Cytotoxicity of Denture Base Resins: Effect of Water Bath and Microwave Postpolymerization Heat Treatments

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Purpose: This study compared the effect of two postpolymerization heat treatments on the cytotoxicity of three denture base resins on L929 cells using ^{3}H-thymidine incorporation and MTT assays. ***Materials and Methods:*** Sample disks of Lucitone 550, QC 20, and Acron MC resins were fabricated under aseptic conditions and stored in distilled water at 37°C for 48 hours. Specimens were then divided into three groups: *(1)* heat treated in microwave oven for 3 minutes at 500 W; *(2)* heat treated in water bath at 55°C for 60 minutes; and *(3)* no heat treatment. Eluates were prepared by placing three disks into a sterile glass vial with 9 mL of Eagle's medium and incubating at 37°C for 24 hours. The cytotoxic effect from the eluates was evaluated using the ^{3}H-thymidine incorporation and MTT assays, which reflect DNA synthesis levels and cell metabolism, respectively. ***Results:*** The components leached from the resins were cytotoxic to L929 cells when ^{3}H-thymidine incorporation assay was employed. In contrast, eluates from all resins revealed noncytotoxic effects as measured by MTT assay. For both MTT assay and ^{3}H-thymidine incorporation, the heat treatments did not decrease the cytotoxicity of the materials tested. ***Conclusion:*** Resins were graded by ^{3}H-thymidine incorporation assay as slightly cytotoxic and by MTT assay as noncytotoxic. Cytotoxicity of the denture base materials was not influenced by microwave or water bath heat treatment. *Int J Prosthodont 2004;17:340–344.*

Acrylic resin is the most commonly used material for dentures.[1] Heat-polymerized denture resins may leach residual monomers and other chemically reactive, toxic components that can cause adverse reactions in the oral mucosa adjacent to dentures. These responses have been attributed to leached residual methyl methacrylate (MMA) monomer, which can be present in denture base resins and is leached from them into saliva.[2,3] Leaching of formaldehyde, methacrylic acid, and benzoic acid from acrylic resin dental materials has also been detected.[4–7] The amount of residual monomer present is dependent on the type of denture base resin, type of polymerization reaction, duration of polymerization cycle, and thickness of resin.[1,8–12]

Methods for reducing the residual monomer contents of polymerized acrylic resins have been described in the literature. Baker et al[13] conclude that autopolymerized appliances should be immersed in water for 24 hours before being worn to minimize the possibility of residual monomer release. Others[14] demonstrate that curing autopolymerized resin in water is the key factor for reducing the quantity of residual monomer. Austin and Basker[15] show the effect on residual monomer content of introducing shortcuts in the recommended polymerization cycles. Others[3] observe that incubation in water for 60 minutes at 50°C reduces the subsequent leaching of MMA and formaldehyde, decreasing the resin's cytotoxic potential. Blagojevic and Murphy[11] report that the residual monomer content of an autopolymerizing resin is reduced

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Table 1 Materials Used

Product	Powder:liquid ratio	Polymerization cycle
Lucitone 550, Denstply	21 g:10 mL	90 min at 73°C, then 100°C boiling water for 30 min
QC 20, Dentsply	23 g:10 mL	Boil water, insert flask, return to boil, boil for 20 min
Acron MC, GC	30 mL:9 mL	Microwave for 3 min at 500 W

by almost 25% after microwaving. Another investigation showed that microwave irradiation of autopolymerized specimens increases the flexural strength of an acrylic resin repair material. This effect is related to the lowest residual monomer level obtained as a result of a higher degree of polymerization.

Biocompatibility of dental materials has been evaluated by in vitro and in vivo studies and human clinical studies.[2,4] Testing of dental materials by cell culture methods is relatively simple to perform, reproducible, and cost effective, and such tests can be carefully controlled. Different parameters, such as inhibition of cell growth, cytolysis, effects on membrane or cytoplasmic markers, and changes in metabolic activity, are used to monitor cytotoxic effects of dental materials.[16] The measurement of DNA synthesis by ^{3}H-thymidine incorporation[17–21] and analysis of the metabolism of yellow methyltetrazolium salt (MTT) by mitochondrial dehydrogenases of active cells into blue formazan crystals[18,20,22–24] are biologic assays commonly used for cytotoxicity testing.

The purpose of the present study was to compare the cytotoxicity of three denture base acrylic resins by evaluating the effects of heat treatments on the materials by ^{3}H-thymidine incorporation and MTT assay. The hypothesis that heat treatments could decrease the cytotoxicity of acrylic resin materials was tested.

Materials and Methods

Sample Fabrication

The materials used in this study are shown in Table 1. Nine specimens of each resin were individually fabricated under aseptic conditions in sterile aluminum molds 10 mm in diameter by 1 mm thick. Samples were produced according to the manufacturers' protocols. After polymerization, excess flash from the processing was removed using a sterilized trimming bur, and samples were stored in distilled water for 48 hours at 37°C. Specimens were randomly divided into three groups (n = 3): *(1)* heat treated in a microwave oven for 3 minutes at 500 W[25,26]; *(2)* heat treated in a water bath at 55°C for 60 minutes[3]; and *(3)* not heat treated. Prior to cytotoxicity tests, disks were ultrasonically cleaned in distilled water for 20 minutes and exposed to ultraviolet light for another 20 minutes to kill microorganisms that may have contaminated the disks during fabrication.[27]

Eluate Preparation and Cell Culturing

Eluates of the materials were prepared by placing three disks into a sterile glass vial (Costar, Corning) with 9 mL of Eagle's medium (Institute Adolfo Lutz) supplemented with antibiotic (80 µg/mL gentamycin) and fetal bovine serum and incubating for 24 hours at 37°C. Medium without disks was also incubated and diluted as above to serve as the negative control.

Mouse fibroblast cells (L929) were propagated in Eagle's minimum essential medium supplemented with 80 µg/mL gentamycin and 7.5% v/v fetal bovine serum. The culture was maintained at 37°C in an atmosphere of 5% CO_2 in 95% air.

Cytotoxicity Assays

The ^{3}H-thymidine incorporation and MTT assays were used to determine the cytotoxicity of the materials.

A 100-µL aliquot of L929 mouse fibroblasts (1×10^4 cells/mL) in Eagle's medium was seeded into a 96-well culture plate and incubated for 24 hours at 37°C. Then, the culture medium was replaced by 20 µL of medium containing 0.25 µCi of ^{3}H-thymidine (Amershan Pharmacia Biotech). An additional 50 µL of eluate and 50 µL of fresh medium were added to each well of a 96-well culture plate and incubated for a further 24 hours at 37°C. The cells were then harvested onto fiber filters using a multichannel automated harvester (Unifilter 96 GF/C, Packard Instrument), and the incorporated radioactivity was measured using a scintillation counter (Unifilter 96 GF/C). Four wells were used for each experimental group. All tests were repeated twice.

The MTT colorimetric assay was performed as described elsewhere.[28] L929 mouse fibroblasts in 100 µL of Eagle's supplemented medium (1×10^4 cells/mL) were seeded into 96-well culture plates and incubated for 24 hours at 37°C. After 24 hours, the culture medium was replaced by 50 µL of eluate and 50 µL of fresh medium, added to each well of the 96-well culture plate, and incubated for a further 24 hours at 37°C. Subsequently, 10 µL of MTT (Sigma Chemical) solution were added to each well and incubated for 3 hours at 37°C. After this time, 100 µL of acid-isopropanol were added to each well and mixed thoroughly to dissolve the dark blue crystals. Cellular viability was determined by

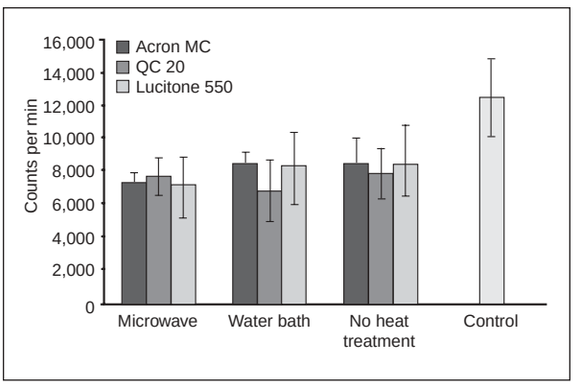

Fig 1 Mean and standard deviation of ^{3}H-thymidine incorporation assay results for all experimental and control groups.

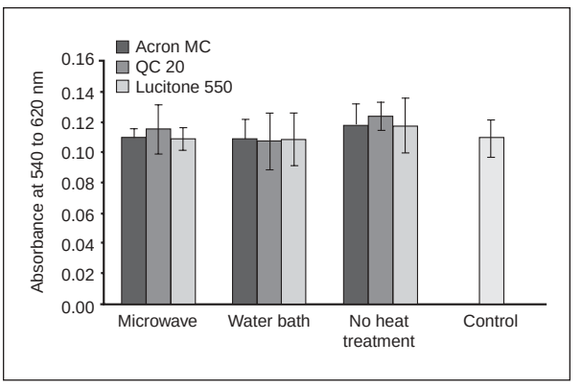

Fig 2 Mean and standard deviation of MTT assay results for all experimental and control groups.

calculating the difference between absorbance at 540 nm and at 620 nm using a spectrophotometer (Labsystems Multiscan Ascent, Thermo Labsystems). Four wells were used for each experimental group. All tests were repeated twice.

Statistical Analysis

Statistical analysis of the data was performed by using two-way analysis of variance (ANOVA). Levels of $P < .05$ were considered to be statistically significant, indicating cytotoxicity on the basis of material and heat treatment. The results were also evaluated in accordance with ISO standard 10993-5,[29] which describes less than 25% inhibition as noncytotoxic, 25% to 50% inhibition as slightly cytotoxic, 50% to 75% inhibition as moderately cytotoxic, and more than 75% inhibition as highly cytotoxic.

Results

With the ^{3}H-thymidine incorporation assay, for all materials no significant differences ($P > .05$) were found between the heat-treated groups (water bath and microwave) and those without heat treatment (Fig 1). The mean quantity of isotope incorporated into cellular DNA for all experimental groups was statistically smaller than for the control. All acrylic resins were graded by the ^{3}H-thymidine incorporation assay as slightly cytotoxic (inhibition level of between 25% and 50%).

For all materials the difference between the water-bath and non–heat treated groups was statistically significant with the MTT assay ($P < .05$; Fig 2). In addition, no significant difference ($P < .05$) was detected between all experimental groups and the control. All denture base acrylic resins were graded by the MTT assay as noncytotoxic (inhibition level of less than 25%).

Discussion

Many toxicity tests of biomaterials using in vitro cell culture models have been used. ^{3}H-thymidine incorporation measures the number of cells synthesizing DNA, but this technique has a number of disadvantages, including the need for expensive special equipment and the production of radioactive waste.[21] In MTT assay, a tetrazolium dye is reduced to a blue formazan product by viable cells. The formazan solution is read spectrophotometrically after the crystals are dissolved by an organic solvent.

In the present study, the ^{3}H-thymidine incorporation assay was more sensitive to resin toxicity than was the MTT method. In the ^{3}H-thymidine incorporation test, all acrylic resins were graded as slightly cytotoxic. In contrast, MTT results graded all acrylic resins as noncytotoxic. This suggests that the toxic substances eluted from the acrylic resin denture base materials caused inhibition of DNA synthesis by L929 cells. Furthermore, the extracts did not affect the reduction of the tetrazolium salt (MTT) of active mitochondria. Our results are in agreement with those of others,[18] who found the ^{3}H-thymidine incorporation assay to be more sensitive to resin toxicity than the MTT method. According to Wagner et al,[21] MTT assay can only be used as a screening method, not for precise quantification of proliferation of canine lymphocytes.

The ^{3}H-thymidine incorporation results indicated that the degree of cytotoxicity differed among experimental and control groups. This may be attributed to a variety of potentially toxic substances eluted from denture base resins that are unlikely to be influenced by heat treatments. These substances include formaldehyde, MMA, methacrylic acid, plasticizers, organic additives, benzoic acid, and biphenyl and phenyl benzoate.[4–7,30–33]

The cytotoxicity of denture base resins has been widely evaluated,[2,27,34–37] and the present investigation evaluated the effects of heat treatments on the cytotoxicity of these

materials. Immediately after polymerization, two processes reduce the concentration of residual monomer: diffusion from the polymer and further polymerization at the site of polymer radicals in the matrix.[38] Tsuchiya et al[3] report that acrylic resin dentures should be immersed in hot water (50°C) for 60 minutes before insertion to decrease their cytotoxic potential, especially for autopolymerized rebasing and denture base materials. Therefore, this procedure was selected as one of the heat treatments evaluated in the present study. The choice of microwave heat treatment was based on previous studies[10,39] that showed that residual monomer levels decrease with microwave irradiation. Microwaves act only on the monomer, which decreases in the same proportion as the degree of polymerization increases.[39] Blagojevic and Murphy[11] showed that microwaving an autopolymerized resin reduces residual monomer by nearly 25%. However, in that study, the cytotoxicity of the denture base acrylic resins was not influenced by the heat treatments as assessed by either the ^{3}H-thymidine incorporation or MTT assays; the hypothesis that heat treatments could decrease the cytotoxicity of acrylic resin base materials was rejected.

This result may be related to the immersion of the specimens in water for 48 hours prior to cytotoxicity evaluation. It has been hypothesized that after the first 24 hours of storage, the concentration of residual monomer may be reduced as a result of leaching into water.[13,38] During polymerization, the produced radicals are consumed by oxygen, and thus the degree of inhibition should be proportional to the concentration of oxygen.[14] Expelling oxygen by immersing the resin in water probably diminished the oxygen effects and resulted in a higher degree of polymerization. Consequently, no significant effect on the cytotoxicity was observed when the denture base resins were subjected to the heat treatments. Despite the fact that the mechanisms involved in the reduction of residual monomer content have not been fully elucidated, several studies demonstrate that the cytotoxic effect can be minimized if the prostheses are stored in water for 24 hours.[4,27,33,40] Therefore, some authors suggest that soaking processed prostheses in water may be beneficial in reducing intraoral monomer release.[3,41] Futher research is needed to establish the effects of heat treatments on the cytotoxicity of acrylic resin denture materials immediately after sample fabrication and to identify the individual components of the eluate responsible for the observed cytotoxicity.

Denture base acrylic resins polymerized by conventional (Lucitone 550), rapid-boil (QC 20), and microwave (Acron MC) techniques all demonstrated equivalent cytotoxicity. Similar findings were observed by others.[37] The different curing conditions likely promoted a consistent degree of conversion of all materials, as observed by Bartoloni et al.[12] Moreover, the immersion of specimens in water for 48 hours before eluate preparation may account for the similar cytotoxicity observed for all materials.

The data from this study cannot necessarily be extrapolated to clinical scenarios. However, in vitro analysis provides a method of investigating cytotoxicity in a simplified system that minimizes the effect of confounding variables.[27] In addition, the use of different assessment methods provides more complete information on the toxicity of resins.[18] Because all acrylic resins were graded as slightly cytotoxic after 48-hour water storage, denture bases fabricated from these materials should be immersed in water before placement to minimize adverse reactions in the oral mucosa.

Conclusions

Within the limitations of this in vitro study, the following conclusions may be drawn:

- All tested materials were graded by the ^{3}H-thymidine incorporation assay as slightly cytotoxic.
- All tested materials were graded by the MTT assay as noncytotoxic.
- The cytotoxicity of the tested denture base acrylic resins was not decreased by either water bath or microwave postpolymerization heat treatments.

Acknowledgments

The authors wish to thank Dr Vehid Salih, Eastman Dental Institute for Oral Health Care Science, for reviewing the manuscript. This investigation was supported by the São Paulo Council of Research (FAPESP; grant No. 01/14004-0), Brazil.

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