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## CLINICAL INVESTIGATION

# In vitro Cytotoxicity of Glass and Carbon Fiber-Reinforced Heat-Polymerized Acrylic Resin Denture Base Material

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**Abstract:** Acrylic resin dentures may have cytotoxic effects on oral tissues. However, there is sparse data about the cytotoxic effect of fiber-reinforced acrylic resin materials. The aim of this in vitro study was to determine the cytotoxicity of a heat-polymerized acrylic resin denture base material reinforced with glass, carbon fibers and unreinforced acrylic resin denture base material. 100 acrylic resin discs were assigned to 5 experimental groups (n=20) and 1 control group. Group NOF did not receive any fiber. Roving type glass (Vetrolex) and carbon fibers (Type Tenox J) were introduced into the acrylic resin specimens of Group RGF and Group RCF with the "embedding" technique. Particulate glass and carbon fibers were impregnated into the specimens of Group PGF and PCF with the "blending" technique. Gingival fibroblast cells were exposed onto the test specimens and the cytotoxicity was determined by succinic dehydrogenase activity (MTT method) after 24 and 72 hours incubation. Data were analyzed with the 1-way-Anova test. Cell proliferation rates after 24 and 72 hours incubation ranged as follows: NOF (89%-83%)> RGF (80%-77%), PGF (80%-76%), RCF (79%-75%), PCF (77%-73%). All groups displayed significant cytotoxicity compared to control cell culture. Fiber reinforced groups were significantly more cytotoxic than the unreinforced group. No significant difference of cytotoxicity was found between the fiber-reinforced groups.

**Key Words:** Cytotoxicity, denture base materials, glass fiber, carbon fiber, cell culture.

## Introduction

The number of denture wearers is increasing as the number of elderly people continually growing, and polymethyl methacrylate (PMMA) is still the most frequently used material in denture base fabrication (1). Despite its satisfactory aesthetic properties, ease of processing and accurate fit, PMMA resin has the potential to elicit irritation, inflammation and allergic reaction in the oral mucosa. Toxic compounds such as formaldehyde, methyl methacrylate, methacrylic acid, benzoic acid, dibutyl phthalate, phenyl benzoate, phenyl salicylate and dicyclohexyl phthalate existing in the chemical composition of the acrylic resin can cause hypersensitization and allergy in dental laboratory personnel and denture wearer prior and after the polymerization (2). Released residual methyl methacrylate is one of the principal factors affecting the biocompatibility and cytotoxic potential of an acrylic resin

denture base. The content and release of residual methyl methacrylate from denture polymers and their cytotoxic effects have been assessed in earlier studies (3-7). It was stated that residual monomer release is dependent on the temperature and time of the polymerization, the processing method and the type and thickness of the acrylic resin (8-10). Previous studies involved the cytotoxic effects of acrylic resins in relation with the pH of the oral environment, the immersion time in oral fluids and the chemical structure of the acrylic material used (11-13).

There are only few studies that evaluated the cytotoxicity of fiber-reinforced acrylic resin dentures. In their in-vitro study performed with high performance chromatography, Miettinen and Vallittu (14) reported that the use of glass fiber reinforcement in heat-polymerized acrylic resin increased the release of residual methyl methacrylate. Yilmaz et al (15) tested the effect

of glass fiber reinforcement on the residual monomer content of a heat-polymerized and an autopolymerized denture base resin, and stated that glass fiber reinforcement increased the residual monomer content of the both denture base resins. Vallittu and Ekstrand (16) investigated the cytotoxicity of fiber-PMMA composite used in dentures with agar diffusion test and determined that neither the unreinforced PMMA nor the fiber-PMMA composite was cytotoxic. Ekstrand et al (17) assessed the cytotoxicity of leachable elements from carbon-graphite fibers subjected to different surface treatments. They used the agar overlay technique to determine the cytotoxicity and found that fibers with cleaned surfaces were less cytotoxic than the non-treated ones.

The cytotoxicity of fiber-reinforced acrylic resin dentures is a controversial issue because it is difficult to achieve adequate impregnation of reinforcing fibers with resins because of the high viscosity of the polymer/monomer mixture or dough (18-20). This problem was solved by the treatment of fibers with silane coupling agents (2) or by impregnation of the fibers with relatively low viscosity polymer powder and monomer liquid mixture (21-22). The viscosity of the mixture can be changed by altering the powder/liquid ratio. Reduced viscosity should theoretically improve the impregnation of the fibers into the resin by increasing their wettability (18-23). Effective impregnation allows the fibers to make contact with polymer matrix of the resin. This is necessary both for the fibers to bond to the polymer matrix and for the strength of the composite (20, 23, 24). However, the high proportion of the monomer liquid in the mixture may increase residual methyl methacrylate content in the fiber-polymethyl methacrylate composite (14). Therefore, it appears that the fiber impregnation method could affect the cytotoxicity of fiber-reinforced

acrylic resins, and this fact has not yet been thoroughly clarified. The purpose of this study was to determine the cytotoxic effects of a glass, carbon fiber-reinforced and unreinforced heat-polymerized acrylic resin denture base material on gingival fibroblasts.

**Materials and Methods**

One hundred acrylic resin discs were prepared and assigned to 5 experimental groups (n = 20) (Table). Group NOF did not include any fiber. The “embedding technique” was used to impregnate unidirectional roving glass fibers (Vetrolex RC, 14-800-P109, Ciba Composites, Birmingham, UK) into the acrylic resin specimens of Group RGF, and to impregnate unidirectional roving type carbon fibers (Type Tenox J, s131-800-TEX, Ciba Composites, Birmingham, UK) into the acrylic resin specimens of Group RCF. The “particle blending technique” was used to impregnate particulate roving glass fibers into the acrylic resin specimens of Group PGF, and to impregnate particulate roving carbon fibers into the acrylic specimens of Group PCF. An untreated cell culture was used as control.

**Specimen preparation**

Cast brass discs, 1.5 mm thick and 10 mm in diameter, used for preparing the test specimens were flaked into a Type II dental stone (Moldano, Bayer, Germany) such as to have five discs in each flask. All flasks were compressed in a hydraulic pressing apparatus (KaVo Elektrotechnisches, D-7970, Werk Germany) under a pressure of 100 bars. A heat-polymerized acrylic resin denture base material (QC-20, Dentsply Int Inc, Waybridge, Surrey, UK) was prepared according to the manufacturer’s instructions.

Table. Classification of the test groups.

TEST GROUP	(n)	TYPE OF FIBER	FIBER ADDING METHOD
NOF	20	NO FIBER	NONE
RGF	20	ROVING GLASS FIBER	EMBEDDING
PGF	20	PARTICLE GLASS FIBER	PARTICLE BLENDING
RCF	20	ROVING CARBON FIBER	EMBEDDING
PCF	20	PARTICLE CARBON FIBER	PARTICLE BLENDING

### Preparation and impregnation of fibers

The fiber content was calculated from the initial weight of the specimens to an accuracy of 0.01 mg using a digital weighting device (Mettler, Zurich, Switzerland). The weight of fibers was determined with a ratio of 10% by weight of the polymer matrix and impregnated with appropriate matching polymer/monomer mixture (18). The weight of the test discs was 0.245g and the weight of the incorporated fibers was calculated as 0.025 g.

The particle blending and embedding methods were used to incorporate fibers into the resin bulk. In the particle blending method, the unidirectional roving glass and carbon fibers were cut with a lancet into small particles ( $0.3 \pm 0.1$  mm). The calculated mass of fibers was first mixed thoroughly with a predetermined volume of methyl methacrylate liquid, and then the required mass of powder was added to the mix and stirred so that the fibers would be randomly oriented to give isotropic properties to the composite. The polymer/monomer ratio was 10g/8ml for all specimens (18). This higher monomer ratio ensured better impregnation of the fibers into the acrylic resin bulk (18-23). The fibers were dipped in the monomer before being introduced in the resin bulk to provide penetration of the acrylic resin into the fiber roving. The acrylic resin dough was then pressed and two trial closures were made in the mould to remove excess resin before heating.

In the fiber embedding method, roving type glass and carbon fibers were cut and shaped to fit the circular form of the specimens and were horizontally embedded into the center of the acrylic resin dough after the first test pressing. Following two trial closures to remove the excess resin, all flasks were closed, pressed and heated for 30 minutes at 75 °C and an additional 30 minutes at 100 °C. After the completion of polymerization, the excessive acrylic resin tips of the specimens were removed with tungsten carbide burs and all specimens were immersed in distilled water at 24 °C for 24 hours. At the end of this period, all specimens were subjected to ethylene oxide gas sterilization for 1 hour at 60 °C.

### Cell Culture and MTT Assay

The human gingival fibroblast (GF) cells were used for the cytotoxicity test. Healthy human gingival tissue was obtained from volunteer patients undergoing extraction

from the third molar region for orthodontic reasons, and specimens were never mixed. All participants in the study gave informed consent to the experimental procedures and the local ethic committee consent was obtained. Immediately after removal, the tissue was placed in Hanks salt solution containing penicillin/streptomycin (Sigma, St. Louis, MO) and amphotericin B (Sigma, St. Louis, MO). Thereafter, biopsies were stored at 4 °C for no longer than 6 hours. Specimens were minced into small pieces and fibroblasts were cultivated with 5% CO<sub>2</sub> at 37 °C in Dulbecco's Modified Eagle's medium (DMEM, F-12, Biochrom, Berlin, Germany) and 10% fetal calf serum (FCS) containing penicillin/streptomycin and amphotericin B (25-27). Cells were plated in 24-well tissue culture trays ( $10^4$  cells/cm<sup>2</sup>). The passage number was 3.

For the cytotoxicity test, the test specimens were placed in the center of 24-well tissue culture trays. After 24 and 72-hour incubation periods, the test specimens were removed from the culture wells and the cytotoxicity of the materials was assessed using the MTT (3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide) method, which has previously been described (28, 29). MTT (5 mg/mL in Hanks balanced salt solution) was added to each well, and the microplates were further incubated at 37°C for 4 hours. After the incubation period, 100 µL of acidified isopropanol (0.04 N HCl in isopropanol) was added to the cultures and mixed thoroughly to dissolve the dark blue crystals of formazan. The solubilized reaction products were transferred to a 96-well plate, and the absorbance values of each well were determined with a microplate enzyme-linked immuno-assay (ELISA) reader equipped with a 570-nm filter. Survival rates of the controls were set to represent 100% viability. Each specimen was tested three times and untreated cell cultures were used as controls. The results were expressed as "percentage cell viability" determined as:

$$100 - [A_{\text{experimental well}} / A_{\text{positive control well}}] \times 100.$$

### Statistical Analysis

Data were analyzed with a 1-way ANOVA test to determine differences between the groups ( $\alpha = .05$ ). A value of  $P < .05$  was considered statistically significant.

### Results

Cell proliferation rates of the experimental groups compared to control cell culture after 24 and 72 hour incubation (Figure) ranged as follows: Cell culture control (100%)> NOF (89%-83%)> RGF (80%-77%), PGF (80%-76%), RCF (79%-75%), PCF (77%-73%). At the end of 24 and 72 hours incubation periods, all experimental groups displayed significantly lower proliferation rates than the cell culture control ( $P < 0.001$ ). The fiber-reinforced groups displayed significantly lower proliferation rates than Group NOF ( $P < 0.001$ ). No significant difference was found between the fiber-reinforced groups. The average decrease in proliferation rates was about 19% at the end of the first 24 hours, and 4.2% at the end of 72 hours periods. The cytotoxic effects observed at the end of the initial 24 hours were approximately fivefold greater than those observed at the end of 72 hours.

### Discussion

Biologic and toxicologic properties of dental materials are important in relation to their clinical usage. In vitro cytotoxicity tests are a necessary screening step in the testing of new materials used in humans (13). Human gingival fibroblast (HGF) cells were obtained as primary cultures from explants of biopsies. Primary cultures have a more normal phenotype and they correlate to in vivo response more accurately. Furthermore, the use of HGF permits enhanced relevance because such cells are exposed to denture base resins when ulceration of epithelium occurs after denture insertion.

In the present study, the cytotoxicity of a glass and carbon fiber reinforced heat-polymerized acrylic resin material was investigated. It was found within the limitations of the study, that all tested experimental groups displayed mild cytotoxicity compared to control cell culture at the end of 24 and 72 hours incubation periods. It was also noticed that fiber-reinforced groups were more cytotoxic than the unreinforced group. Our findings are in correlation with those of Miettinen and Vallittu (14), and Yilmaz et al (15) who reported that the use of glass fibre reinforcement in heat-cured denture PMMA increases the release of residual monomer from the material.

However, Vallittu and Extrand (16) reported that fibre-polymethyl methacrylate composites were not cytotoxic to oral epithelial cells. It may appear at first sight that this statement is contradictory to the findings of the present study. Nevertheless, it is thought that the observed cytotoxicity of the present study is not inherent to the fibers used, but the fiber impregnation methods. Adequate impregnation of fibers with resin is generally considered difficult because of the high viscosity of polymer/monomer mixture dough (18-20). This problem was solved with the impregnation of the fibers with a relatively low viscosity of polymer powder and monomer liquid mixture (2, 21, 22). Reduced viscosity should theoretically improve impregnation of fibers with the resin. It was stated the high proportion of the monomer liquid in the mixture to improve impregnation of the fibers into the resin could increase the residual monomer content and increase the cytotoxic effects (14, 15). To effectively determine the cytotoxic potential of glass and

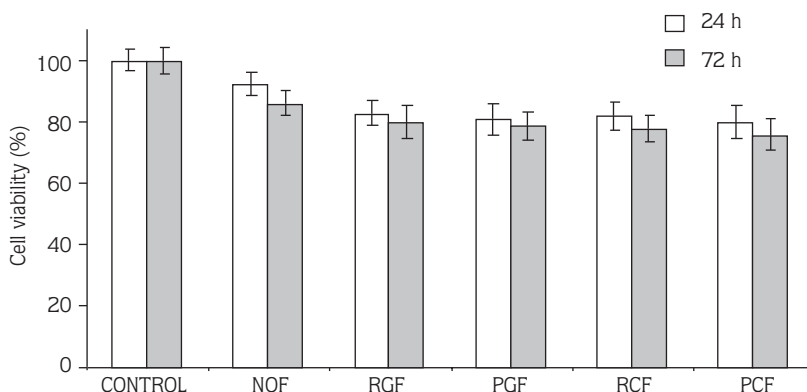


Figure. Cell viability rates of the test groups after 24 and 72 hours incubation periods. Data depict the mean ± SD of three separate experiments.

carbon fibers, the particle blending method was used in Groups PGF and PCF, in which the fibers were randomly dispersed in the acrylic resin bulk and surface, to provide a direct contact of the fibers to the cells so as to easily affect their viability. However, no significant difference of cytotoxicity was found between RGF and RCF test groups in which fibers were incorporated within the specimens with the embedding method. Moreover, the particle blending method is not clinically practicable due to its potential to cause roughness, unaesthetic appearance, and mechanical irritation to oral tissues.

In the present study, the average decrease in proliferation rates was about 19% at the end of the first 24 hours, and 4.2% at the end of 72 hours incubation periods. The cytotoxic effects observed at the end of the initial 24 hours were approximately fivefold greater than those observed at the end of 72 hours. This finding is in accordance with that of Sheridan et al (11) who reported that the cytotoxic effect of acrylic resins was greater in the first 24 hours after polymerization and decreased with time for all the resins evaluated in their study. The authors concluded that the longer the denture is soaked, the less likely are the cytotoxic effects to occur. Lefebvre et al (12) observed the effects of substances released from 4 light-polymerizing denture base resins on hamster oral epithelial cells. Their findings indicated that components released by light-or heat-polymerized acrylic resins may produce toxic effects on oral epithelial cells, leading to greater cellular inhibition in the initial 24-hour period on the basis of cell numbers. It was also reported that most unbound substances are liberated from polymerized resins within the first 24 hours (13).

To minimize tissue reactions for allergic patients, acrylic resin bases which do not include colorant components or resins with decreased residual monomer content must be preferred (30). It was suggested that allergic reactions of the underlying soft tissues against denture base material was the major etiologic factor of denture sore mouth and that it was rarely observed with

well-polymerized dentures. It was also stated that the chemotoxic effect of an irritant heat-polymerized acrylic resin denture can be eliminated by repeating the heating process (31). It has been reported that the level of cytotoxicity differs in different systems and cell lines (3). In this study, human gingival fibroblasts were used to simulate the gingival tissues.

The in vitro cytotoxicity data obtained in the present study are relevant yet not directly transferable to in vivo conditions. Nevertheless, the in vivo methods could play an important role in analyzing the biocompatibility of denture base resins. It is considered that with the advent of non-PMMA denture base resin materials, there could be a significant decrease in oral allergic reactions (33). The mechanisms of cell death caused by fiber-reinforced acrylic resins still have to be elucidated. The cytotoxicity of recently introduced reinforcing-fibers in prosthetic field is to be considered in future investigations.

## Conclusion

It was determined that glass and carbon fiber-reinforced heat-polymerized acrylic resin was found moderately cytotoxic by decreasing the proliferation of gingival fibroblasts by approximately 20%. No difference in cytotoxicity was found between fiber-reinforced groups and the fiber impregnation methods. The unreinforced acrylic resin was significantly less cytotoxic than the reinforced groups. The average decrease in proliferation rates was about 19% at the end of the first 24 hours, and 4.2% at the end of 72 hours incubation periods.

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