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Long-term cytotoxicity of resin-based dental restorative materials

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SUMMARY Highly filled composites, Ormocers (organically modified ceramics) and 'smart' materials have been developed to overcome the polymerization shrinkage problems of conventional composite materials. The purpose of the current study was to investigate the effect of longer-term (up to 8 weeks) ageing of these resin-based dental restorative materials and determine the effect of post-curing on cytotoxicity. Twelve discs of each material (Colombus/IDR, Definite/Degussa, Ariston pHc/Vivadent) were either light-cured (Lc) or light-cured and post-cured (Pc). For cytotoxicity testing, the discs were placed in contact with cell culture medium (DMEM) and incubated at 37 °C. Extracts from composite materials were collected after 24 h and weekly over a time period of 8 weeks. Cytotoxicity of the eluates to cultured fibroblasts (Balb/c3T3) were measured by the succinic dehydrogenase (SDH) activity (MTT assay) and the results expressed in percentage of negative controls (Teflon discs). The results showed that ageing significantly influenced the cytotoxicity of the materials. Except for Ariston pHc, materials were less cytotoxic after 8 weeks of ageing than they were in early intervals and post-curing was not generally useful in reducing cytotoxicity. The Ariston pHc was initially moderately toxic, but then become highly cytotoxic for 5 weeks before returning to initial levels. The current study demonstrated the importance of assessing the cytotoxicity of resin composite materials at multiple times.

KEYWORDS: cytotoxicity, cell-culture, ageing, resin composites.

Introduction

Resins composites have been used with increasing frequency as posterior restorative materials because of demand for both aesthetic restorations and worries about adverse effects of mercury from amalgam. However, pulpal sensitivity problems are more probable to occur with resin composite materials because of gap formation secondary to polymerization shrinkage which occurs for many traditional materials (Bergenholtz, 1998). Although some clinical techniques have been used to minimize the effects of polymerization shrinkage, this problem remains a significant liability for current resin materials (Davidson & Feilzer, 1997).

Several newer strategies to overcome the effects of polymerization shrinkage have been introduced in the last few years. First, indirect composite restorations have been proposed. This technique reduces the effects of shrinkage by allowing the majority of the shrinkage to occur before the restoration is bonded (Dietschi & Spreafico, 1998). Materials used in this manner generally have higher filler contents and allow significant post-curing to improve the degree of conversion of the material. The chemistry of these materials are identical to traditional resin composite materials. A second strategy for reducing the adverse effects of polymerization shrinkage is the use of new resin matricies which shrink less. One class of these materials recently introduced in dentistry is the Ormocers (organically modified ceramics). Originally developed for electronic applications (Wolter *et al.*, 1994), these materials rely on an inorganic alkoxysilane network which is chemically attached to traditional methacrylate groups (Watts, 1996). One recent study by Watts (1998) has confirmed that

Ormocers exhibit less shrinkage than conventional Bis-GMA materials. A third strategy for dealing with the adverse effects of polymerization shrinkage uses the so-called 'smart' materials. The rationale for these materials has been based on the assumption that marginal gap formation resulting from polymerization shrinkage cannot be completely avoided clinically. Thus, these materials are formulated to release ions including fluoride under acidic conditions. The released ions presumably can limit the amount of bacteria in marginal gaps or on restoration surfaces (Heintze, 1999).

Whereas the cytotoxicity of conventional dental composites and their components have been studied (Hanks *et al.*, 1991; Ratanasathien *et al.*, 1995), little is known about the toxicity of 'smart' materials, Ormocers, and materials used for indirect composite restorations. Several factors may increase the risk of cytotoxicity of these materials. There have been reports that most toxic effects from composites occur during the first 24 h of testing and correlate with the early leaching of residual monomers (Ferracane & Condon, 1990). However, other studies have reported that resin-based restorative materials may leach sufficient components to cause cytotoxicity as late as 2 weeks (Wataha *et al.*, 1999a). Although some Ormocers performed well in these 2-week studies, there have been no reports on the longer-term cytotoxicity of these materials. Long-term effects may be more relevant to the clinical use of these and other materials as they remain in contact with living tissues for years. Smart materials may have an increased risk for cytotoxicity because of the mass they release for therapeutic or preventative purposes (Heintze, 1999). On the contrary, highly filled materials used for indirect resin restorations should exhibit less cytotoxicity because of their lower leachable resin content (Sölderholm *et al.*, 1996).

Other factors may influence the cytotoxicity of these materials. Caughman *et al.* (1991) reported that both the resin content and monomer conversion contributed to composite cytotoxicity. Thus, post-curing may reduce the cytotoxicity of composite materials. Mohsen *et al.* (1993) determined that ageing decreased the cytotoxicity of urethane dimethacrylate composites, but used ethanol–water solutions for ageing. Data which relate cytotoxicity to ageing beyond 2 weeks in more biologically relevant solutions have not been reported.

Therefore, the purpose of the current study was to investigate the effect of longer-term (up to 8 weeks) ageing of resin-based dental restorative materials and determine the effect of post-curing on cytotoxicity. A 'smart' material, an Ormocer material, and a highly filled material used for indirect composites were compared and the repeatability of the cytotoxicity assay was measured. The hypothesis for the current study was that ageing and post-curing should reduce cytotoxicity of these materials.

Materials and methods

Three resin-based materials were tested (Table 1). Ariston pHc* was chosen as the 'smart' material, Definite[†] as the Ormocer material, and Colombus[‡] as the highly filled material for indirect restorative procedures. Sample preparation was performed aseptically to prevent the risk of biological contamination during the cytotoxicity testing (Wataha *et al.*, 1999a). Samples were not polished to prevent the need for cleaning the samples after polishing and provide a condition relevant to that seen by pulpal cells. The unpolymerized material was packed into a Teflon*** mould (1 mm thick × 10 mm diameter) and covered on both sides with Mylar sheets to avoid oxygen inhibition. The material was light-cured for 60 s from each side with a composite curing unit[§] with an output of at least 500 mW cm^{-2}. After curing, the specimens ($n = 12$) were removed from the mould aseptically and used immediately. Half of the specimens ($n = 6$) were further post-cured using an MPA 2000 unit[¶] at 110 °C for 180 s. Post-curing was performed to test the hypothesis that higher conversion of the monomers would result in less leaching and less cytotoxicity. Controls were Teflon discs.

Specimens were aged for 24 h, 1, 2, 3, 4, 5, 6 or 8 weeks in Dulbecco's modified eagle's medium (DMEM)** to test the ability of ageing to reduce cytotoxicity of the materials. The DMEM was maintained at pH 7·34 by adding 25 mM HEPES. Each specimens was immersed in 1 mL of sterile DMEM under aseptic conditions and stored at 37 °C in 5% CO_2 and 95% air. The ratio of the surface area of the discs to the volume of medium was 1·88 cm^2 mL^{-1} and was within the range 0·5–6·0 cm^2 mL^{-1} as recommended by

Table 1. Materials, manufacturers, and approximate resin composition*

Material	Lot no.	Manufacturer	Approximate resin composition
Colombus	97380052	IDR, Paris, France	Bis-GMA (9%) UDMA (4·5%) TEGDMA (4·5%) Ethoxyl. BPA-DMA (4·5%) Barium glass (74%) Pyrogenic silica (3%) Catalyst, stabilizer, pigments (0·5%)
Ariston pHc	04594	Vivadent, Schaan, Lichtenstein	Dimethacrylates (20·8%) Alkaline glass Ba–Al–fluorosilicate glass Ytterbiumtrifluoride (YbF3) Silicon dioxide Catalyst, stabilizer
Definite	CHB 222	ADS, Degussa, D	Ormocer resin, DMA (23%) Barium glass (68%) Silica (5%) Apatite (3%)

*According to manufacturers' information.

the International Standards Organization. The ageing times were selected to extend intervals used in previous reports (Wataha *et al.*, 1999a) and to obtain an indication of the trend of cytotoxicity over time. A 24-h interval was used based on previous reports indicating that the majority of leaching from composites is complete after 24–48 h (Ferracane & Condon, 1990). Eight weeks was considered to be more relevant for materials which are used for long-term in the mouth. After each ageing interval, the specimens were transferred into fresh DMEM. The extracts were then used to assess cytotoxicity.

The cell-culture testing was performed using Balb/c 3T3 mouse fibroblasts[††]. Cells were maintained in DMEM with 5% foetal calf serum**, 25 mmol L^{-1} of HEPES buffer**, 1 µg mL^{-1} of gentamycin**, 125 units mL^{-1} of penicillin**, 125 µg mL^{-1} of streptomycin**, and 2 mmol L^{-1} glutamine**. Twenty-four hours prior to the addition of the composite extracts, the cells were plated at 25 000 cell cm^{-2} in 24-well format[‡‡] in 1 mL of medium per well. The cells were incubated at 37 °C in 5% CO_2/95% air to allow attachment of the fibroblasts to the bottom of the

wells. After 24 h, the medium above the cells was replaced with the composite or Teflon extracts, and the cells were incubated for 24 h before assessing cellular activity. The entire set of experiments was repeated to assess the reproducibility of the assay as applied to these materials.

Cellular activity was assessed by measuring mitochondrial succinic dehydrogenase (SDH) activity. This activity was measured by means of the MTT colorimetric assay (Wataha *et al.*, 1992). Succinic dehydrogenase activity was quantified by dissolving the MTT-formazan into 0·1 N NaOH (6·25%) in dimethyl sulphoxide and reading the optical density of the resulting solution at 550 nm with a microplate reader[§§]. Cytotoxicity was expressed as a percentage of the Teflon negative controls. Differences between groups were determined by analysis of variance with Tukey multiple comparison intervals ($\alpha = 0.05$).

Results

Ageing significantly influenced the cytotoxicity of the materials (Fig. 1, series 1). In general, the materials became less cytotoxic with ageing. Colombus and Definite were indistinguishable from controls after 5 weeks

[††]ATCC CCL 163, clone A31, American Type Culture Collection, Rockville, MD, USA.
[‡‡]Costar, Cambridge, MA, USA.

[§§]MR 5000, Dynatech Laboratories, Embrach-Embraport, Switzerland.

Series 1   **Series 2**
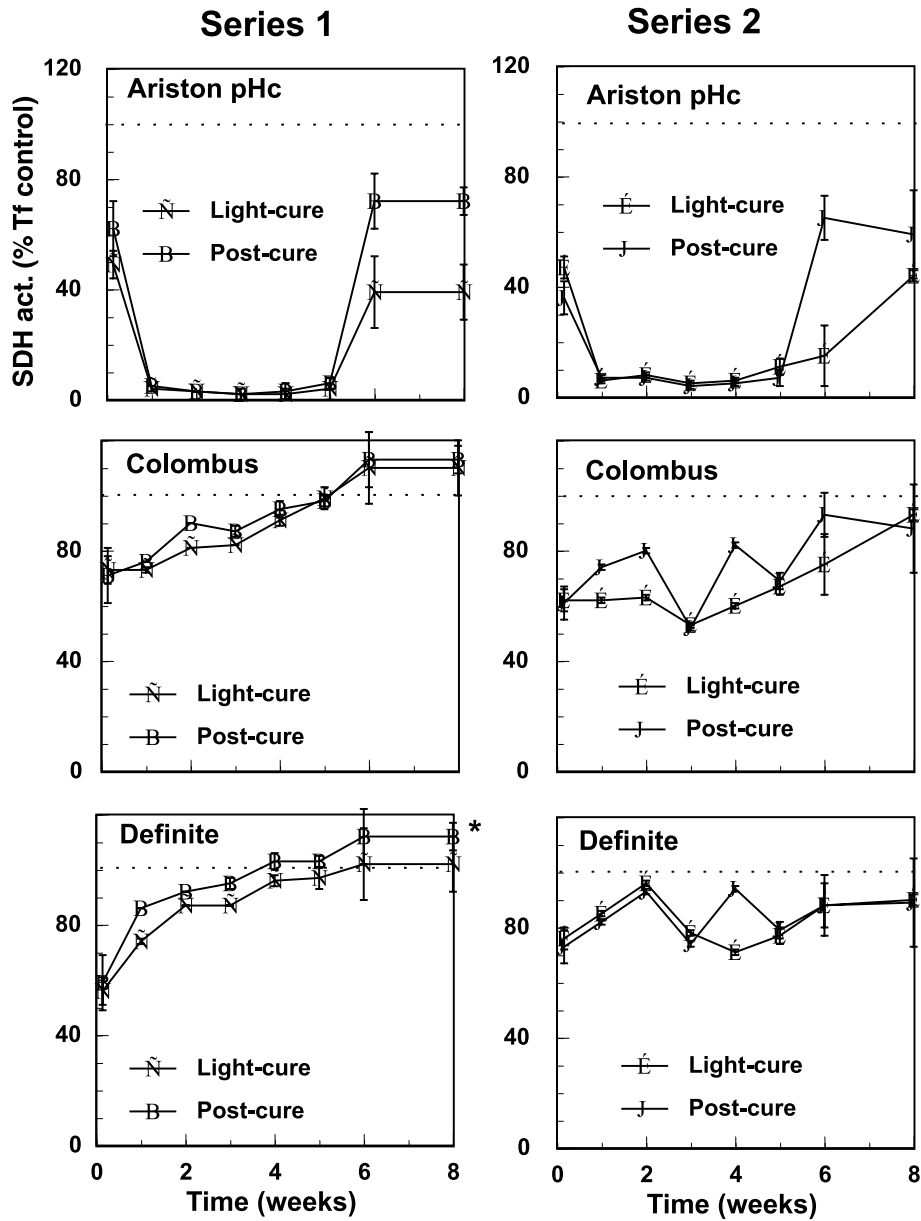
Fig. 1. Cytotoxicity of Ariston pHc, Colombus and Definite as a function of ageing time with either light-curing or light-curing and post-curing. Cytotoxicity was measured by succinic dehygrogenase (SDH) activity and expressed as a percentage of the Teflon controls defined at 100% (dashed line). The experiments were replicated completely (series 1 and 2). There were six replicates per condition. Asterisks indicate a statistically significant difference between the light- and post-cured condition at that ageing time (ANOVA, Tukey intervals, $\alpha = 0.05$).

of ageing. The Ariston pHc was an exception, being initially moderately toxic, but then becoming highly cytotoxic for 5 weeks before returning to initial levels. Post-curing had varied effects on cytotoxicity (Fig. 1, Series 1). Although post-cured materials were generally less toxic than light-cured, these differences were minor except for Ariston pHc at 24 h, 6 and 8 weeks.

In general, the materials were more cytotoxic after 24 h of ageing than after 8 weeks (Fig. 2). At 24 h, the Colombus was slightly but statistically less cytotoxic than Definite or Ariston pHc when the materials were light-cured. After post-curing, there were no statistical differences at 24 h between the three materials. At 8 weeks, the Colombus and Definite were the same
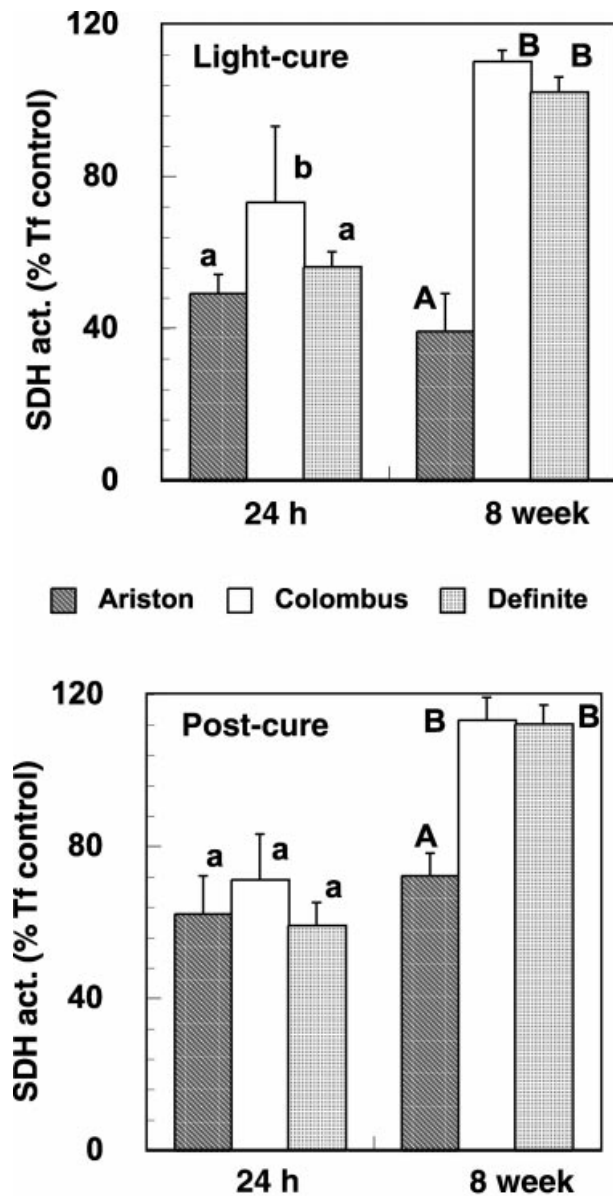
Fig. 2. Comparison of cytotoxicity of Ariston pHc, Colombus and Definite at 24 h and 8 weeks of ageing after either light-curing or light-curing and post-curing. Cytotoxicity was measured by succinic dehygrogenase (SDH) activity and expressed as a percentage of the Teflon controls. There were six replicates per condition. Different letters indicate a statistically significant difference between the materials at 24 h or 8 weeks (ANOVA, Tukey intervals, α = 0·05).

regardless of curing method and were both significantly less toxic than the Ariston pHc.

The assay appeared repeatable, but there were some discrepancies in the two experimental series (Fig. 1). In general, all materials had the same cytotoxic trends over time, and the curing method behaved similarly. However, the absolute cytotoxic percentages varied by as much as 20% between series. Furthermore, the second series had somewhat less consistency week-to-week.

Discussion

The results of the current study agreed closely with those of previously published studies for Definite (Willershausen *et al.*, 1999; Wataha *et al.*, 1999a). A comparison between studies could only be made at 1 and 2 weeks for Definite, as the previous study did not age specimens beyond 2 weeks and did not study all materials (Table 2). The agreement between the two studies is interesting given that the methods used to test cytotoxicity were different. In the previous study, cytotoxicity was evaluated by direct contact between the composite specimen and the cells. In the current study, the cytotoxicity was measured using extracts of the specimens. Such close agreement between direct and indirect methods was surprising given other reports with metallic alloys which showed differences in cytotoxicity between these methods (Wataha *et al.*, 1999b). Each method retains advantages and disadvantages. The indirect method requires only one set of specimens for multiple time points, as the specimen can be extracted repeatedly. However, the direct method allows a more legitimate comparison between ageing intervals as each set of specimens is statistically independent. The important point is to be aware that these two strategies may or may not give the same results depending on the materials and times of the measurement.

The current study demonstrates the importance of using multiple time points to study the cytotoxicity of a material *in vitro*. The time trends shown in Fig. 1 give a better indication of the *in vitro* cytotoxicity than could be obtained from any one time interval. The results for Ariston pHc show the difficulty in using a single time point to assess cytotoxicity because this material was initially far less toxic than in several subsequent intervals. Furthermore, the use of a test which measures cellular function rather than morphology is critical. In the current study, cells were visible and apparently healthy under light microscopy during the 1–5-week intervals for Ariston, but the SDH assay showed unequivocally that the cells were severely compromised (Fig. 1).

The lack of significant reduction in the cytotoxicity of the materials after post-curing was somewhat

Table 2. Comparison cytotoxicity of Definite measured using direct and indirect techniques

Method	At 1 week	At 2 weeks
Direct*	80	91
Indirect[†]	75	93

Results are percentage of SDH activities of controls.
*From Wataha *et al.* (1999a).
[†]From current study.

surprising because our hypothesis was that post-curing should reduce leachable and therefore cytotoxicity (Fig. 1). This result could have been caused by the relatively complete polymerization during the light-cure procedure. The samples were only 1 mm thick and were cured for 60 s from each side under a Mylar sheet which should have limited oxygen inhibition (Rueggeberg & Margeson, 1990). Such ideal conditions may not occur clinically and post-curing may have more of an effect under non-ideal conditions. The light-curing conditions were chosen to assure a relatively thorough cure of the composites as cytotoxicity of uncured composites are known to be severe (Hanks *et al.*, 1991).

Although the trends between the two experimental series were similar, there were discrepancies (Fig. 1). The absolute percentages and week-to-week variation were not as good in series 2. These results show that the basic procedure used in the current study is sound, but is also sensitive to many potential technical problems. For example, variation in the extraction process might alter the results as the medium can easily be transferred with the disc to the next interval. Other technical variables such as suctioning technique during the medium change and MTT assay, and even plating of cells can all contribute to the variability observed between series 1 and 2.

The severe cytotoxicity observed for Ariston pHc in weeks 1–5 was surprising because this 'smart' material should have released relatively little mass under controlled pH conditions (pH = 7·34). Although a material designed to release ions as therapeutic agents might appear cytotoxic *in vitro*, the clinical release of these ions might be acceptable. However, for Ariston this release should not have occurred significantly at pH 7·34, and to be clinically useful, any 'therapeutic' release should have continued past 8 weeks. Thus, the value of the 'smart' material strategy appeared questionable. The current study did not differentiate between cytotoxicity caused by release of 'therapeutic' agents versus leaching of constituents of the composite matrix.

In summary, the current study demonstrated the importance of assessing the cytotoxicity of resin composite materials at multiple times. Post-curing was not generally useful in reducing cytotoxicity of these resin-based dental restorative materials. Most materials were less cytotoxic after 8 weeks of ageing than they were in early intervals. The current study did not measure the release of components from these materials, but such measurement would be helpful to better understand the biological risks of these types of materials.

Acknowledgments

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