after curing with standard,

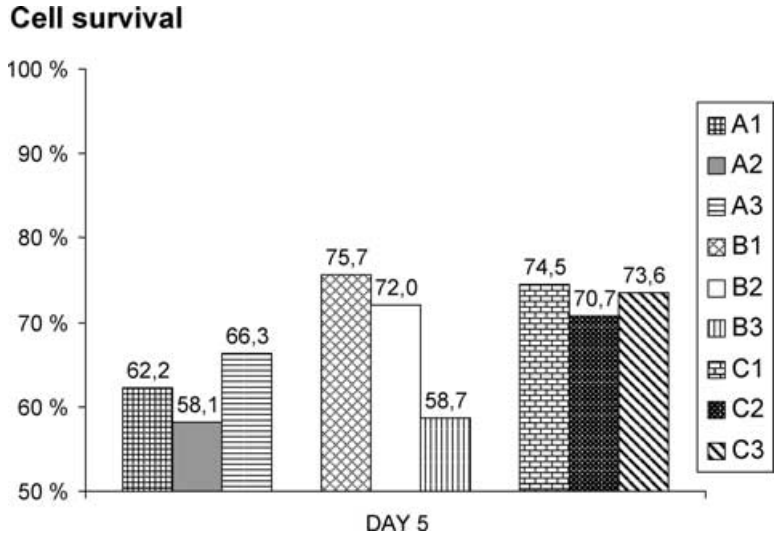

Figure 5 Ratio (%) of OD values of extract-treated cells to the control cells by MTT assay at 5 days.

soft-start or fast cure methods. They concluded that specimens cured by a fast cure method showed highest solubility and sorption. This finding could explain the toxicity of Flowline® samples cured with fast cure at 2 days in the present study. The samples of Flowline® samples cured with standard and soft-start cure methods caused cytotoxic effects on the cells at 3 and 5 days incubation like the others. This could be because of elution time and the amount or nature of cytotoxic substances that can leach out of resin. The materials used in this study have formulation differences. Flowline® differs from other tested composite resins with TEGDMA content. However, except in second-day results, the toxicity findings were similar for all three composites. During the period of experiment, the level of cell viability was above 50% for each group.

The aim of this *in vitro* cytotoxicity study was to determine the effects of the curing methods on the composite's cytotoxic effects. For this purpose, soft-start cure method, which is a relatively new technique, was tested. Soft-start curing unit (Elipar Free Light®), which has blue LEDs as a light type, was used for polymerization of composites. The use of LED technology for the polymerization of the light-activated dental materials was proposed by Mills (1995). Blue LED sources produce significantly greater depth of cure and a degree of monomer conversion than those obtained with a halogen source (Jandt *et al.* 2000). However, the findings of the present study revealed that curing methods did not have significant effect on the cytotoxicity of composite materials.

Conclusions

The current study demonstrated that the composite resins tested in this study were cytotoxic after 48 h pre-incubation, and this toxicity disappeared after pre-incubation in a biological medium for 7 days. Further studies about the elution time and the release of components from these materials polymerized with the different curing methods would be helpful to better understand the biological risks of these materials.

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