Cytotoxicity evaluation of methacrylate- and silorane-based composite resins

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ABSTRACT

Objectives: The objective of this study was to investigate and compare the cytotoxic effects of four composite resin materials with different content.

Material and Methods: Two traditional methacrylate-based (Clearfil AP-X, RefleXions), as well as a self-adhering methacrylate-based (Vertise Flow) and a silorane-based (Filtek Silorane) composite resin were tested in the experiment. Ten cylindrical specimens were made of each material, using a mould (2mm. thick and 8 mm. in diameter). An agar diffusion method was employed, and cytotoxicity rankings were determined using lysis index scores. For statistical analysis, Kruskal-Wallis and Mann-Whitney U-tests were used.

Results: Amongst the composite resins, the silorane-based composite was found to be less cytotoxic than the methacrylate-based composite resins, which all had the same cytotoxicity ranking.

Conclusions: The silorane-based composite resin was considered more biocompatible than the methacrylate-based composite resins.

Keywords: Cytotoxicity, methacrylate-based composite resin, silorane-based composite resin, self-adhesive composite resin.

INTRODUCTION

Restorative composite resins have undergone continuous development during the most recent decades.[1] The clinical success of these materials, however, depends not only on their physical and chemical properties, but also on their biological safety.

The composition of dental composites is chemically complex since they contain a great variety of different monomers and additives.[2,3] Resin composites typically consist of a methacrylate-based resin matrix (mass fraction of about 25–30%), glass or ceramic fillers (mass fraction of about 70–75%), and a filler-matrix coupling agent.[4] The conventional organic matrix of resin composites is generally based on methacrylate chemistry, especially cross-linking dimethacrylates.[5] For the organic monomer matrix, bisphenol A glycol dimethacrylate (Bis-GMA), triethylene glycol dimethacrylate (TEGDMA), and urethane dimethacrylate (UDMA) are widely used in dental composites.[6] The organic polymerized matrix seems to be responsible for most of the reported undesirable effects, and the filler does not appear to play a major role in the biocompatibility of dental composites, despite the organic component.[7]

The state of the art of the composition of dental composites has been changing rapidly in the past few years. Current changes are more focused on the polymeric matrix of the material, principally to develop systems with reduced polymerization shrinkage and to make them self-adhesive to the tooth structure.

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Recently, Weinmann et al.[1] reported a new monomer system named "*silorane*", with the combination of hydrophobic siloxane and low-shrinkage ring-opening oxirane. The silorane-based composite provides verified lower shrinkage than typical dimethacrylate-based resins, likely due to the epoxide curing reaction, which involves the opening of an oxirane ring. This new resin composite has been shown to have good properties, such as low shrinkage,[1] lower contraction stress,[9,10] low water absorption and water solubility[11] and good polishing.[12]

The latest news is the development of self-adhering flowable composite resins. These formulations are based on traditional methacrylate systems, but they incorporate acidic monomers typically found in dentin bonding agents such as glycerol phosphate dimethacrylate (GPDM), which may be capable of generating adhesion through mechanical and possibly chemical interactions with the tooth structure. These materials are currently recommended for use as liners and in small restorations, and are serving as the entry point for universal self-adhesive composites.[8]

The cytotoxicity evaluation of dimethacrylate-based resin composites has been widely studied,[13,18] while few studies have been carried out on silorane-based resin composites[19,20] and apparently no previous research has been established on self-adhering resin composites. Hence, it is worth comparing the cytotoxicity of newly introduced resin composites: self-adhering resin composites based on adhesive/methacrylate co-monomers and posterior restorative resin composites based on silorane.

The aim of this study was to investigate and compare the cytotoxic effects of two traditional methacrylate-based (Clearfil AP-X, RefleXions), as well as a self-adhering methacrylate-based (Vertise Flow) and a silorane-based (Filtek Silorane) composite resin were tested in the experiment.

MATERIALS AND METHODS

Specimen preparation

Four different composite resins were tested in the experiment: Clearfil AP-X, RefleXions, Vertise Flow, and Filtek Silorane. Table 1 shows the components and details of the composite resins that were used. Ten cylindrical specimens were made of each material, using a mould (2 mm. thick and 8 mm. in diameter), according to ISO 10993 recommendations. The mould was placed onto a glass plate and the composite material was condensed into the mould from the top, while the flowable material (Vertise Flow) was delivered directly via syringe. A thin Mylar strip was placed on top of the specimen, followed by a 1-mm glass slide on top of the mould to extrude excess composite material and standardize the distance between the composite specimens and the curing light's tip. Each specimen was cured for 20 seconds using a Quartz-tungsten-halogen light (Hilux, Benlioglu, Ankara, Turkey) according to the manufacturers' instruction. The output of the curing unit was measured with a curing radiometer to ensure light intensity at a constant value of 550 mW/cm^2. All procedures were carried out aseptically. After curing, the specimens were kept in the dark at room temperature for 24 h before testing, and then they were sterilized with ethylene oxide gas.

Cultivation of L929 mouse fibroblast cells

A mouse connective tissue fibroblast cell line, L929 (ATCC cell line, NCTC clone 929), was cultured in Dulbecco's minimum Eagle medium (DMEM) (Sigma, St. Louis, MO,USA) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO,USA) and 2 mM/ml L-glutamine. No antibiotics were added to the cell culture medium. The cultures were cultivated in an incubator at 37 °C and 5% CO2, until the cell monolayer attained confluence, after

Table 1. Test materials, types, lot numbers, manufacturers and components.

Test materials	Type	Lot number	Manufacturer	Components
Clearfil AP-X	Micro hybrid	1038AA	Kuraray Medical INC. Okayama, Japan	Silanated bariumglass, Silanated colloidal silica, silanated silica, Bis-GMA, TEGDMA, dl-Camphorquinone
Reflexions XLS	Nanohybrid composite	1000005127	Bisco, Inc., Schaumburg, IL, USA	Ethoxylated Bis-GMA Glass Filler Amorphous Silica
Vertise Flow	Self-adhering flowable composite	34402	Kerr Corporation, Orange, CA	GPDM, methacrylateco-monomers Prepolymerized filler, barium glass, nano-sized colloidal silica, nano-sized ytterbium fluoride
Filtek Silorane	Posterior Composite	N338417	3 M Espe AG Seefeld, Germany,	3,4-Epoxycyclohexylethylcyclo-polymethylsiloxane,bis-3,4 epoxycyclohexylethylphenylmethylsilane

approximately 7 days. Assays were always performed in the exponential growth phase of the cells.

Agar diffusion method

The agar diffusion tests were performed according to international standards (International Standard ISO 10993- 5 1999). Briefly, the cultures were harvested using 0.25% trypsin solution (Gibco, Germany). Stock cultures were seeded in 35-mm diameter cell culture petri dishes (Nunc, Wiesbaden, Germany), at a density of $1\times l0^6$ cells per petri dish, and subcultured once a week. After the formation of the confluent cell layer, the medium was removed and replaced with complete medium containing 1.5% agarose (FMC BioProducts, Rockland, ME, USA). After solidifying the agarose, the cells were stained with a vital dye (neutral red; Sigma). During the experimental procedures, the cells were protected from light to prevent cell damage elicited by photo-activation of the stain. Test specimens were placed on the agar surface so that the bottom surface of each specimen was in contact with the agar. A phenol-impregnated blotting paper was used as positive control and a DMEM-impregnated blotting paper as negative control. After an exposition period of 24 h at 37°C, the cell responses were evaluated by inverted microscope observation. In this study, cell lysis was scored as follows: 0 = no cell lysis detectable; 1 = less than 20% cell lysis; 2 = 20–40% cell lysis; 3 = > 40 to < 60% cell lysis; 4 = 60–80% cell lysis; 5 = more than 80% cell lysis. For each specimen, one score was given, and the median score value for all parallels from each specimen was calculated for the lysis zone. Cytotoxicity was then classified as follows: 0–0.5 = noncytotoxic; 0.6–1.9 =

mildly cytotoxic; 2.0–3.9 = moderately cytotoxic; 4.0–5.0 = markedly cytotoxic. The median (rather than the mean) was calculated to describe the central tendency of the scores, given that the results were expressed as an index in a ranking scale.

Statistical tests were performed using SPSS（Version 14.0, SPSS Inc., Chicago, IL, USA). Data were analysed statistically using Kruskal-Wallis and Mann-Whitney U-test. The level of significance was set at p=0.05.

RESULTS

Means and standard deviations of cytotoxicity scores for each group are given in Table 2. Statistical results of the Kruskal-Wallis for cytotoxicity scores of the groups are represented significant differences among the composite resin groups (*KW*= 23.49 and p=0.018).

Filtek Silorane demonstrated statically significant difference between all of the composite resins tested. According to Mann-Whitney U test, there were significant difference between Group 4 and Group 1(p=0.005); Group 4 and Group 2 (p=0.005); Group 4 and Group3 (p=0.005). However, there were no differences between Group 1 and Group 2 (p=0.805), Group 1 and Group3 (p=1.00); Group 2 and Group 3 (p=0.805).

Table 2. Mean and standard deviation for all groups. Means with different superscript are statistically different at p<0.05.

	Mean + Standard Deviation
Clearfil AP-X	0.70 ± 0.67^{a}
Reflexions XLS	0.80 ± 0.78^{a}
Vertise Flow	0.70 ± 0.67^{a}
Filtek Silorane	0.00 ± 0.00^{b}

DISCUSSION

The aim of this study was to compare the cytotoxicity of four resin composites (Clearfil AP-X, RefleXions, Vertise Flow, and Filtek Silorane). Therefore, these materials were chosen according to the differences in their compositions.

One widely accepted definition of biocompatibility is ‘the ability of a material to elicit an appropriate biological response in a given application’.[21] In the development of any restorative biomaterials, biocompatibility must be considered in addition to durability, aesthetics and ease of clinical manipulation.[22] With the rapid development of new composite biomaterials in restorative dentistry, it is imperative to develop reliable and clinically relevant cytotoxicity screening tests.[23]

Biocompatibility is measured with 3 types of biological tests: *in vitro* tests, animal tests, and usage tests.[22,24] *In vitro* tests are simple, reproducible, cost-effective, relevant, and suitable for evaluating basic biological properties of dental materials.[25] To date, there are a variety of different *in vitro* test models for cytoxicity screening of biomaterials. These include: ‘direct contact tests’, where the biomaterial contacts the cell system directly without barriers;[27,28] ‘indirect contact tests’, where there is a barrier between the biomaterial and the cell system (i.e., an agar layer or a Millipore filter);[29,31] and ‘extract tests’, whereby eluants from composite biomaterials are exposed to the cells.[29,32] In the present study, the agar diffusion test (ADT) was chosen to evaluate cytotoxicity.

Fibroblasts were used for cytotoxicity testing since they are an ISO-approved cell type and are the most common cell type in the pulp, which would be the target of any chemical components that may be released from the composites if the odontoblastic

layer has been destroyed.[33] As well, they are easy to cultivate, given their favourable doubling time of 24 h.

In the present study, we used the L929 mouse fibroblast cell to test the cytotoxicity of four different resin composites. The results of cytotoxicity testing of the materials showed that methacrylate-based composites (Clearfil AP-X, RefleXions, Vertise Flow) were mildly cytotoxic (less than 20% cell lysis) and the silorane-based composite resin (Filtek Silorane) was noncytotoxic to the cells.

Both resin content and percentage of monomer conversion of dental materials were considered as potential causes of cytotoxicity.[34] Previous studies reported that Bis-GMA, TEG-DMA, and UDMA have been shown to be cytotoxic in sufficient concentrations.[2,14,20]

Under the conditions of the present study, while there was no significant difference among methacrylate-based composite resin groups, RefleXions showed somewhat higher cytotoxicity than the others. RefleXions composite resin contains ethoxylated bisphenol A dimethacrylate (Bis-EMA), according to the manufacturer's information. A previous study by Hanks et al.[14] demonstrated cytotoxic effects of some resin components on DNA and protein synthesis on 3T3 fibroblasts and found Bis-EMA as the most toxic, followed by UDMA and Bis-GMA; TEGDMA was slightly less toxic. Also, Ergun et al.[35] found that resin composites that consist of Bis-EMA agent showed the lowest cell survival rate values, and they reported that this agent might be the most toxic among all of the dimethacrylates they studied.

Some studies have shown that flowable materials were more cytotoxic than traditional composites.[17,33] They claimed that the difference in cytotoxicity between the flowable composites and their traditional composites could be related to the difference in the chemical composition of these materials and the increase in mass release. However, in this paper, although Vertise Flow has a flowable composition, it did not induce a higher cytotoxic effect then other methacrylate-based composite resins. Vertise Flow is based on traditional methacrylate systems, but incorporates acidic monomers typically found in dentin bonding agents, such as glycerol phosphate dimethacrylate (GPDM), which may be capable of generating adhesion through mechanical and possibly chemical interactions with the tooth structure.[8] Our finding is also in agreement with Ulker et al.,[30] who evaluated the cytotoxicity of self-adhesive composite resin cements. They demonstrated that the self-adhesive composite resin cement that contains GPDM caused the lowest cytotoxic effects.

Finally, Filtek Silorane is a material consisting of a new monomer technology that uses a combination of a siloxane backbone along with oxirane molecules and a cationic ring-opening polymerization process resulting in a polysilorane polymer.[36] Among the materials tested, it was the only one based on a different monomer technology and was found to be noncytotoxic in the present study. Information on the biocompatibility of the silorane-based composite is minimal compared with the vast amount of data reported on classical methacrylate-based materials. Krifka et al.[20] investigated the cytotoxicity generated by dental composites in human pulp cells and reported that unlike all other resin-based composite resins, silorane-based composite Hermes, a precursor of Filtek Silorane, did not test as being cytotoxic. The findings of the present study are in agreement with these previous reports. Also, Castañeda et al.[37] had evaluated the tissue compatibility of Filtek Silorane after implantation in subcutaneous connective tissue of isogenic mice and claimed that it had shown tissue compatibility in vivo.

The change in the chemical structure of the composite and the variation in the ratio

of filler and monomer have a significant effect on the element release and cytotoxicity level of the materials. [17] Eick et al.[38] found that siloranes were stable and insoluble in biological fluids simulated using aqueous solutions containing either epoxide hydrolase, porcine liver esterase or dilute HCl. Alternatively, silorane-based composites are another approach for the reduction of polymerization shrinkage and the prevention of biologically adverse effects caused by restorative resin materials, probably due to the low solubility of individual compounds in water.[1]

Additionally, the mechanisms of cytotoxicity are related firstly to the short-term release of free monomers occurring during the monomer–polymer conversion.[7] Many *in vitro* studies have shown that an unpolymerized monomer oxygen inhibition layer was observed on the surface,[39,40] which has been implicated in increased resin toxicity.[41] Yesilyurt et al.[6] reported that there was no oxygen inhibition layer on the surface of Filtek Silorane after polymerization. This meant that the number of unreacted monomers on the surface would be lower than the methacrylate-based composite resins. Silorane-based composite resins are promising well for reducing the release of uncured components and, consequently, their biocompatibility.

CONCLUSION

Within the limitations of this *in vitro* study, the following conclusion could be drawn: The cytotoxic effects in the cell culture showed dependence on the type of resin composite. While Filtek Silorane was non-cytotoxic, the other composite resins caused mildly cytotoxicity. Before initiating any restorative procedure, it is important to select the most appropriate dental materials. Attention should be given not only to the handling characteristics of the materials, but also to the possible cytotoxic effects that they may cause to the oral mucosa and teeth.

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