Original article

Biocompatibility of denture base acrylic resins evaluated in culture of L929 cells. Effect of polymerisation cycle and post-polymerisation treatments

Janaina Habib Jorge[1], **Eunice Teresinha Giampaolo**[1], **Carlos Eduardo Vergani**[1], **Ana Lúcia Machado**[1], **Ana Cláudia Pavarina**[1] and **Iracilda Zeppone Carlos**[2]

[1]Department of Dental Materials and Prosthodontics, Araraquara Dental School, São Paulo State University, São Paulo, Brazil; [2]Department of Clinical Analysis, Araraquara Pharmaceutical School, São Paulo State University, São Paulo, Brazil

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Biocompatibility of denture base acrylic resins evaluated in culture of L929 cells. Effect of polymerisation cycle and post-polymerisation treatments

Objective: The purpose of this study was to evaluate the effect of two post-polymerisation treatments and different cycles of polymerisation on the cytotoxicity of two denture base resins.
Materials and methods: The resins tested were Lucitone 550 and QC 20. Discs of resins were fabricated following the manufacturer's instructions. Lucitone 550 was processed by long cycle or short cycle. The resin QC 20 was processed by reverse cycle or normal cycle. The specimens were divided into groups: (i) post-polymerised in microwave for 3 min at 500 W; (ii) post-polymerised in water-bath at 55°C for 60 min and (iii) without post-polymerisation. Eluates were prepared by placing three discs into a sterile glass vial with 9 ml of Eagle's medium and incubated at 37°C for 24 hours. L929 cells were seeded into 96 well culture plates and DNA synthesis was assessed by ^{3}H-thymidine incorporation assay.
Results: The results were submitted to two-way ANOVA and Tukey HSD test. QC 20 specimens polymerised by the normal cycle and submitted to microwave post-polymerisation were graded as moderately cytotoxic. Similar results were observed for Lucitone 550 processed by long cycle without post-polymerisation. The other experimental groups were graded as not cytotoxic. After water-bath post-polymerisation, specimens of Lucitone 550 processed by long cycle produced significantly lower inhibition of DNA synthesis than the other groups.
Conclusion: The long cycle increased the cytotoxicity of Lucitone 550 and water-bath post-polymerisation reduced the cytotoxicity of Lucitone 550 processed by long cycle.

Keywords: cytotoxicity, acrylic resin, polymerisation cycle, cell culture.

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Introduction

Since the 1930s, a variety of resins have been introduced into dental treatments for the construction of dental prostheses and their efficacy has been based on physical, chemical and biological properties[1]. However, one of the major factors limiting the use of these materials is their biocompatibility. Biocompatibility can be defined as the acceptance (or rejection) of artificial material by the surrounding tissues and by the body as a whole[2].

The majority of denture bases used consist of polymethylmethacrylate. Many authors have discussed the polymerisation process involved in converting monomer to polymer, because adequate polymerisation is a crucial factor in maximising the physical properties and biocompatibility of acrylic denture base resins[3–5]. The acrylic resins may be classified by polymerisation mode and include those that are heat-polymerised, auto-polymerised, microwave polymerised and visible light polymerised. Heat-polymerised denture base resins may leach out residual monomers and other chemically

reactive, toxic components[6–11] that can cause adverse reactions in the oral mucosa adjacent to the dentures[10,12].

To ensure the safety of these materials, *in vitro* cytotoxicity tests have been developed as a preliminary screening test to evaluate material biocompatibility[13–15]. Testing of dental materials by cell culture methods is relatively simple to perform, reproducible, cost-effective and can be carefully controlled[15]. One of the biological assays suggested for cytotoxicity testing is ^{3}H-thymidine incorporation test, which measures the number of cells synthesising DNA[16]. Studies have shown that the ^{3}H-thymidine incorporation assay was more sensitive to resin toxicity than other tests[1,17,18].

Methods for reducing the residual monomer content of polymerised acrylic resins have been described in the literature. Several authors have indicated the use of water-bath post-polymerisation[10,19,20] and others the microwave post-polymerisation[20,21] for reducing the residual monomer contents. Therefore, a previous study investigated whether the cytotoxicity of denture base acrylic resins could be reduced by water-bath and microwave post-polymerisation treatments. Unexpectedly, it was observed that the cytotoxicity of the three denture base resins was not decreased by either water-bath or microwave post-polymerisation treatments[18]. The low efficacy of the post-polymerisation treatments was attributed to the immersion of the specimens in water for 48 hours before the cytotoxicity assay.

This study investigated the effect of polymerisation cycles and water-bath and microwave post-polymerisation treatments on the cytotoxicity of two denture base resins tested without previous water immersion. The hypothesis for the current study was that post-polymerisation treatments could decrease the cytotoxicity of acrylic base resins when the specimens were not stored in distilled water.

Materials and methods

Sample fabrication

The materials used in this study, together with the manufacturer, composition and mixing proportions of polymer to monomer are listed in Table 1. Triplicate samples of each resin were fabricated under aseptic conditions in sterile aluminium moulds 10 mm in diameter and 1 mm thick. Samples were produced according to the manufacturer's protocol. The following polymerisation cycles were employed: Lucitone 550 was processed for 9 hours at 71°C (long cycle) or 90 min at 73°C and then in 100°C boiling water for 30 min (short cycle). The resin QC 20 was processed by placing the flask in boiling water, removing heat for 20 min, returning to boil, boiling for 20 min (reverse cycle) or by boiling water, inserting the flask, returning to boil, boiling for 20 min (normal cycle). After polymerisation, excess flash from the processing was removed using a sterilised trimming bur. To assess the biological effect of the post-polymerisation methods, specimens were divided into three groups: (i) post-polymerisation in a microwave oven for 3 min at 500 W[22,23]; (ii) post-polymerisation in a water-bath at 55°C for 60 min[10] and (iii) no post-polymerisation. Prior to cytotoxicity tests, discs were ultrasonically cleansed in distilled water for 20 min and exposed to ultraviolet light for another 20 min to kill microorganisms that may have contaminated the discs during fabrication[24].

Eluate preparation

Generally, the exposure pattern of tissues to resin materials occurs both directly and indirectly. Directly tissue-material contact exposure occurs in tissues, such as opened dental pulp, oral mucosa, skin cells and blood cells. Indirect resin-tissue contact occurs when the tissue is exposed to components released from the resins into the local

Table 1 Materials used in this study.

	Composition			
Brand name	*Powder (P)*	*Liquid (L)*	*Manufacturer*	*P/L ratio (g/ml)*
Lucitone 550	MMA	PMMA	Dentsply Int. Inc., Chicago, IL, USA	0.42/0.2
QC 20	MMA	PMMA	Dentsply Int. Inc., Chicago, IL, USA	0.46/0.2

MMA, methyl methacrylate; PMMA, poly methyl methacrylate.

environment, such as when oral mucosa are exposed to chemicals released into the saliva. In this study, eluates of the materials were prepared by placing three specimens of each acrylic denture base resin, immediately after sample fabrication, into a sterile glass vial (Costar; Corning Inc., Corning, NY, USA) with 9 ml of Eagle's medium supplemented with an antibiotic (80 μg/ml of gentamicin) and fetal bovine serum and then incubated for 24 hours at 37°C. Medium without discs was also incubated and diluted as above and served as the negative control. This test was realised according to ISO 10993-5[25].

Cell culture

Mouse fibroblast cells (L929) were propagated in Eagle's minimum essential medium (Institute Adolfo Lutz, São Paulo, Brazil) supplemented with 80 μg/ml of gentamicin and 7.5% v/v fetal bovine serum. The culture was maintained at 37°C in an atmosphere of 5% CO_2/95% air.

Cytotoxicity assays

DNA synthesis in fibroblasts was assessed by measuring the incorporation of ^{3}H-thymidine (Amershan Pharmacia Biotech do Brazil Ltda., São Paulo, Brazil). L929 mouse fibroblasts (1×10^4 cell/ml) in 100 μl of the Eagle's medium were seeded into 96 well culture plates and incubated for 24 hours at 37°C in an air atmosphere containing 5% CO_2. After 24 hours of incubation, the culture medium was replaced by 20 μl medium containing 0.25 μCi of ^{3}H-thymidine. Additional 50 μl of eluate and 50 μl of fresh medium were added to each well of a 96-well culture plate and incubated at 37°C in an air atmosphere containing 5% CO_2. Isotope incorporation into DNA was measured after 24 hours of incubation. After 24 hours of exposure to ^{3}H-thymidine, the cells were then harvested onto fibre filters using a multichannel automated harvester (Unifilter 96 GF/C; Packard Instrument Company, Meriden, CT, USA) and the incorporated radioactivity was measured using a scintillation counter (Unifilter 96 GF/C; Packard Instrument Company, Meriden, CT, USA). Six wells of a 96-well culture plate were used for each experimental group.

Statistical analysis

Statistical analysis of the data was performed by using two-way analysis of variance (ANOVA). Tukey HSD test was also used. Levels of $p < 0.05$ 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[25], which states inhibition of <25% counts as non-cytotoxic, 25–50% as slight, 50–75% as moderate and >75% as highly cytotoxic.

Results

The results from 2-way ANOVA are shown in Table 2. It can be seen that significant differences were found for material ($p = 0.0015$), post-polymerisation treatment ($p = 0.0067$) and for material × post-polymerisation treatment interaction ($p < 0.001$). Figure 1 shows the effects of eluates of the two materials of each post-polymerisation treatment and polymerisation cycle group on DNA synthesis. QC 20 polymerised by normal cycle and post-polymerised in a microwave oven for 3 min at 500 W was graded as moderately cytotoxic. A

Table 2 Two-way ANOVA.

	Source	*df*	*Mean square*	*F*	*p*
Treatment	3.90 E + 07	2	1.95 E + 07	5.42	0.0067
Material	6.22 E + 07	3	2.07 E + 07	5.76	0.0015
Treatment × material	1.39 E + 08	6	2.32 E + 07	6.44	0.0000
Error	2.34 E + 08	65	3.60 E + 06		

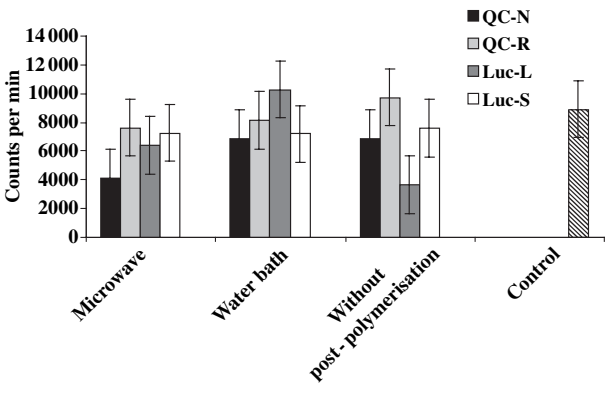

Figure 1 Mean and standard deviation of ^{3}H-thymidine incorporation assay results for all experimental and control groups.

similar result was obtained for Lucitone 550 processed by long cycle, without post-polymerisation treatment. The other experimental groups were graded by the ^{3}H-thymidine incorporation assay as non-cytotoxic (inhibition level <25%).

The water-bath post-polymerisation reduced the cytotoxicity of Lucitone 550, when the specimens were processed by long cycle ($p < 0.001$). Without post-polymerisation treatment, Lucitone 550 specimens polymerised by long cycle were more cytotoxic than those polymerised by short cycle ($p = 0.032$).

Discussion

Biocompatibility of dental materials has been evaluated in a variety of ways. The clinical relevance of tests for the assessment of cytotoxicity of dental materials is widely recognised. Different assays and different cell types cultured *in vitro* are being used to test dental materials[26–28]. In the present investigation, the cytotoxicity of acrylic resins was assessed by incorporation of radioactive ^{3}H-thymidine, which is based on the DNA synthesis activity in a dividing cell population that remains viable after exposure and incubation of the eluates from the denture base resins. This technique has some disadvantages, including the need of expensive special equipment and the production of radioactive waste[16]. However, studies showed that ^{3}H-thymidine incorporation assay was more sensitive to resin toxicity than other tests[1,17,18].

The aim of this *in vitro* study was to evaluate the effects of post-polymerisation treatments on the cytotoxicity of two denture base resins, polymerised by different polymerisation cycles suggested by their manufacturer. The cytotoxicity was represented by the number of viable cells present after exposure to denture base resin eluate.

In this study, different processing techniques were used for the two resins evaluated, using the methods recommended by the manufacturer. Despite the differences in polymerisation cycle, when the number of viable cells of the eluates from the specimens without post-polymerisation was compared with that of the control, no significant differences were observed between the cases of the QC 20 polymerised by long and reverse cycles and Lucitone 550 processed by short cycle. This result may be attributable to the fact that the QC 20 resin is a rapid polymerising material, in which the monomer is modified by the addition of a chemical activator (dimethyl-*p*-toluidine) that begins to decompose in the presence of benzoyl peroxide when mixing powder and liquid. This would lead to a more complete polymerisation (a high degree of conversion of monomer to polymer) and consequently to lower cytotoxicity. On the other hand, a significantly lower number of viable cells was observed when Lucitone 550 was processed by long cycle. In this cycle, the temperature used for the polymerisation of the resin Lucitone 550 (71°C) was below the glass transition temperature (Tg) of the matrix phase (97–100°C)[29]. It is likely that the monomer had a poorer ability to polymerise because of lower molecular chain motions and immobilisation of monomer in the glassy polymer[30]. It has been recommended that the polymerisation cycle of heat-polymerised acrylic resins should include a terminal boiling treatment for at least 1 hour to achieve maximum monomer conversion[4]. The long polymerisation cycle for Lucitone 550 (for 9 hours at 71°C) did not include a terminal boil, which probably resulted in higher residual monomer levels and, consequently, increased cytotoxicity. This hypothesis can be confirmed by the results from Urban *et al.*[20], who observed that the short cycle promoted lower amount of residual monomer (0.08%) for Lucitone 550 compared with the long cycle (0.24%).

In the current study, it was hypothesised that water-bath and microwave post-polymerisation treatments could decrease the cytotoxicity of the resins. This hypothesis was partially confirmed. For specimens of Lucitone 550 processed by long cycle, the water-bath post-polymerisation reduced the cytotoxicity when compared with the group without post-polymerisation treatment, resulting in a higher number of vial cells. These results are in agreement with those of Tsuchiya *et al.*[10], who reported that acrylic resin dentures should be immersed in hot water (50°C at 60 min) before insertion to decrease their cytotoxic potential, especially for autopolymerised rebasing and denture base materials. The use of water-bath post-polymerisation may have enhanced the leaching of residual monomer and other toxic substances before eluate preparation[31,32]. It is well known that the increase in temperature of storage media results in higher diffusion rates[31,33]. The possible toxic substances pre-leached include formaldehyde, methacrylic acid, plasticisers, organic additives, benzoic acid, and biphenyl and phenyl benzoate[6–8,11,34–37].

Microwave post-polymerisation treatment was based on the studies by Blagojevic & Murphy[38], Yunus *et al.*[21] and Urban *et al.*[20], who observed that the residual monomer levels of autopolymerising resins can be decreased by microwave irradiation.

The results of the present investigation showed that microwave post-polymerisation improved the biocompatibility of Lucitone 550 polymerised by long cycle. Microwave irradiation probably provided an additional degree of conversion to the polymer, reducing the residual monomer. In addition, the possible decrease in residual monomer by microwave heating could have been as a result of monomer volatilisation[39]. The microwave post-polymerised specimens were graded as non-cytotoxic whereas the specimens without post-polymerisation were moderately cytotoxic. An earlier study showed that the cytotoxicity of Lucitone 550 was not decreased by microwave post-polymerisation at 500 W for 3 min. Differences in methodology may help explain this contrasting result. In the earlier study, the specimens were stored in water for 48 hours at 37°C before post-polymerisation. In the present investigation, however, the specimens were not pre-leached in water before eluate preparation.

Contrary to the expectations, the cytotoxicity of the QC 20 polymerised by the normal cycle was detrimentally affected by microwave post-polymerisation treatment. Similar findings were observed by Campanha *et al.*[17], who tested auto-polymerised acrylic resins. The specimens were microwaved in dry conditions and, therefore, the reduction in residual monomer by the mechanism of diffusion into water was not present. Therefore, the observed adverse effect could be the result of leaching of toxic compounds during eluate preparation[35]. Moreover, the cytotoxicity could be the result of formaldehyde formation on the superficial layer of the specimens during post-polymerisation[8]. The presence of this substance in dental acrylic resin materials has been well documented[8,10,40].

The data of this study cannot necessarily be extrapolated to clinical scenarios. However, *in vitro* analysis provides a method of investigating cytotoxicity in a simplified system that minimises the effect of confounding variables[24]. From the results of this study, it can be suggested that microwave irradiation and water-bath post-polymerisation treatments could be used to reduce the cytotoxicity of some acrylic resins. In addition, this study showed that the cytotoxicity of the resins might vary under different polymerisation cycles. *In vivo* studies and human clinical studies should be undertaken to clarify the effects of acrylic resins on oral tissue. Furthermore, future research is recommended to identify the individual components of the eluate that were responsible for the observed cytotoxicity.

Conclusions

Within the limitations of this *in vitro* study, the following conclusions were drawn:

1. The long cycle increased the cytotoxicity of Lucitone 550;

2. Water-bath post-polymerisation reduced the cytotoxicity of Lucitone 550 processed by long cycle.

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Correspondence to:
Dr Janaina Habib Jorge, Department of Dental Materials and Prosthodontics, Araraquara Dental School, R. Humaitá no. 1680, Araraquara, SP, Brazil.
Tel.: +55 16 201 6410
Fax: +55 16 201 6406
E-mail: eunice@foar.unesp.br and janainahj@bol.com.br