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Biocompatibility and Cytotoxicity of Two Different Polymerized Denture Base Resins Cultured on Human Mesenchymal Stem Cells

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Abstract:

Background: Traditionally for decades most oral maxillofacial prosthesis were fabricated using heat cured acrylic resin with or without Co-Cr metal base, however, recently polyamide materials that are processed thermally not chemically have been widely promoted for use as the perfect alternative to conventional hard acrylic dentures. The aim of this study was to assess the reaction of human mesenchymal stem cells (hMSCs) toward the biocompatibility of two different polymerized denture base resins.

Materials and Methods: A total of 20 disc-shaped specimens (25 mm diameter and 3 mm thickness) were prepared according to the manufacturer's instruction as follows: Group (I) 10 disc-shaped heat cure acrylic resin samples polymerized conventionally in compression flasks, and Group (II) 10 disc-shaped thermoplastic acrylic resin samples polymerized using the injection molding technique. The cells used for this study were human bone marrow mesenchymal stem cells. Cytocompatibility was evaluated by WST-1 assay and live/dead fluorescent staining. The cells were observed under fluorescent microscope and photographed at 10× magnifications.

Results: An increase of the survival cell rate was observed in samples of both groups with no statistically significant difference. Although the live/dead viability/cytotoxicity assay showed a green fluorescence cell in Group I and II with fewer scattered red fluorescence cell in Group II, the difference was not significant in both groups indicating that the materials of both groups were compatible with hMSCs.

Conclusions: The current in vitro study concluded that the polymerization methods used in each group had no detrimental or negative effect on the biocompatibility of denture base resins. Our finding highlighted on the use of fluorescent live/dead viable/cytotoxicity assay to study the biocompatibility and the viability behavior of the two-denture base resins. These findings are also support by WST-1 assay.

Key Words: Acrylic resin, biocompatibility, cytotoxicity, maxillofacial prosthesis, polyamide

Introduction

Polymer (resin) is the most universal class of biomaterials with numerous applications in the biomedical field including tissue engineering, implantation of medical devices and prostheses. Proper selection of denture resins in prosthesis is important, as patients use the prostheses for long periods of time. Acrylic resin consists of two components: The powder (polymer) which contains the pigment that gives color to the resin and the liquid (monomer) which is clear, flammable, and volatile at room temperature, however, the monomer is considered to be cytotoxic and possibly genotoxic.[1]

The majority of denture bases are constructed using heat-cure acrylic resins that release certain toxic chemicals such as formaldehyde, methyl methacrylate, and benzoic acid, possibly eliciting serious reactions in the surrounding tissues.[2] The mechanical properties, as well as biologic features of denture base resin, are highly influenced by its monomer-polymer conversion. During the polymerization of denture base resin, the conversion of monomer to polymer is not completed resulting in residual monomers presence within the denture base polymers, which is leachable into water and saliva. Substances leached from denture base resin can cause adverse reactions in the oral mucosa adjacent to the denture.[3,4]

The countless variations in the components, structure and purity level of resins available in the market, as well as the pigments added to the powder and the monomer conversion rate in addition to the manipulative variables, may all affect the physical and biochemical properties as well as the toxicity of the resins.[5-9]

Nowadays, metal free restoration is in high demand in the replacement of lost structure due to oral diseases and conditions as well as prosthesis due to patient's more esthetic concern. Recently polyamide materials formed thermally processed have been widely promoted for use as denture base. It is perfect alternative to conventional hard acrylic dentures. Polyamide is a thermoplastic material that is, flexible, highly comfortable, and resistant to fracture. They offer many advantages over conventional powder and liquid resin systems due to their favorable creep resistance as well as high fatigue

properties and excellent wear characteristics. In light of their numerous clinical applications, the biological and toxicological properties of polyamides are critical. Among the various testing methods, which have been reported to determine the biocompatibility of biomedical materials, cytotoxicity test conducted *in vitro* on cell or tissue culture is necessary for testing the new materials used in humans to overcome the potential hazards of toxicity.[10] The current research utilizes the use of live/dead and WST-1 cytotoxicity assay to evaluate the response of human mesenchymal stem cells (hMSCs) with regard to the biocompatibility of two denture base resins.

Materials and Methods

Preparation of specimens

Twenty disc-shaped specimens (25 mm diameter and 3 mm thickness) were prepared as follows:

Group I consisted of 10 discs fabricated with heat cure acrylic resin (vertex – Dental B.V, Zeist, Netherlands), produced according to the manufacturer's instructions and polymerized using conventional compression flask technique. Using the lost wax technique, where the wax patterns were invested in stone mold flask then acrylic was packed and polymerized in water bath curing tank for 90 min at 70°C followed by 30 min at 100°C. The flask was then allowed to bench cool.

Group II included 10 thermoplastic acrylic resin discs (Bre. flex polyamide, Bredent, Gmbh. Co.K.G. Senden, Germany) fabricated using the injection molding technique. The wax patterns (Dental wax, Lordell trading, New south wales, Australia) were invested in a stone mold in an injection molding flask. The wax was eliminated and then the molten thermoplastic resin material was injected according to manufacturer's instructions. To plasticize the resin, an injection-molding machine was used at an injection pressure of 720-750 KPa, 220°C for 15 min.

On completion of processing, the acrylic resin specimens of both groups were then deflasked and polished to the desired dimension.

Sterilization of specimens

All specimens of both groups were sterilized by exposure to ultra violet light for 1 h to kill microorganisms that may have contaminated the discs during fabrication.[11]

Cell culture media

The cells used for this study were hMSCs (hMSCs, Lonza, Germany). These cells were grown as monolayer cultures in T-75 flasks (Nunc, Germany).

The disc-shaped samples were placed in complete culture media (Dulbecco's Modified Eagle Medium, GE Health, Germany) with 15% fetal bovine serum (FBS, Sigma, Germany) and 1% antibiotic (PAA, Germany). These were then incubated at 37°C with 5% CO_2 for 24 h. After the incubation period, the extracts were filtered through 0.22 μm filters and the samples collected were then tested to evaluate cytotoxicity as follows:

Control group: hMSCs cultured in complete culture media for 24 h.

Test groups (I, II): hMSCs cultured in extracted media of samples of Group I and II for 24 h.

WST-1 cytotoxicity assay

Cytotoxicity was evaluated by WST-1 assay (Roche applied science, Germany) in accordance with the manufacturer's instructions. This colorimetric assay is based on the cleavage of WST-1 tetrazolium salt via the mitochondrial dehydrogenases.[12]

hMSCs at passage 8 were plated into 96 well microplates at a seeding density of 1.7×10^3 cells in a volume of 100 μl per well of complete culture medium at 37°C, 5% CO_2 for 24 h. Next day, normal media were replaced by 100 μl of the extracts and incubated for 24 h.

Wells containing normal media served as control.

After exposure period, extract medium was changed with fresh medium, and finally, 10 μl of WST-1 solution was added to all wells. The same process was performed in the control wells. Aluminum foil was used to cover the culture plates and protect cells from light to allow incubation in the dark for 4 h at 37°C and 5% CO_2. On removal, well plates were shaken thoroughly for 1 min on a shaker.

Optical density was measured on a spectrophotometer plate reader (Multiskan MCC340, Labsystems, Germany) at 450 nm. WST-1 assay was repeated in three separate experiments to ensure reproducibility. The control cells survival rates of 100% were set to represent proliferation.

Live/dead fluorescent cell viability staining

This is a two – color fluorescent stain where the live calls are stained (green) by Calcein-AM stains while Ethidium homodimer III (EthD-III) – a membrane-impermeable DNA dye - stains dead cells red. This measures two parameters of cell viability, the integrity of the plasma membrane and the activity of intracellular esterase. The existence of intracellular esterase activity distinguishes the live cells as a result of enzymatic conversion of Calcein-AM to the Calcein resulting in the production of an intense uniform green fluorescence in live cells. Meanwhile, cells with damaged membranes are permeable to EthD-III, which on binding to nucleic acids produces the red fluorescence indicating dead cells.

To determine cell viability, live-dead cytotoxicity kit (Promokine, Germany) was used according to the manufacturer's instructions. Cells were seeded at a density of 7×10^4 on pre-coated chamber slides for 24 h. On the following day, hMSCs

were cultured either in Group I or Group II media. Following an incubation period of 72 h growth medium was discarded and replaced with sufficient volume of Calcein-AM/EthD-III staining solution to fully cover the cell monolayer on which the cells were incubated for 30-45 min at room temperature in the dark. After the staining, the cells were washed with phosphate buffered saline to reduce the background and the cells were then examined under fluorescent microscope and photographed at 10× magnification.

Statistical analysis

Statistical analyses were attained using Statistical Software Package (SPSS, version 20 Chicago, IL, USA). Descriptive statistics as means and standard deviations were used. For normally distributed data, comparison between the means of the control group and the test groups at 24 h were analyzed using *F* test (ANOVA) and *post-hoc* test (LSD).

Results

The survival cell rate of hMSCs of Group I and Group II after 24 h of incubation period was higher than the control group with more survival cell rate of hMSCs in Group I. Comparison showed no statistical significance between control group, Group I and Group II or between test Groups I and II (Table 1).

In the live/dead viability/cytotoxicity assay, the numbers of viable (green) and nonviable (red) cells were manually counted from the images. There were no detectable changes in cell viability in both groups. Cell viability was not affected by exposure to the media of Group I or II.

Figure 1a and b displays the results of live/dead viability/cytotoxicity assay. Green fluorescence cell was observed in Group I and II with fewer scattered red fluorescence cell in Group II, the difference was not significant in both groups indicating that the materials of both groups were compatible with hMSCs. This result supports the WST-1 results.

Representative images were selected from the results of one set experiment between two experiments.

Table 1: Comparison between the control group and the test groups after 24 h incubation.

Case no.	Control	Test groups	
		Group I (heat cure)	Group II (thermoplastic)
Mean±SD	2.02±0.55	2.26±0.87	2.09±0.91
F(p)	0.589 (0.558)		
Sig bet. groups	p_1=0.292, p_2=0.741, p_3=0.468		

F: *F*-test (ANOVA) for comparing between the three studied groups. *Post-hoc* test P_1: *P* value for comparing between control and Group I. *Post-hoc* test P_2: *P* value for comparing between control and Group II. *Post-hoc* test P_3: *P* value for comparing between Group II and Group III

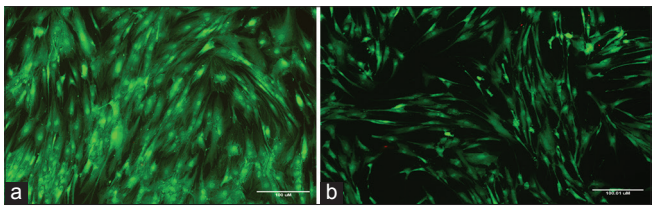

Figure 1: Green fluorescence in live cells of Group I (a) and Group II (b). Group II clearly shows green fluorescence in live cells with fewer bright red dead fluorescence cells at 10× magnification.

Discussion

In this study, two denture base resins were tested to evaluate of cytocompatibility using WST-1 assay and fluorescent live/dead assay.

The use of different assays and different cell types cultured *in vitro* to investigate the cytocompatibility of denture base resins have been widely reported including the measuring of both cells proliferation and viability.[3]

In light of its sensitivity and accuracy, WST-1 assay was used as a tool for the evaluating of cell cytotoxicity and cell proliferation as well as the estimation of the number of viable cells in cultures.[13,14] This is due to the convenience of the assay where adherent cells are cultured in a microplate, incubated with WST-1 and monitored using a spectrophotometer. The assay measures the transformation of the tetrazolium salt WST-1 to formazan by cellular dehydrogenases. The formazan generated is dark yellow in color, measured at 450nm and is directly correlated to cell number.[12,15,16]

Fluorescent live/dead assay is a newly developed powerful investigation tool for the visualization of distinct components of cells by incorporation of fluorescent markers which support the WST-1 assay finding and is considered to be more sensitive than WST-I assay in the evaluation of the cytotoxicity of resin monomers. In addition, fluorescent microscope combined with fluorescent live/dead assay provides greater resolution and imaging of biological structures than conventional imaging.[17-19]

hMSCs are commonly used in the protocol line of cytotoxicity tests. The hMSCs are multipotent self-renewing progenitor cells with the ability to secrete growth factors. These cells have the ability to differentiate into several cell types such as chondrocytes, adipocytes and osteoblasts, with easy isolation and expansion.[20]

Cytotoxic WST-1 assay test was carried out in this study following 24 h of incubation period since reports of cytotoxic effect of acrylic resin was greater in the first 24 h.[2,21]

The results of this study revealed that the variance in the survival cell rate of hMSCs was not significant between Group I and Group II. This result might be due to the temperature and protocol used for polymerization of heat cure acrylic resin (90 min at 70°C followed by another 30 min at 100°C) in Group I. The short curing cycle of heat polymerized acrylic

resins in Group I included a terminal boiling treatment for 30 min used to achieve maximum monomer conversion promoting lower amounts of residual monomer.[11,22,23] This is in contrast with the other polymerization cycles, which did not include a terminal, boil cycle where higher residual monomer level was detected and consequently resulted in increased cytotoxicity.[24,25]

The results of live/dead viable/cytotoxicity assay under fluorescent microscope confirmed the WST-1 cytotoxicity assay results. Live/dead cytotoxicity assay showed a green fluorescent cell in Group I and II. From our results, there was no significant difference in the live/dead cell count, which proves that the material was compatible with the cells.

Conclusion

The choice of the resin used for the fabrication of denture bases as well as the method of polymerization are critical and could be detrimental to the oral tissues considering how the denture base resin materials are in close and continuous contact with the oral tissues. The current *in vitro* study concluded that the polymerization method used in both groups had no effect on the cytotoxicity or biocompatibility of denture base resins. The use of hMSCs is recommended for better screening of the cytotoxic effect of denture base resins.

Our finding highlights, the benefits of using fluorescent live/dead cytotoxicity assay in distinguishing the biocompatibility and the viability behavior of two different polymerized denture base resins.

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