Cell death induced by eluates from hypoallergenic denture base acrylic resins in NIH-3T3 fibroblast cells

Gulay Kansu, Tugba Kalyoncuoğlu, Pembegul Uyar, Esra Uzun

Department of Prosthodontics, Faculty of Dentistry, Ankara University, Ankara, Turkey

Biology Department, Selçuk University, Konya, Turkey

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Abstract

Background/purpose: The use of a hypoallergenic denture base material for dental patients is susceptible to trigger allergic reactions. The aim of this study was to compare the cytotoxicity at the cellular level of hypoallergenic denture base materials and polymethyl methacrylate denture base materials polymerized in different ways.

Materials and methods: Comparisons were done with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) results of eluates, which were derived from all test materials by immersing for 24 and 48 hours in various ratios of the test materials' weights to the volume of Dulbecco’s modified Eagle’s medium without supplements (0.2, 0.4, and 0.8 g/mL) and incubating NIH-3T3 cells for 24 and 48 hours (N = 9). Then the apoptotic effects of the materials were detected, using the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay.

Results: The 24-hour eluates of all materials exhibited strong cytotoxicity, and cytotoxicity increased with time but the difference was not statistically significant. In terms of cell death rates, the materials were ranked as follows: QC 20, Puran HC, Acron MC, Promysan Star. As a result of the apoptotic index of the materials, the ratio of necrotic cells was found from highest to lowest: QC 20, Puran HC, Acron MC, and Promysan Star.

Conclusion: It was determined that the test materials have cytotoxic effects on fibroblasts, and polymethyl methacrylate-based test materials have a more necrotic effect on cells; however, other test materials induced apoptosis at a higher range.

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**Introduction**

The cases of allergies due to denture base materials are increasing, and as a consequence, dentists are confronted with more patients reporting allergic reactions to polymethyl methacrylate (PMMA)-based denture materials. Adverse reactions may be induced by residual methyl methacrylate monomer (MMA). Therefore, presumably hypoallergenic resins, such as diurethane dimethacrylate, polyurethane, polyethylenterephthalate, and polybutylenterephthalate, have replaced MMA or the content of the residual monomer has been reduced. However, hypoallergenic denture base materials are not definitely risk-free. The reactions of cells in contact with the material surface with regard to the mechanisms of cell death remain to be elucidated. This is an important aspect, since the future of biomaterial science—as outlined by Ratner—depends on the capacity to produce the desired interfacial (i.e., tissue/biomaterial) reaction. It is important to conduct a death-inducing potential test of a biomaterial in different cell lines because it is known that different cell types may exhibit a wide spectrum of responses to the same toxic stimulus with regard to susceptibility, kinetics of the toxic effect, or mode of cell death. Two main forms of cell death have been described: apoptosis and necrosis.

Apoptosis is an active and physiological process characterized by cell shrinkage, detachment from neighboring cells, condensation of nuclear chromatin followed by nuclear fragmentation, and preservation of the structural integrity and most of the functions of the plasma membrane and of the cellular organelles. In vivo, apoptotic cells are rapidly phagocytized without triggering an inflammatory reaction. In vitro, in the absence of professional phagocytes, the apoptotic cells eventually swell, and finally cell lysis occurs. This late stage of in vitro apoptosis was named "secondary necrosis", and more recently "apoptotic necrosis".

Necrosis is a passive and degenerative process occurring as a result of the cell’s exposure to gross injury. It is characterized by mitochondrial swelling, dissolution of the nucleus, rupture of the plasma membrane, and release of the cytoplasmic constituents. Necrosis triggers an inflammatory reaction in the tissue and often results in scar formation.

It is clear that the biocompatibility of various denture materials were studied plenty of times. However, no information or any research about the biocompatibility of hypoallergenic denture base materials has been made available so far. The objective of the present study was to compare two different types of polymerized hypoallergenic denture base materials and PMMA-based denture base materials with different incubation times and different concentrations in terms of apoptosis and necrosis based on cytotoxicity at the cellular level.

**Materials and methods**

**Denture base polymers**

Four commercially available denture base polymers were chosen for this study (Table 1). Acrylic specimens, which were 12 mm in diameter and 1 mm in thickness, were fabricated under aseptic conditions in stainless steel molds according to the manufacturers’ instructions and the ISO recommendation.

QC 20 (Dentsply de Trey, England) and Puran HC (Novodent EST) were processed using conventional procedures for heat-polymerized-resin based materials. Acron MC (GC Industrial Corp., Japan) was polymerized with microwave irradiation at 500 W for 3 min. Promysan Star (Pedrazzini Dental Germany), a heat-polymerized base material and the injection molding process applied in the study, was processed by the manufacturer as its polymerization technique requires special equipment and methods.

Puran HC and Promysan Star were purported to be hypoallergenic denture base materials.

**Preparation of eluates**

The specimen disks were used immediately after fabrication. Eluates were prepared following the ISO requirements, by placing the extraction medium, DMEM (Dulbecco’s modified Eagle’s medium), with a 2% mix of penicillin/streptomycin/fungizone (PSF; Biochrom, UK) and the specimens (N = 9) in Costar Petri dishes (60 mm diameter) (Costar, Cambridge, MA, USA) at 37°C in a rotary shaker for 100 rpm for 24 and 48 hours. Fetal bovine serum (FBS) and L-glutamine (Qiagen, Australia) were added before exposure to the cells. The ratios between the weights of the test specimens and the volume of medium used for extraction were 0.2, 0.4, and 0.8 g/mL. For the negative control, DMEM without supplements incubated

<table>
<thead>
<tr>
<th>Name of product</th>
<th>Material</th>
<th>Type of processing</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC 20</td>
<td>Thermoplastic resin based on polymethyl methacrylate (PMMA)</td>
<td>Heat polymerized</td>
<td>Dentsply De Trey, England</td>
</tr>
<tr>
<td>Puran HC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Di urethane dimethacrylate (thermoplastic resin based on polyester)</td>
<td>Molding technique, preferentially under high temperature (boiling water)</td>
<td>Novodent Est., Lichtenstein</td>
</tr>
<tr>
<td>Acron MC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Light-curing polyethyl methacrylate (PEMA)</td>
<td>Microwave polymerized</td>
<td>GC Industrial Corp., Japan</td>
</tr>
<tr>
<td>Promysan Star&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Polybutylenterephthalate</td>
<td>Injection die casting</td>
<td>Pedrazzini Dental Technologie, Germany</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hypoallergenic denture base resin.
Cell culture

The NIH-3T3 mouse fibroblast cell line was obtained from the Foot-and-Mouth Disease Institute of the Ministry of Agriculture and Rural Affairs of Turkey, Turkey (ATCC Grade, HUKUK no: 950217) and maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine (Qiagen, Australia), and 100 units/mL PSF (Sigma, St. Louis, MO, USA). Continuous cell lines, such as 3T3 mouse fibroblasts, are routinely used for the testing of cytotoxic properties of dental materials because of their reproducible growth rates and biological responses.6,8

MTT test

In our study, comparison of cytotoxic values were carried out with the 3-4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT; Sigma) test using the eluates derived from all test materials in different concentrations (0.2, 0.4, and 0.8 g/mL) extracted at different times (24 and 48 hours) and by immersing in different incubation durations (24 and 48 hours) to reveal the effects of monomers released from the materials. DMEM without supplements, incubated under the same conditions as the test samples, were completed with 10% FBS and 2 mM L-glutamine before it was used as a negative control. The MTT test was carried out following the ISO requirements, ISO 10993-5, which deals with cell culture test methods.

Microscopic detection of DNA fragmentation in NIH-3T3 cells by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling method

The cells were cultured on sterilized cover glass in six-well dishes and incubated in the medium with and without test materials. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed using ApoAlert DNA Fragmentation Assay Kit (Clontech Laboratories Inc., USA) with several modifications. The fluorescent signals of fluorescein depending on DNA damage and of propidium iodide depending on nuclei in the cells were observed using a fluorescence microscope (Olympus CKX 41).

The method used is based on the biochemical property of terminal deoxynucleotidyl transferase (TdT), which catalyzes a template-independent addition of deoxyribonucleotide triphosphate to the 3′-OH ends of double- or single-stranded DNA. After completing the protocol, slides were photographed at low magnification (20×) on a color slide film using a fluorescent microscope with a 520-nm filter (for propidium iodide staining) and a 590-nm filter (for fluorescence staining).

Apoptotic cells were counted from four randomly chosen fields per slide. Therefore, propidium iodide nuclear staining (red) represents the total number of cells per field; these were counted first, followed by apoptotic cell counts (yellow to bright green) in the same fields. Results for apoptotic cells are given as a percentage of the total propidium iodide-stained cells.

After the counting of apoptotic cells, randomly selected specimens in groups were photographed under the fluorescent light microscope at high magnification (×40) to obtain better images.

Statistical analysis

The cell activities were initially compared with each other by considering two different incubation periods (24 and 48 hours) and two different extract periods (24 and 48 hours) for four basic groups (QC 20, Puran HC, Promysan Star, Acron MC) and three different concentrations (0.2, 0.4, and 0.8 g/mL) during the cytotoxic evaluation of at least three independent experiments.

Next, the live cell ratio released according to the four main groups and three different concentrations of these groups for two different incubation periods (24 and 48 hours) and two different extract times (24 and 48 hours) were compared accordingly.

For the four main groups, apoptotic indices after the 24-hour incubation time of the 24-hour extracts of the 0.8 g/mL concentration were compared during the evaluation of the apoptotic ratio (Table 2).

We applied analysis of variance to determine the statistical differences between the variables and the Duncan test to find out among which groups the differences occur.

<table>
<thead>
<tr>
<th>Material (0.8 g/mL 24-h extract–24-h incubation)</th>
<th>MTT results (%)</th>
<th>Apoptotic index results (%)</th>
<th>Final results</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC-20 (Group 1)</td>
<td>35.80</td>
<td>21.6</td>
<td>Necrosis →↑</td>
</tr>
<tr>
<td>Puran HC (Group 2)</td>
<td>50.89</td>
<td>14.5</td>
<td>Necrosis →↓</td>
</tr>
<tr>
<td>Promysan Star (Group 3)</td>
<td>82.97</td>
<td>6.2</td>
<td>Necrosis →↑</td>
</tr>
<tr>
<td>Acron MC (Group 4)</td>
<td>65.64</td>
<td>10.1</td>
<td>Necrosis →↓</td>
</tr>
</tbody>
</table>

MTT = 3-4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide.
Results

MTT cell proliferation test results

In the cytotoxic evaluation of our study, regardless of incubation and concentration durations, it was determined that all materials have death effect on cells at particular rates.

In this study, it is proven that the extracts attained after 24 and 48 hours in Group 1 (QC 20), which is conventional heat polymerized, reflect the highest cytotoxic effect independent of the concentrations used after the two incubation periods. Group 2 (Puran HC), Group 4 (Acron MC) and Group 3 (Promysan Star) also showed cytotoxic effects in this particular order, at different levels (Figs. 1 and 2).

Apoptotic and necrotic cell death

It was determined that all of the materials induced apoptosis based on the TUNEL test results of the apoptotic evaluations within the selected incubation period. The results obtained in the TUNEL test of Groups 2, 3, and 4 test specimens indicate that NIH-3T3 cells die mainly by apoptosis and in a lower proportion by necrosis. Only the response to eluates of conventional heat polymerized denture base resin that includes PMMA (Group 1) demonstrated a higher ratio of necrotic cell death (Table 2).

Comparison of the apoptotic index results of the 24- and 48-hour extracts of the test materials revealed that the apoptotic index value of the 48-hour extracts was higher than that of 24-hour extracts, although not at a statistically significant level. The apoptotic index value of all evaluated materials showed the following trend: QC 20 > Puran HC > Acron MC > Promysan Star. Whereas QC 20, which is the denture base material comprising PMMA, results in more necrosis, the other materials’ processes result in more apoptosis (Figs. 3–6).

Discussion

In modern dentistry, MMA is the mainstream material in denture bases. Acrylic monomers—acylates, methacrylates, urethane acrylates, and epoxy acrylates—are used in dentistry not only to make prostheses, but also in dental composite resins, dentin bonding materials, and glass ionomers. It was reported that heat and autopolymerized denture base materials include toxic substances such as formaldehyde, benzoic acid, methyl methacrylate, and methacrylic acid. Although various methods have been used to initiate the polymerization of denture base resin, the conversion of monomer to polymer is not complete, and some unreacted MMA is left as residual nonpolymerized MMA in the denture base that continues to be released into water as well as saliva. Acrylic monomers, especially methacrylates, have been among the most common occupational contact allergens in dental personnel. It has been shown that MMA monomer is also the primary irritant

Figure 1 Effects of 24-hour extracts at different concentrations and incubation times on cell survival in NIH-3T3 cells. Cytotoxicity was measured with the MTT assay. NIH-3T3 cells were precultured in 96-well microplates for overnight and then incubated with 0.2, 0.4, and 0.8 g/mL of QC 20, Puran HC, Acron AC and Promysan Star extracts for 24 and 48 hours. The percentage of cell growth in the negative control group was designated as 100%. This statistically significant difference between QC 20 data compared to Promysan Star. MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Figure 2 Effects of 48-hour extracts at different concentrations and incubation times on cell survival in NIH-3T3 cells. Cytotoxicity was measured with the MTT assay. NIH-3T3 cells were precultured in 96-well microplates for overnight and then incubated with 0.2, 0.4, and 0.8 g/mL of QC 20, Puran HC, Acron AC and Promysan Star extracts for 24 and 48 hours. The percentage of cell growth in the control group was designated as 100%. This statistically significant difference between QC 20 data compared to Promysan Star. MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
that causes eczematous reactions on oral mucosa and skin in patients.\textsuperscript{16} It has been proved in several studies that MMA is cytotoxic. In another study, MMA was also found to induce papilloma and fibroma in terms of sequential histopathological changes on hamster cheek pouches.\textsuperscript{17}

Many authors reported that the amount of residual monomer is affected by the composition of denture base materials and preparation conditions.\textsuperscript{13,18–22} Miettinen and Vallittu\textsuperscript{20} compared the residual monomer content released from heat-polymerized and autopolymerized denture base materials and concluded that the autopolymerized materials released considerably more residual MMA compared with heat-polymerized materials (1–2\% wt). Moreover, these authors showed that the residual monomer content could be reduced when the polymerization time was extended. Dogan et al\textsuperscript{21} studied the effects of varying polymerization times and temperatures on the residual monomer content of polymer/monomer based denture base materials. The authors showed that increased temperatures and extended polymerization times were accompanied by a decrease in the residual monomer content.

However, the polymerization conditions alone are not enough to explain the effect of each material. To determine the causes for the differences, the chemical composition and the leachable compounds of each brand should be identified, and the apoptogenic and necrogenic potential of each component should be assessed.\textsuperscript{7}

Tsuchiya et al\textsuperscript{12} demonstrated that the residual monomer content of denture base materials is lowered to a quarter of the initial value. It has been advocated that the prosthesis should be immersed in water at 50°C for 60 minutes, to reduce the amount of released monomer and
Therefore the toxic potential of denture base resins, especially for autopolymerized resins. This is particularly important when hard autopolymerized reline resins are used. By immersing the prosthesis in heated water, monomer molecules diffuse more rapidly, reaching the remaining free radicals and leading to a complementary polymerization reaction or other chemicals released in the medium that may alter their cytotoxic potential.14,22–25

Therefore, it is recommended that dentists soak the acrylic resin prostheses in water for at least 24 hours before placing them in the patient’s mouth. For this reason, using eluates is appropriate for the cytotoxic tests.

Until today, denture base resins are usually composed of prepolymerized polymethacrylate powder particles, which are mixed with monomers of MMA and a cross-linking agent.17,26 In recent studies, PMMA and its derivatives have been in general comparatively evaluated.13,17,24,27 However, there is not enough information about the biocompatibility of MMA-free or modified MMA denture base materials, which are made of hypoallergenic acrylic. For this reason, in our study the cytotoxic properties of hypoallergenic denture base materials were comparatively evaluated with traditional PMMA denture base materials using different incubation periods and different concentrations.

Geurtsen et al28 compared the cytotoxic effects of 35 different dental resin monomers on 3T3 cell series and three different human primer cell types. Consequently, it has been reported that there were no differences between primer cells and 3T3 cell series with regard to sensitivity to cytotoxic effects. Likewise, in most studies it has been indicated that there were no differences between primer cells and continuous cell lines with regard to sensitivities of cytotoxic effects.29–32

In one study, the cytotoxic effects of resin composite monomers on cultures of human gingival fibroblasts were examined; MTT assay was compared with LDH (lactate dehydrogenase) test and was found to be more sensitive than the LDH assay.33

In this study, the reason for selecting the extraction test method is the contact between primarily oral mucosa and components released from dental materials into the saliva.34 With the cytotoxicity evaluations, the acute (>24 hours) and subacute (<24 hours) effects could be determined.35

Because of the contamination risks of test samples with cultured cells in the long term and the reduction of environmental components, studies are generally of limited durations.36 For this reason, in this study subacute cytotoxicities were evaluated based on 24- and 48-hour incubation periods.

However, it is revealed that even if the main substance of the prosthesis base materials among the experimental groups are sometimes relatively the same, the monomer and the concentrations used are different, and the contrasts in the polymerization methods may be put forward as the reasons behind the results of the study. The rising cytotoxicity effect along with the rising concentration in all materials reflect parallel data of the cytotoxicity research on the L929 mouse fibroblasts extracts attained from the denture based materials, conducted by Cimpan et al,7 who used different methods. However, the numbers of experiments in the literature that detail the cytotoxic effects of different concentrations attained from the different denture base materials are limited. From this point of view, the outcome of the research shall serve as a reference for further in vitro studies.

According to the results of this study, the cytotoxicity findings for all experiment groups within all concentrations at the end of the 48-hour incubation period were numerically higher than the cytotoxicity findings at the end of the 24-hour incubation period; however, the observed differences were not statistically significant and cell death was higher in some groups in the first 24 hours. In Table 2, for the four main groups, apoptotic indices after the 24-hour incubation time of the 24-hour extracts of the 0.8 g/mL concentration were compared during the evaluation of the apoptotic ratio. These results are compatible with those of other studies that reveal cytotoxicity results in the first 24 hours.14,17,37

It is important to know which type of cell death is induced, apoptosis or necrosis. The reactions of cells in contact with the material surface with regard to the mechanisms of cell death remain to be elucidated.1 First, Cimpan et al7 explored the effects of different auto- and heat-polymerized PMMA-based denture base polymers that affect the clonogenicity and induce death by apoptosis and/or necrosis in L929 fibroblasts during 24 and 48 hours. The annexin V method and light and electron microscopy were used.7 Consequently, a higher amount of MMA is released from autopolymerized PMMA than from heat-cured PMMA, providing evidence that two distinct modes of cell death—apoptosis and necrosis—were involved in the cytopathogenic effects induced by PMMA-based denture base polymers on L929 murine fibroblasts. Furthermore, apoptosis was demonstrated to be the major mode of cell death. The tested denture base polymers augmented cell death and decreased the clonogenicity of L929 cells in the order of potency autopolymerized to heat polymerized.7

The apoptotic mechanism responsible for cell death is explained with the alterations in several classes of cell lipids. Eluates often contain benzoyl peroxide, which is commonly used as polymerization initiator in denture base polymers. Peroxidation of cellular lipids by benzoyl peroxide may be a major mechanism of toxicity. Peroxides can induce activation of phospholipase A2, uncoupling of oxidative phosphorylation with concomitant effects on ATP production, and alterations of calcium homeostasis.7 These findings might explain our results wherein necrotic effects have been found at significantly higher levels in Group 1 samples, which were PMMA-based denture base materials, compared to those of the other groups.

Our results also indicate that tested denture base materials have a cytotoxic effect on fibroblasts, and PMMA denture base materials have a mostly necrotic effect on cells, but other tested denture base materials mostly induce apoptosis. These findings are similar to the results reported by Cimpan et al.7

As part of the results of our study, the basic death type of various base materials, which are launched as hypoallergenic and declared not to contain PMMA in vitro, is apoptosis, and it is concluded that apoptotic death does not cause inflammation in vivo. Thus, in our opinion, these materials are promising and safe to use for prosthetic treatments especially for allergic patients.
Conflicts of interest

The authors declare that there are no conflicts of interest that could influence their work.

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