

An *In Vitro* Comparison of Cytotoxic Effects of Injection Molding, Computer-Aided Design and Computer-Aided Manufacturing Milling, and Rapid Prototyping Denture Base Resins

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Abstract

Background: Residual monomer, resulting from incomplete conversion of monomers into polymer, has the potential to cause irritation, inflammation, and an allergic response of the oral mucosa. Different processing techniques have an influence on the biocompatibility of denture base acrylic resins. This study aims to compare the *in vitro* cytotoxicity of polymethyl methacrylate denture base acrylic resins processed by injection molding technique, computer-aided design and computer-aided manufacturing (CAD/CAM)-milled and rapid prototyped technique using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. **Materials and Methods:** A total of 45 acrylic specimens of 10 × 1 mm discs were fabricated. Study was conducted between four groups: Group I - Control, Group II - Injection molded samples (SR Ivocap High Impact, Ivoclar Vivadent) (*n* = 15), Group III - CAD/CAM-milled specimens (Ruthinium, India), Group IV - CAD/CAM-Rapid prototyped samples (NextDent system). MTT assay was performed for all samples at 24 and 48 h. Wilcoxon signed rank test was used to compare means between paired data. For multiple group comparison, Kruskal–Wallis analysis of variance was used, followed by the Mann–Whitney U test for individual pairwise comparisons. **Results:** Cytotoxicity of CAD-CAM-rapid prototyped samples was found to be statistically the highest, followed by injection samples, and the least was observed in CAD/CAM-milled samples at 24 and 48 h, respectively. **Conclusion:** CAD-CAM-rapid prototyped samples exhibit the least biocompatibility followed by injection molded and CAD/CAM-milled samples.

Keywords: Computer-aided design and computer-aided manufacturing rapid prototyped, Computer-aided design and computer-aided manufacturing-milled, Cytotoxicity, Injection-molded, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, Polymethylmethacrylate.

INTRODUCTION

Acrylic resins are widely used in the fabrication of denture bases and have been cytotoxic as a result of substances that leach from the resin.^[1-3] The primary eluate is residual monomer. Numerous reports suggest that residual monomer may be responsible for mucosal irritation and sensitization of tissues. This information is important, not only to assess the biologic effects of such materials, but also to enable a comparison among the different polymerization methods, thus assisting the clinician in selecting a material with minimal cytotoxicity.^[4-6]

Resins used for the manufacture of denture bases have displayed various degrees of *in vitro* cytotoxicity and *in vivo* allergic responses, probably caused by unreacted components remaining after the polymerization process. Residual monomer

concentration varies with the methods and the conditions of polymerization. The variations in chemical composition and purity of the commercially available resin systems, the degree of conversion of their constituent monomers, and manipulative variables may all affect the biologic and physical properties of the acrylic resins. Nevertheless, acrylic resins are still widely used for the fabrication, relining, and repair of prostheses even

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though no biologic testing is required for their use in dental practice because they are considered to be low-risk materials for patients' health.

It is prudent to undertake *in vitro* cytotoxicity analysis to assess the levels of biocompatibility of any dental material before introducing them to the oral environment. According to the recommendations by ISO 10993-5 "Biological evaluation of medical devices-tests for *in vitro* cytotoxicity," (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay can be used to quantify the cell viability by monitoring the metabolic activity of the cell. One of the first assays to be developed specifically for the 96-well cell plate format, the MTT reduction assay is suitable for high throughput screening. The cell viability is calculated as a function of the cell metabolism that can be monitored by its ability to convert the yellow MTT into a formazan product that is purple with absorbance at approximately 570 nm.^[7-9]

MATERIALS AND METHODS

The present *in vitro* study was conducted to comparatively evaluate the cytotoxicity of polymethyl methacrylate (PMMA) denture-base acrylic resins processed by Injection molding and computer-aided design and computer-aided manufacturing (CAD/CAM)-milled and rapid prototyped techniques using an MTT assay (IEC/SVDCH/2226). In this study, 45 denture base samples were processed following three different processing techniques: Injection molding, CAD/CAM-milled, and rapid prototyped techniques. Under each group, 15 samples were fabricated to the desired dimensions of 10 mm × 1 mm discs.

Materials used in the study

The following materials were used for the present *in vitro* study. These include Polyvinylsiloxane putty impression material (Dentsply Sirona Aquasil Soft Putty), CAD-CAM Wax blank (Shiva Products, Vasai Virar, Thane, Maharashtra, Premeasured Capsules of Resin and Monomer (SR Ivoclar High Impact, Ivoclar Vivadent), Pre-polymerized CAD-CAM milling denture base material (Ruthinium® Dental Products Pvt. Ltd., India), and Pre-polymerized printing denture base material (Nextdent Denture 3D+, Nextdent® 3D systems, The Netherlands).

Preparation of samples

A total number of 45 denture base samples were processed using Injection molded, CAD/CAM-milled, and rapid Prototyped techniques. Under each group, 15 samples were fabricated to the desired dimensions of 10 mm × 1 mm discs.

- Designing of standard tessellation language (STL) file: The disc specimen of 10 mm diameter and 1 mm thickness was designed virtually using Blender 3.4 software and then exported as STL files
- Fabrication of injection molded specimens: Wax pattern of dimensions 10 mm × 1 mm was milled using the Icam V5 smart software and subjected to injection molding process
- Fabrication of CAD/CAM-milled specimens: Pre-polymerized CAD-CAM resin blanks were used in the

fabrication of CAD-CAM specimens. A CAD-CAM resin blank was milled in a computer numerical control (CNC) machine to the desired dimensions of 10 mm × 1 mm into 15 discs

- Fabrication of CAD/CAM-rapid prototyped specimens: Pre-polymerized NextDent® (translucent pink) acrylate ester-based resin was used in the fabrication of rapid prototyped specimens. The STL file was imported into the Nesting software, and a total of 15, 10 × 1 mm discs were designed. The CNC-generated CAM file was exported to the NextDent® 5100 3D printer and the resin was stirred in the LC-3DMixer before pouring it in the resin tray of the printer. 3D-printing was done with an incremental layer thickness of 50 µm in a 0° horizontal orientation using digital light processing method. The photopolymerized resin specimens were cleaned by rinsing in 96% isopropyl alcohol for 3 min. All 15 acrylic specimens were then exposed to an extra curing step in a photocuring unit for 10 min, and the acrylic denture base specimens were obtained
- Verification of dimensions: The dimensions of 10 × 1 mm for the specimens were verified with a Digital Vernier Caliper.

Testing of specimens

MTT calorimetric assay

The *in vitro* cytotoxicity test was carried out under ISO 109935 (cytotoxicity of medical devices). 3T3 L-1 Cells (1×10^5) were plated in a 96-well plate (supplemented with ~100 µL cell culture media - Dulbecco's modified eagle medium) in each well along with acrylic specimens. Cell culture without acrylic resin discs was taken as the control group. After treatment was over, 10 µL of MTT solution was added to each well and mixed properly. Cells were then incubated for 4 h in the dark at 37°C in a carbon dioxide incubator. After the incubation period, the media from each well was removed and the formazan crystals were dissolved with 100 µL dimethyl sulfoxide (DMSO) in each well by mixing properly. Then, the mixture was again incubated in the dark at 37°C for 30 min.

Calculating cell viability percentage using microplate reader

The microplate reader measured the absorbance of the dissolved formazan reagent at 595 nm. The formazan crystals were solubilized with DMSO, and the absorbance was measured at 545 nm at 24 and 48 h, respectively. The percent cell viability was calculated using a control and tabulated.

Calculation

$$\text{Viable cells (\%)} = \frac{\text{OD of drug treated sample}}{\text{OD of control sample}} \times 100$$

Statistical analysis

The optical density (%) was tabulated using MS Excel® (Microsoft Corp., New Mexico, USA) and analyzed using

Statistical Software, IBM Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corp., Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp). Shapiro–Wilk normality test revealed that data followed a non-normal distribution; hence, non-parametric tests were used to analyze the data. The Wilcoxon signed-rank test was used to compare means between paired data. For multiple group comparison, Kruskal–Wallis analysis of variance (ANOVA) was used, followed by the Mann–Whitney U test for individual pairwise comparisons, and $P < 0.05$ was considered statistically significant.

RESULTS

In this study, comparison of mean rank for optical density was done using Kruskal–Wallis ANOVA test (Table 1), and the Mann–Whitney U test was done for individual pairwise comparisons (Tables 2 and 3). The Wilcoxon signed rank test was used to compare means between paired data (Table 4). All the samples were subjected to MTT assay under recommendations by ISO 10993-5 “Biological evaluation of medical devices-tests for *in vitro* cytotoxicity.”^[5,10]

At 24 h, the optical density values were found to be higher in Group I (42.53%), followed by Group III (39.47%), Group II (26.67%), and Group IV (13.33%) (Table 1). These results are in contrast to study by Srinivasan *et al.*^[11] which stated that milled resins were equally biocompatible as conventional heat-cured resins in an *in vitro* setting with human primary osteoblasts and mouse embryonic fibroblasts. The results were similar at 48 h.

With respect to individual pairwise comparisons at 24 h (Table 2), statistically significant difference was found between Group I and Group IV (0.000), Group III and Group IV (0.000), respectively, which is consistent with the findings observed by Burgers *et al.* which stated that residual monomers leached from the 3D-printed resins into the surrounding cell culture medium and affected biocompatibility.^[12]

At 48 h, a statistically significant difference was found between Group I and Group IV (0.000), Group II and Group IV (0.014), Group III and Group IV (0.000) (Table 3) respectively, which is contrary to the data observed by previous studies that stated, milling and 3D printing did not have a significant influence on the biocompatibility of splint resins.^[13-16]

Table 1: Comparison of mean rank for cytotoxicity values (%) between control group (Group I), injection molded samples (Group II), CAD/CAM-milled samples (Group III) and CAD/CAM-rapid prototyped samples (Group IV) at 24 and 48 h

Sample	n	Mean rank (%) 24 h	P-value	Mean rank (%) 48 h	P-value
Control (Group I)	15	42.53	0.000*	44.10	0.000*
injection Molded (Group II)	15	26.67		28.57	
CAD/CAM-milled (Group III)	15	39.47		40.13	
CAD/CAM-rapid prototyped (Group IV)	15	13.33		9.20	

* $P < 0.05$ – Statistically significant. Significance level fixed as 5% ($\alpha = 0.05$). CAD/CAM: Computer-aided design/computer-aided manufacturing

Table 2: Multiple group comparison of mean rank for cytotoxicity values (%) between control group (Group I), injection molded samples (Group II), CAD/CAM-milled samples (Group III) and CAD/CAM-rapid prototyped samples (Group IV) at 24 h

Mean rank (%) at 24 h	Control (Group I) (42.53%)	Injection molded (Group II) (26.67%)	CAD/CAM-milled (Group III) (39.47%)	CAD/CAM-rapid prototyped (Group IV) (13.33%)
Control (Group I) (42.53%)	-	0.077	1.000	0.000*
Injection molded (Group II) (26.67%)	0.077	-	0.268	0.219
CAD/CAM-milled (Group III) (39.47%)	1.000	0.268	-	0.000*
CAD/CAM-rapid prototyped (Group IV) (13.33%)	0.000*	0.219	0.000*	-

* $P < 0.05$ - Statistically significant. Significance level fixed as 5% ($\alpha = 0.05$). CAD/CAM: Computer-aided design/computer-aided manufacturing

Table 3: Multiple group comparison of mean rank for cytotoxicity values (%) between control group (Group I), injection molded samples (Group II), CAD/CAM-milled samples (Group III) and CAD/CAM-rapid prototyped samples (Group IV) at 48 h

Mean rank (%) At 48 h	Control (Group I) (44.10%)	Injection molded (Group II) (28.57%)	CAD/CAM-milled (Group III) (40.13%)	CAD/CAM-rapid prototyped (Group IV) (9.20%)
Control (Group I) (44.10%)	-	0.089	1.000	0.000*
Injection molded (group II) (28.57%)	0.089	-	0.418	0.014*
CAD/CAM-milled (Group III) (40.13%)	1.000	0.418	-	0.000*
CAD/CAM-rapid prototyped (Group IV) (9.20%)	0.000*	0.014*	0.000*	-

* $P < 0.05$ - Statistically significant. Significance level fixed as 5% ($\alpha = 0.05$). CAD/CAM: Computer-aided design/computer-aided manufacturing

Table 4: Individual pairwise comparative evaluation of mean rank for cytotoxicity values (%) of control group (Group I), injection molded samples (Group II), CAD/CAM-milled samples (Group III) and CAD/CAM-rapid prototyped samples (Group IV) at 24 and 48 h

	Control (group I)	Injection molded (Group II)	CAD/CAM-milled (Group III)	CAD/CAM-rapid prototyped (group IV)
24 h	42.53	26.67	39.47	13.33
48 h	44.10	28.57	40.13	9.20
P-value	0.004*	0.005*	0.003*	0.011*

* $P < 0.05$ - Statistically significant. Significance level fixed as 5% ($\alpha = 0.05$). CAD/CAM: Computer-aided design/computer-aided manufacturing

On comparing the optical density values amongst the four groups at 24 and 48 h (Table 4), there was a statistically significant difference was observed which was consistent with the previous studies done by Burgers *et al.*, Bural *et al.*, Dawood *et al.*, Huettig *et al.* that reported that rapid prototyped samples showed highest amount of cellular toxicity followed by injection molded samples and least was found to be in CAD/CAM milled samples due to more amount on monomer release in rapid prototyped samples. [4,17-19]

DISCUSSION

Glossary of prosthodontic terms 10 defines biocompatibility as “capable of existing in harmony with the surrounding biologic environment.” The polymerization process and degree of polymerization affect the biocompatibility of acrylic resins.^[12,20] The traditional processing techniques involve compression molding and the injection molding technique. Recent advancements in digital technology have led to the development of CAD-CAM dentures. In the milled method, a pre-polymerized PMMA disc was used to mill the denture base.^[12] Although acrylic resins are biocompatible, they are cytotoxic as a result of substances that leach from the resin.^[21-23] Residual monomer, resulting from incomplete conversion of monomers into polymer, has the potential to cause irritation, inflammation, and an allergic response of the oral mucosa. Clinical signs and symptoms most frequently reported include erythema, erosion of the oral mucosa, and a burning sensation on the mucosa and tongue.^[21,24,25] Hence, it is prudent to undertake *in vitro* cytotoxicity analysis to assess the levels of biocompatibility of any dental material before introducing them to the oral environment.

According to the recommendations by ISO 10993-5 “Biological evaluation of medical devices-tests for *in vitro* cytotoxicity,”^[5] the MTT tetrazolium reduction assay can be used to quantify cell viability by monitoring the metabolic activity of the cell. It is based on the quantification of mitochondrial succinate dehydrogenase enzyme activity and measures the conversion of water-soluble tetrazolium salt to insoluble formazan. The cell viability is calculated as a function of the cell metabolism that can be monitored by its ability to convert the yellow MTT into a formazan product that is purple in color with absorbance at approximately 570 nm.^[7,17,26]

Bural *et al.*, Azzari *et al.*, Urban *et al.*, and Kurt *et al.* reported that the *in vitro* cytotoxicity of denture base acrylic resins

was described mostly after 1–2 days of elution. Potentially toxic substances such as formaldehyde, methyl methacrylate, methacrylic acid, benzoic acid, dibutyl phthalate, phenyl benzoate, phenyl salicylate, and dicyclohexyl phthalate, are eluted from denture base resins in 1–2 days.^[4,10,20,27] Hence, in the present study the cell viability percentage was calculated at 24 h and 48 h, respectively.

Apart from conventional resins, CAD/CAM resin blocks are pre-polymerized to a very high degree of pressure and temperature using sophisticated equipment, thus producing a highly condensed resin mass with less residual monomer content.^[18,28,29] Layer-by-layer polymerization of resins by 3D printing has been associated with high rates of residual monomers.^[11,28] Hence, it seemed possible that residual monomers leached from the 3D-printed resins into the surrounding cell culture medium and affected biocompatibility in the present study. The present study aimed to comparatively evaluate the *in vitro* cytotoxicity of PMMA denture base acrylic resins processed by injection molding technique, CAD/CAM-milled, and rapid prototyped technology using the MTT assay at 24 and 48 h. A null hypothesis stated that there was no statistically significant difference between injection molded, CAD/CAM-milled, and rapid prototyped groups at 24 and 48 h. However, the present study had certain limitations. The *in vitro* cytotoxicity data obtained in the present study are relevant yet not directly transferable to the *in vivo* situation. Nevertheless, the *in vitro* methods could play an important role in analysing the biocompatibility of denture base resins. The need for improved biocompatibility testing could be addressed using tissue-engineered human oral mucosa with fibroblasts and keratinocytes in multilayer cell culture models that simulate organotypic conditions.^[4,5,19,23,27]

CONCLUSION

Within the limitations of the present *in vitro* investigation, it was concluded that the cytotoxic effects of rapid prototyped samples were higher than CAD/CAM milled and injection molding samples; however, the cytotoxicity of all resins was below a critical threshold. Nonetheless, denture base acrylic resins should be selected carefully for clinical application as individual biocompatibility may vary significantly, possibly due to different chemical compositions. Further *in vitro* and *in vivo* investigations are needed to validate the findings of the present study.

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