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ORIGINAL ARTICLE

In vitro evaluation of long-term cytotoxic response of injection-molded polyamide and polymethyle metacrylate denture base materials on primary fibroblast cell culture

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Abstract

Objective. This study investigated the long-term cytotoxic response of thermoplastic polyamide and conventional polymethyle metacrylate (PMMA) denture base materials. ***Materials and ethods.*** Twenty discs were prepared for each polyamide, heat and cold cured PMMA denture base resins (totally 60) and divided into four sub-groups ($n = 5$). Cytotoxicity was assessed with the direct cell contact method using cell viability and neutral red (NR) uptake assay. Each sub-group was tested at initial and after being aged for 24 h, 1 week and 8 weeks with artificial saliva according to ISO 10993 standards. ***Results.*** There were no significantly difference among the materials and control groups after initial, 24 h and 1 week testing. In 24 h testing, only Deflex was more toxic according to the Control group ($p < 0.05$). After 8 weeks of aging with artificial saliva, all materials were significantly cytotoxic when compared to the control group. QC20 was more toxic than Deflex and SC Cold Cure ($p < 0.05$). There were significant differences between the 8 week aging group and the initial, 24 h and 1 week testing for all materials ($p < 0.05$). ***Conclusions.*** Cytotoxicity of all tested denture base materials increased significantly after the long-term aging. Therefore, long-term aging may be useful to determine a dental material's toxicity. Polyamide denture base material had a similar toxicity profile with conventional heat- and cold-cured PMMA.

Key Words: *denture base materials, cytotoxicity, long-term aging, cell culture*

Introduction

A variety of resins have been used for construction of removable dental prostheses from the first use in the 1930s to date [1]. The material commonly used for the fabrication of complete dentures is polymethyl methacrylate (PMMA). Despite its popularity, it is still far from ideal in fulfilling the mechanical requirements of a prosthesis. The most important disadvantage of a PMMA denture base is high fracture incidence [2]. Various polymers have been developed for use as denture base resins to overcome some mechanical disadvantages of PMMA, such as polyamides, epoxy resin, polystyrene, vinyl acrylic, rubber graft copolymers, polycarbonate and nylon [3].

Polyamide resin was proposed as a denture base material in the 1950s [4]. Nylon is a generic name for certain types of thermo-plastic polymers belonging to the class known as polyamides. Polyamide is a thermoplastic polymer based on a condensation reaction between a diamine and a dibasic acid [5]. A heat injection molding technique was used to fabricate the polyamide denture base. This technique improves the physical properties of denture base materials such as dimensional stability and polymerization shrinkage [6]. To date, a few reports have assessed some properties of the lately developed nylon-based flexible resin system for the denture base construction. They have lower flexural strength and low elastic modulus; but higher impact strength than the conventional PMMA [7]. Injection-molded dental polymers are monomer-free, more flexible than PMMA and recommended for the fabrication of removable dentures as well as occlusal splints and

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night-guards, providing patients with comfortable long-term use. This is often recommended for geriatric and disabled denture wearers.

Beyond the physical properties, one of the major factors limiting the use of the denture base materials is their biocompatibility. These materials are used in direct contact with oral tissues for a long time. Biological factors of the oral environment such as pH, thermal changes, moisture, micro-organisms and enzymes could change the chemical and physical properties of these materials [8]. However, it is shown that substances leaching from denture base resins can cause irritation of oral tissue, inflammation or even an allergic reaction in patients and dental staff [9]. In this context, between 0.7–2% of patients and dental practitioners display allergic reactions [10].

The variations in chemical composition and purity, the degree of conversion of their constituent monomers and manipulative variables can affect the biological properties of the denture base materials [11]. Studies showed that the most toxic effects of denture base materials occur during the first 24 h of exposure and these effects correlate with the early leaching of residual monomers [12]. However, other studies have reported that resin-based restorative materials may leach sufficient components to cause cytotoxicity as late as 2 weeks [13]. As there is no long-term cytotoxicity data for denture base materials, the relevance of the aging tests could not be determined. Long-term aging may be more relevant to the clinical use of these and other dental materials, as they remain in contact with living tissues for many years [14].

Data regarding the *in vitro* cytotoxic effect of heat-cured and auto-polymerized PMMA on cell culture systems have been reported [15,16], but the knowledge on long-term cytotoxicity is inadequate. There isn't any published data about cytotoxicity of polyamide-based denture materials, although the mechanical properties have been reported [7,17]. The aim of this study was to evaluate and compare the short- and long-term cytotoxicity of the polyamide-based denture base material with the conventional pressure-pack and auto-polymerized PMMA by an *in vitro* direct cell contact method. The working hypotheses were that there would be differences between the cytotoxicity of both the different denture base materials and short- and long-term (up to 8 weeks) follow-up.

Materials and methods

Cell culture

Human amnion fibroblasts (HAFs) were acquired from a pregnant woman through routine amniocentesis procedures. The remaining cells were used for bioassay, after the karyotype results had been reported. HAFs were grown in 25 cm^2 culture flasks for 14 days in 3.5 ml of BIOAMF-1 medium (Biological Industries, Israel) containing fetal calf serum (20%) (PAN Biotech, Aidenbach, Germany), L-glutamine, penicillin, streptomycin and amphotericin-B (PAN Biotech) and then removed using tripping/EDTA-C (Multicell, Quebec, Canada). HAFs were centrifuged at 1000 rpm for 10 min and supernatant was poured. Pellet was re-suspended in 10 ml of BIOAMF-1 medium and cultured in four 96-well sterile microtiter plates at a density of 1×10^4 cells/well. After 5 days, 100 µl of fresh medium was added to each well. The plates were maintained at 37°C and 5% CO_2 for 5 days. The specimens were placed in the wells and incubated at 37°C and 5% CO_2. After 72 h incubation at 37°C and 5% CO_2, cytotoxicity tests were applied to all wells. The not treated five wells were used as a control group. We set off 12 working groups and four control groups, including five wells in each.

Specimen preparation

The materials used in this study are listed in Table I. All materials were prepared according to manufacturers' recommendations. Initially, cylindrical wax pattern discs (5.5 mm in diameter and 3 mm thick) were prepared using a cylindrical stainless steel matrix. The discs were invested with type 4 of dental stone in stainless steel or injection flasks for PMMA or polyamide. The wax was eliminated with boiling water. The heat-cured PMMA specimens were packed and processed using a conventional pressure-pack technique (100°C, 20 min). Self-cured PMMA specimens polymerized at room temperature. To fabricate the Deflex specimens, the injection flasks were injected after the polyamide resin was plasticized at 270°C. All flasks were allowed to bench cool for 2 h. To avoid extra cleaning procedures, no grinding or polishing was performed after the excess flashes were removed. All specimens were washed twice with liquid soap under rinsed distilled water and sterilized

Table I. Used materials, manufacturers and other properties.

Trade name	Manufacturer	Composition	Polymerization method	Mixing ratio powder/liquid
Deflex	Nuxen S.R.L., Buenos Aires, Argentina	Polyamide	Injection molded	—
QC-20	Dentsply Ltd, England	PMMA	Heat-cure	24 g/10 ml
SC cold cure	Imicryl Diş Mlz, Konya, Turkey	PMMA	Auto-polymerized	0.6 g/0.4 g

by ethylene oxide gas sterilization (40% humidity, 50°C, 2 h).

In total, 60 discs were prepared and divided into three groups (20 discs for each dental material) and each group was divided into four sub-groups ($n = 5$). The first sub-group of each material was used for initial cytotoxicity testing and the others were aged for 24 h, 1 week and 8 weeks with artificial saliva (1.0 ml/well; 4.1 mM KH_2PO_4, 4.0 mM Na_2HPO_4, 24.8 mM $KHCO_3$, 16.5 mM NaCl, 0.25 mM $MgCl_2$, 4.1 mM citric acid and 2.5 mM $CaCl_2$, pH = 6.7) to examine the changes in toxicity [18]. The aging times were selected to extend intervals used in previous reports [13,14]. The 8 week aging was considered to better reflect the materials intended for long-term use in the mouth. The artificial saliva was sterilized by 20 μm filters (Millipore Ireland B.V., Tullagreen Carrigtwohill, Country Cork, Ireland) before using. Each specimen was aged by immersion in 200 μl of artificial saliva in the 96-well plates at 37°C. The ratio of the surface area of the discs to the volume of artificial saliva was 4.95 cm^2/ml, as recommended (0.5–6.0 cm^2/ml) by ISO 10993 standards [19].

Cytotoxicity testing

The cytotoxicity was assessed using cell viability and neutral red (NR) uptake assay. The NR uptake test is based on the fact that only live cells are able to take up the NR dye and accumulate it in the lysosomes. The test can thus determine the amount of live cells in a culture. After the 72-h incubation, 100 μl of 1.5% neutral red dye (1.5%; 39.4 ml Hank's Balanced Salt Solution + 0.6 ml NR) was added to each well. The plates were incubated for 2.5 h at 37°C. NR and medium in the wells were poured and 100 μl of 1% formic acid (1%; 12 ml dH_2O + 0.12 ml formic acid) was added to the each well. After 5 min incubation formic acid was removed and 100 μl of 1% acetic acid (1%; 12 ml dH_2O + 0.12 ml acetic acid) was added to each well. Following cell washing, the cell viability was assessed by reading the optical density of the resulting solution at 550 nm using a spectrophotometer (MicroQuant, Bio-Tek Instruments, Winooski, United States). Cytotoxicity was calculated as a percentage of the corresponding control group.

Statistical analysis was performed using SPSS 17 (SPSS, Chicago, IL) software. Whether the cell viability percentage measurements were normally distributed or not was determined by using the Shapiro Wilk Test. The results were analyzed using one-way analysis of variance (one-way ANOVA). The post-hoc Tukey HSD test was used as a post-hoc method to determine differences among the groups for each time period and the differences among time periods of each material. Statistical significance was considered to be $p < 0.05$.

Results

According to the initial test results, cell viability of all materials was similar ($p > 0.05$) (Table II). After the 24-h aging, all materials displayed small decreases in terms of cell viability. Deflex was more toxic than the control group ($p < 0.05$). However, any materials were not statistically different from each other ($p > 0.05$). After 1 week, all materials reached the highest viability values, but these differences were not statistically different from initial and 24-h cell viabilities. After 8 weeks, all materials became more toxic when compared with the control group, initial, 24 h and 1 week aging times ($p < 0.05$). QC-20 was the most toxic material after the 8 week aging time and it was significantly different from Deflex and SC Cold Cure ($p < 0.05$).

Discussion

In this study, short- and long-term biological responses of the polyamide denture base material were evaluated and compared with PMMA. According to the results of this study, the cytotoxicity of Deflex polyamide denture base material was consistent with PMMA. All the tested materials had a similar toxicity profile at the initial and short-term aging period, although long-term aging caused a sharp increase in cytotoxicity.

Table II. Cell viability values (percentage of the corresponding control group) and standard deviations.

Materials ($n = 6$)	Initial	24 h	1 week	8 weeks
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Control	100 ± 7.25	100 ± 9.13	100 ± 7.25	100 ± 9.13
Deflex	89.42 ± 7.39	79.26 ± 14.31[d]	92.69 ± 9.56	54.63 ± 8.58[abcde]
QC-20	88.46 ± 7.57	87.78 ± 7.34	96.15 ± 19.47	39.26 ± 4.38[abcd]
SC cold cure	90.38 ± 7.19	88.70 ± 9.76	90.77 ± 6.14	56.67 ± 2.37[abcde]

[a] $p < 0.05$; Statistically significant difference compared with initial (at 8 weeks).
[b] $p < 0.05$; Statistically significant difference compared with 24 h (at 8 weeks).
[c] $p < 0.05$; Statistically significant difference compared with 1 week (at 8 weeks).
[d] $p < 0.05$; p-values represent differences from control group.
[e] $p < 0.05$; p-values represent differences from QC-20 group.

We used unpolished specimens because the unpolished denture surface must interact with oral mucosa when dentures are placed [13]. To investigate the long-term biological response, specimens were aged with artificial saliva for 8 weeks, as accepted in the literature [14].

Different cell lines have been used in cytotoxicity studies. Primary cells (human epithelial cells, human gingival fibroblasts and human lymphoma cell lines) and continuous cells (L929 and Balb/C 3T3) have been mostly used for cytotoxicity evaluation [20,21]. HAFs are primary cells and well-matched the general fibroblast morphology and anatomy and reflect the biological response more closely than continuous cell lines.

Due to suffering from allergies and inflammation of traditional PMMA and developing of new materials, cytotoxicity of denture base materials continues to be a subject of interest. Cytotoxicity of PMMA generally is claimed by residual methyl methacrylate monomer (MMA), which can be released from denture base into water and saliva [22]. Residual monomer content varies with the polymerization methods and conditions and post polymerization treatments [23].

The wearing of new dentures overnight may result in mucosal irritation from these leachable substances because the released MMA after incubation for 24 h can cause cell toxicity *in vitro* [24]. Lefebvre and Schuster [25] showed that cytotoxic effects may occur a few days after polymerization and water immersion of dentures for 24 h, before usage reduces their toxicity. This process reduces the amount of residual monomers by accommodating additional polymerization [26,27]. In the current study, decreasing of cytotoxicity after 24 h and 1 week aging is harmonious with the literature [16,25].

Residual monomer leaching from PMMA is initially high and essentially completed after 14 days [23]. It is estimated that MMA-related cytotoxicity could decrease after the 14 days, and our 1-week cytotoxicity outcomes support this estimation. Residual monomer releasing from PMMA could be continuous. Studies [28,29] showed that residual monomer could be released for up to several years after insertion of prosthesis and loss of it completely may take many years. Another factor related to cytotoxicity of PMMA is other released chemical substances. Lygre et al. [30] reported that there was a number of released substances from PMMA, such as methacrylic acid, benzoic acid, phthalates, dibutylphthalate, dicyclohexyl phthalate, phenyl benzoate and phenyl alicylate. The amount of released particles changes with temperature as well as formaldehyde [12,31]. Formaldehyde toxicity is higher than MMA, even though the amount released was lower [32]. The toxic particles released from PMMA into the medium broke down over time or interfere with other chemicals in the medium that could alter the cytotoxic potential [33]. Our study showed that long-term aging caused a considerable increase inthe cytotoxicity of all materials. However, the toxicity changes between the 1- and 8-week aging should have been researched thoroughly. It is considered that the lately releasing substances are more effective on long-term cytotoxicity of PMMA than MMA. This *in vitro* outcome is very important in oral conditions which have many aggressive features such as pH, thermal changes and occlusal forces. These conditions could change the chemical properties and surface characteristics of the materials over time, contrary to the standard experimental set-up conditions such as constant temperature, stable pH and moisture [34]. However, lately released particles and their cumulative effects on the surrounding tissues and peripheral organs are very important and a specific issue in their future status.

The cytotoxic effects of heat-cured PMMA vs auto-polymerizing PMMA on fibroblast cells were reported by several authors [11,24,35,36]. Auto-polymerizing acrylic resins were more cytotoxic than the heat-polymerized, although some researcher found that the polymerization method did not significantly affect the cytotoxicity of both PMMA acrylic resins. Ata and Yavuzyilmaz [35] reported similar cytotoxicity values between heat- and auto-polymerized acrylic resins initially and 1 week later. Sheridan et al. [33] also observed that the greatest cytotoxic effect was produced by the auto-polymerized acrylic resins. According to our study, all materials exhibited a similar and decreasing toxic response up to the 1 week aging period, as in Sheridan et al. [33]. Auto-polymerized acrylic resin displayed a comparable toxicity profile with other materials at initial, 24 h and 1 week testing, unlike Sheridan et al. [33]. Advanced comparison was not possible, as they did not perform longer than 96 h aging. Lamb et al. [37] revealed that the levels of residual monomer changes with temperature. Specimens polymerized at 22°C had a higher level of residual monomer than polymerized at 55°C. In our study, aging at a higher temperature (37°C) probably decreased the amount of residual monomer and the cytotoxicity.

Polyamide specimens had a similar toxicity profile with the conventional PMMA materials in all tests except for 24 h aging. Deflex is a monomer-free material and does not have a polymerization reaction like PMMA. However, it was significantly cytotoxic according to control group, after 24 h and 8 weeks aging. At present, we do not have enough knowledge about the reasons of Deflex cytotoxicity, which mechanism causes the cell death and how it can be minimized. Studies showed that the toxic effects of releasing particles from the restorative materials originated from the chemical or mechanical effect of the soluble components or ions. Yamamoto [38] indicated that dendritic-shaped particles/surfaces have

more toxic effects than spherical ones. The extended aging period could alter the surface characteristics of the biomaterials and these alterations are thought to be the cause of the increased cytotoxicity after 8 weeks aging. Further research is needed to identify the effect of short- and long-term aging on chemical behavior, particle releasing characteristics, surface roughness and their relation with the cytotoxicity of Deflex. On the basis of our results it can be stated that the Deflex can be used as a denture base material with similar biological safety limits as PMMA.

It is emphasized that the results of the *in vitro* cytotoxicity tests have limitations in point of their applicability to clinical situations. Nevertheless, such tests are important in defining the biological behavior of dental materials and their components. Further *in vitro* and *in vivo* long-term studies are necessary to identify the nature of the interaction between the biomaterials and culture cells, surrounding tissues and body. The special toxic components of the denture base materials and the cytotoxicity mechanisms between cells and denture base materials are needed to be investigated.

In conclusion, according to results of this study, there was no significant difference between different denture base materials so the first part of the working hypothesis was rejected. There was a significant difference between short- (initial, 24 h and 1 week) and long-term (8 weeks) cytotoxicity. Therefore, the second part of the working hypotheses was accepted. The main findings of this study were:

- All materials showed similar toxic effects according to the control group in short-term aging periods.
- All tested materials reached the highest toxicity levels after the 8 week artificial aging time.
- Long-term aging was a more effective method to estimate biocompatibility of denture base materials.
- Polyamide specimens had a comparable toxicity profile with the conventional PMMA denture base materials in all tests.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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