ORIGINAL RESEARCH

Histocompatibility of Thermopolymerized Denture Base Copolymer Processed with a Novel Ring-opening Oxaspiro Comonomer: A Histomorphometric Investigation in Rats

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Abstract

Aim and objective: The aim of the research was to evaluate the histocompatibility of thermopolymerized (TP) novel denture copolymer containing 3,9-dimethylene-1,5,7,11-tetraoxaspiro[5,5]undecane (DMTOSU) comonomer in rats' palatine tissue.

Materials and methods: The rats were randomly categorized into four groups ($n = 6$ per group). G_{CW}: Denture base appliance (DBA) fabricated by short polymerization cycle in a water bath without DMTOSU; G_{TW}: DBA with 20 wt.% DMTOSU polymerized at 70°C for 2 hours followed by short polymerization cycle in a water bath; G_{TA}: DBA with 20 wt.% DMTOSU polymerized at 60°C for 45 minutes followed by 130°C for 20 minutes in an autoclave; and Group NC (negative control): rats with no DBA. The rats were euthanized after 2 weeks and the palatal tissues were subjected to histological examination. Epithelial thickness (ET), connective tissue thickness (CT), and keratin layer thickness (KT) were measured.

Results: G_{CW} exhibited greater ET, CT, and KT than the other groups. The ET and KT of G_{TA} were significantly lesser than G_{TW}. Multiple comparisons exhibited significant differences between the groups, except for G_{TW} and G_{TA} concerning the CT.

Conclusion: The novel denture copolymer containing 20 wt.% of DMTOSU comonomer is histocompatible with rats' palatine tissue.

Clinical significance: As DMTOSU is a double-ring-opening antishrinking oxaspiro monomer, its incorporation in TP-DBR would result in dimensionally accurate and stable dentures without endangering the biocompatibility in the prospective years.

Keywords: Comonomer, Denture, Histocompatibility, Initiation, Ring-opening.

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Introduction

Poly(methyl methacrylate) [P(MMA)] is an extensively employed biomaterial in dentistry for the past 8 decades, predominantly in the field of prosthetic oral rehabilitation. Hence, it is mandatory to achieve clinically palatable physico-chemico-mechanical and biological properties possessed by P(MMA).[1–5] Resin polymerization is a decisive process in dictating the physico-mechanical and biological properties of the material by enabling the monomer to polymer conversion (degree of conversion; DC).[6] A complete DC has never been achieved in any polymeric material. Unreacted monomer (methyl methacrylate; MMA) and its malicious metabolites from the denture base ensue due to the insufficient polymerization.[6–9] These unreacted residual monomers (URMs) and the metabolites persist even after the manufacturer's polymerization instructions are followed and get emancipated in the aqueous ambience of the oral cavity.[4,7–9] Previous researches reported that this URM inflicted mucosal irritation and cytotoxic/allergic reactions.[10–14] Therefore, biocompatibility is one of the significant determinants that curb this material's bioapplication.

Diverse researches have been executed to test novel dental biomaterials in order to check and improve the physico-chemico-mechanical and biological properties.[15–18] In thermopolymerized (TP) P(MMA), the heat activates the polymerization reaction by spontaneous decomposition of the dibenzoyl peroxide (DBPO) and thus, releasing voluminous free radicals. In reality, complete polymerization has never been achieved which ensues URM. Myriad studies have attempted to ameliorate the DC in order to diminish or suppress the cytotoxic effect caused by the URM and its metabolites.[19–21] Noxious metabolites along with URM and their effects on tissues have been demonstrated through animal model experiments, clinical surveys, and cell culture assays.[22,23] A cytotoxicity assay employing appropriate cell cultures is the initial biocompatibility test for a biomaterial, since it is uncomplicated, replicable, reliable, and cost-effective.[24] There are umpteen

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researches in the dental literature concerning initial tests assessing the cytocompatibility of denture base resin (DBR).[25–28] DBR exclusively contacts the oral tissues directly which necessitates *in vivo* animal model experiments to ascertain the histocompatibility of these materials owing to prolonged utility. Repeatedly encountered mucosal reactions were rubor, ulcerations, and burning sensation.[10,11]

Several compositional modifications had been executed in order to alleviate the URM and improve the DC.[29] The development of ring-opening monomers exhibiting antishrinking property with good dimensional accuracy renders the resultant polymer shrink-free.[30] 3,9-Dimethylene-1,5,7,11-tetraoxaspiro[5,5]undecane (DMTOSU), a six-membered oxaspiro double-ring-opening spiro-ortho-carbonate monomer with two symmetrical exocyclic methylene groups, was synthesized and employed as a denture base comonomer in our previous research which improved the DC of the resultant copolymer P(MMA-*Co*-DMTOSU).[31] However, the histocompatibility of the P(MMA-*Co*-DMTOSU) copolymer has not been reported yet in the dental literature. Therefore, the present research aimed to evaluate the histocompatibility of the P(MMA-*Co*-DMTOSU) copolymer in rats by histomorphometric analysis. The null hypothesis is that the P(MMA-*Co*-DMTOSU) denture copolymer would not affect the rat's histocompatibility.

Materials and Methods

The research was conducted in the Swamy Vivekanandha College of Pharmacy, Tiruchengode, Tamil Nadu. The institutional animal ethics committee (IAEC; Reg. No. 889/PO/Re/S/05/CPCSEA; January 30, 2018) approved the protocol of the research (Approval No. SVCP/IAEC/DSc/1/05/2020). The chemicals used for the synthesis of DMTOSU and the chemical integrants of a conventional TP-DBR were procured from Aldrich Co (Sigma-Aldrich, St Louis, Missouri, USA) and used as purchased (Table 1). The synthesis of DMTOSU and proportionating the integrants of TP-DBR were executed by following the steps described in the previous researches.[31,32]

Animal Care and Impression Procedures

Twenty-four adult Albino Wistar rats (*Rattus norvegicus*) of either sex were employed in this research. The International Organization for Standardization (ISO) 10993-2:2006 guidelines were strictly adhered throughout the research. The weight of the rats was periodically noted (350–400 g) before initiating the study to ascertain the growth cessation and then weekly during the study. Rats were housed in polypropylene cages at 23 ± 2°C with 55 ± 10% relative humidity and subjected to 12 hours of bright–dark acclimatizing cycles. Rats were provided with *ad libitum* water and rodent pellets with complete nutrients (Krishi Cattle and Lab Animal Feeds, Bengaluru, India). Rice husk cage bed was replenished with fresh husk three times a week.

Rats were to be anesthetized twice for making palatal impressions and to lute the denture base appliances (DBAs). Anesthesia was achieved for approximately half-an-hour by the intraperitoneal injection of ketamine hydrochloride and xylazine mixture. The impressions were made by two-stage putty wash technique using sterilized wooden stick depressors. Eventually, the rats were placed in the cages to convalesce.

Fabrication and Insertion of DBA

The rats were randomly divided into three groups ($n = 6$ per group) based on the composition of the DBA and polymerization regimen employed. The groups, their compositions, and polymerization regimens are tabulated in Table 2. Apart from this, Group NC ($n = 6$) served as negative control (NC) in which the rats were not affixed with DBA. The mold space needed for the fabrication of DBA was achieved by the modus operandi explained in the previous research.[33] The DBAs covered the hard palate and the occluso-buccal surfaces of the molars till the last antemolar rugae (AMR). The DBAs were checked for smooth

Table 1: Chemicals used in the research

	TP-DBR integrants and functions	
Chemicals for DMTOSU synthesis	*Powder*	*Liquid*
2-Methylene-1,3-propanediol	P(MMA) polymeric powder (molecular weight: 350×10^3 g/mol)	MMA (containing ≤30 ppm mequinol as inhibitor, 99%)
Tetraethyl orthocarbonate	Dibenzoyl peroxide (DBPO) as initiator	Tricyclodecane dimethanol diacrylate (TCDDMDA) as cross-linking agent
		Boron trifluoride diethyl etherate (BFDE) as initiator in cationic ring-opening polymerization
1,3-Dichloro-1,1,3,3-tetrabutyl distannoxane		Di-tert-butyl peroxide (DTBP) as initiator

Table 2: Groups, their composition, and polymerization regimen

Group	*Composition*	*Polymerization regimen*
G_{CW}	**Powder (75 wt.%):** P(MMA) prepolymeric powder with 2 wt.% DBPO **Liquid (25 wt.%):** MMA + 10% TCDDMDA	Thermopolymerized at 74°C for 2 hours followed by 100°C for 1 hour in water bath (short polymerization cycle) **Resultant polymer: P(MMA)**
G_{TW}	**Powder (60 wt.%):** P(MMA) polymeric powder with 2 wt.% DBPO **Liquid (20 wt.%):** MMA + 10% TCDDMDA with **4 mol% BFDE** To the powder-liquid mix, **20 wt.% DMTOSU** was added	Thermopolymerized at 70°C for 2 hours followed by short polymerization cycle in water bath. **Resultant copolymer: P(MMA-Co-DMTOSU)^W**
G_{TA}	**Powder (60 wt.%):** P(MMA) polymeric powder with 2 wt.% DBPO **Liquid (20 wt.%):** MMA + 10% TCDDMDA with 4 mol% BFDE and 4 mol% DTBP To the powder-liquid mix, **20 wt.% DMTOSU** was added	Thermopolymerized at 60°C for 45 minutes followed by 130°C for 20 minutes in digital vertical autoclave. **Resultant copolymer: P(MMA-Co-DMTOSU)^A**

W; Polymer resulted from WATERBATH A; Polymer resulted from AUTOCLAVE

cameo and intaglio surfaces. The appliances were meticulously trimmed, polished, and stored in distilled water for 48 hours at 37°C.

An anteroposterior narrow trough was made using a #6 round bur in the molar capping area to retain the DBA on the molars by self-adhesive resin cement (RelyX U200 Automix; 3M, St Paul, Minnesota, USA). The DBA was secured on the palate without applying inordinate pressure and ensured no gap between the DBA and the palate. Also, there should not be cement encroachment between the appliance and palate. The DBA with the cement was then light-cured (SS White Dental, Thane, India). The rats were housed to recuperate. Sequentially, the diet was converted to paste form to prohibit the debris amass under the DBA and worn for 2 weeks. Eventually, the rats were euthanized by overdosing thiopentone (120 mg/kg).

Histomorphometric Analysis

Each rat was decapitated and the mandible with the tongue was dissected. The DBA was removed punctiliously without compromising the palatal tissue. Residual tissues or organs were removed meticulously and the cranium was fixed in 10% buffered formalin for 48 hours. Ethylenediaminetetraacetic acid was employed for decalcification of the cranium, and end-point decalcification was ascertained with radiographic technique. Eventually, tissue dehydration was achieved by graded series of ethanol. The cranium was then bisected into two halves through the midpalatine section. For sectioning, the tissues were lodged into paraffin wax. Five ribbon sections (5 µm thick) were made by a microtome (Leica RM2245, Nussloch, Germany) per slide/rat and stained conventionally. The slides were examined under a light microscope (Olympus CH20i; Olympus Opto, New Delhi, India). Other tissue remnants and the carcasses were incinerated promptly.

The histomorphometric strata selected for evaluation were epithelial thickness (ET), connective tissue thickness (CT), and keratin layer thickness (KT) in micrometer (µm). For measuring the strata thicknesses, Image-Pro Premier (version 9.0 [9.1.5262.28]; Media Cybernetics, Rockville, Maryland) software was utilized. Histomorphometry was evaluated in a section by acquiring images of standardized sites at the last AMR and midpalatine region by digital microimager (Leica DMD108; 100 magnification). Two random sections of a slide were selected for measurements.[34] Ten measurements were made in each of the standardized sites for a stratum and the mean was noted. This yielded two means per slide/rat. Therefore, a total of 12 mean values per group and stratum were subjected to statistical analysis.

Statistical Analysis

Statistical Package for the Social Sciences (SPSS) software, version 21.0 (SPSS Inc., Chicago, Illinois) was employed for statistical calculations. Kolmogorov–Smirnov test was employed to verify the data distribution and found to be normally distributed ($p > 0.05$). Descriptive statistics, including mean, standard deviation (SD), standard error, maximum, and minimum, were calculated. For comparing the histomorphometric strata, one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests (*post hoc*; $\alpha = 0.05$) were executed. $p < 0.05$ was considered for statistical significance.

Results

The means ± SD concerning KT of NC, G_{CW}, G_{TW}, and G_{TA} were 100.02 ± 1.05, 136.21 ± 1.37, 107.14 ± 1.45, and 102.27 ± 0.85 µm, respectively. The means ± SD concerning ET of NC, G_{CW}, G_{TW}, and G_{TA} were 418.63 ± 2.37, 539.80 ± 3.92, 426.95 ± 2.7, and 412.31 ± 2.92 µm, respectively. The means ± SD concerning CT of NC, G_{CW}, G_{TW}, and G_{TA} were 316.74 ± 1.16, 399.39 ± 1.80, 312.09 ± 1.44, and 312.67 ± 1.49 µm, respectively. The means of the groups regarding the three histomorphometric strata are also depicted in Figure 1. Group G_{CW} exhibited the thickest epithelium, connective tissue, and keratin layer. One-way ANOVA showed a statistically significant difference ($p < 0.05$) among the groups concerning each stratum. Bonferroni multiple comparison tests determined a statistically insignificant difference ($p > 0.05$) between the G_{TA} and G_{TW} groups concerning the CT. All the other comparisons between the groups in each stratum exhibited a statistically significant difference ($p = 0.000$).

Discussion

In the present research, G_{CW} exhibited KT, ET, and CT than the other groups which can be ascribed to the release of URM from the DBA resulting in hyperparakeratosis (Fig. 2). The release of URM is due to

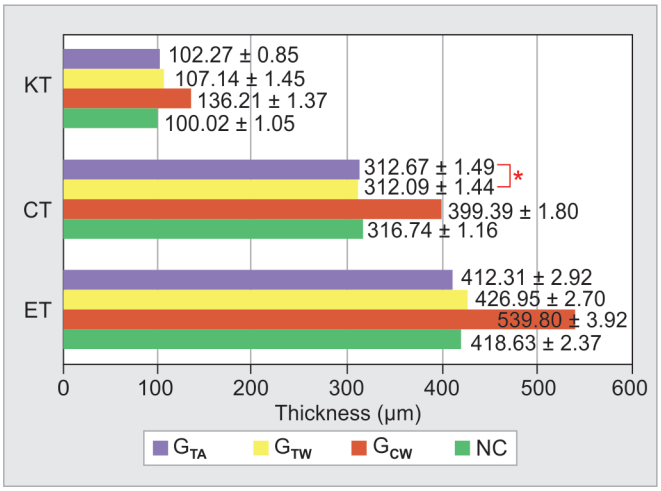


Fig. 1: Results of histomorphometric analysis. *Statistically not significant ($p > 0.05$)



Fig. 2: G_{CW} histological section in hematoxylin and eosin staining (100 magnification). K, keratin layer; E, epithelium; C, connective tissue; P, periosteum; PB, palatine bone; AMR, antemolar rugae

the incomplete polymerization or low DC. In our previous research, the control group demonstrated low DC than the P(MMA-*Co*-DMTOSU)^{A}.[31] Absence of polymorphonuclear (PMN) cell infiltration and presence of matured fibroblasts in the lamina propria can be attributable to the URM's inability to reach the connective tissue that is guarded by intact epithelium and keratin layer. Kapur and Shklar[35] found keratinization with a minimal rise in CT on alveolar mucosa under complete denture and postulated that these tissue aberrations resulted because of chemical insult. Ajay et al.[33] demonstrated an increase in the histomorphometric strata with the control group. The result of G_{CW} of the current research was in accordance with these previous researches. The increase in the ET can also be attributed to the changes related to acanthosis characterized with increased mass of epithelium, increased cytoplasmic and nuclear volume, and epithelial edema.[36]

Polymerization regimen and postpolymerization heat treatments have a great influence on the DC and eventually on the URM release.[6,31,37,38] In the present research, G_{TW} (Fig. 3) exhibited significantly lesser ET, CT, and KT than the G_{CW} which can be attributable to the polymerization regimen employed and the composition. The polymerization of G_{TW} exploited both free radical (Rȯ) and cationic (H^+) initiations. Bailey and Endo[39] demonstrated 4.3% expansion of DMTOSU during polymerization with 1 mol% boron trifluoride diethyl etherate (BFDE) at room temperature and 7% expansion at 70°C. Therefore, in G_{TW}, the initial thermopolymerizing temperatures were held at 70°C for cationic initiation by BFDE. Also, DBPO decomposes to release Rȯ at a temperature >60°C. Hence, with the hybrid Rȯ-H^+ initiations, G_{TW} is expected to have good DC. However, in our previous research, P(MMA-*Co*-DMTOSU)^{W} possessed lesser DC than the P(MMA).[31] This may lead to a misconception of G_{TW} group releasing high URM and eventually less histocompatible. However, in the present research, G_{TW} showed greater histocompatibility than the G_{CW}. This is because the C=C (unsaturated carbon–carbon bond) responsible for less DC is attributable to the pendant C=C of the ring-opened poly(ether carbonate) chain and not to the MMA polymeric chain. G_{TW} exhibited slightly higher ET and KT than the NC. All the above reasons concerning G_{CW} could be applicable to G_{TW} as well to a lesser extent. The decrease in CT of G_{TW} when compared to NC can be attributed to the adaptive response of the connective tissue subjected to masticatory loads under the DBA.

The ET and CT of the G_{TA} (Fig. 4) are significantly thinner than the NC (Fig. 5) and G_{CW}. The KT of G_{TA} is thinner than the G_{CW} and G_{TW}, whereas slightly thicker than the NC. The CT did not differ significantly between the G_{TA} and G_{TW}. The thinner ET in both G_{TW} and G_{TA} than the NC can be attributed to the flatter basement membrane due to adaptive alterations caused by the masticatory load yielding a uniform epithelium.[40] Ajay et al.[33] demonstrated thinner ET, CT, and KT with DBA containing cycloaliphatic comonomer in rats. Maruo et al.[41] studied that the tissue reactions owing to exerted pressure by acrylic appliances on the rat's palate and a decrease in ET were observed. The histomorphometric difference between G_{TA} and G_{CW} can also be attributable to the DC. G_{TA} employed hybrid Rȯ-H^+ initiations as same as the G_{TW}. Nevertheless, G_{TA} was subjected to autoclave polymerization regimen for which 4 mol% of di-tert-butyl peroxide (DTBP) initiator was utilized in addition. The homolytic temperature of DTBP is

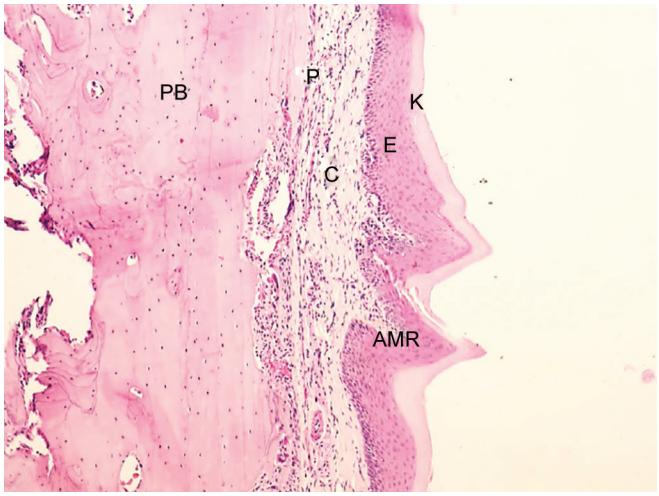


Fig. 4: G_{TA} histological section in hematoxylin and eosin staining (100 magnification). K, keratin layer; E, epithelium; C, connective tissue; P, periosteum; PB, palatine bone; AMR, antemolar rugae



Fig. 3: G_{TW} histological section in hematoxylin and eosin staining (100 magnification). K, keratin layer; E, epithelium; C, connective tissue; P, periosteum; PB, palatine bone; AMR, antemolar rugae

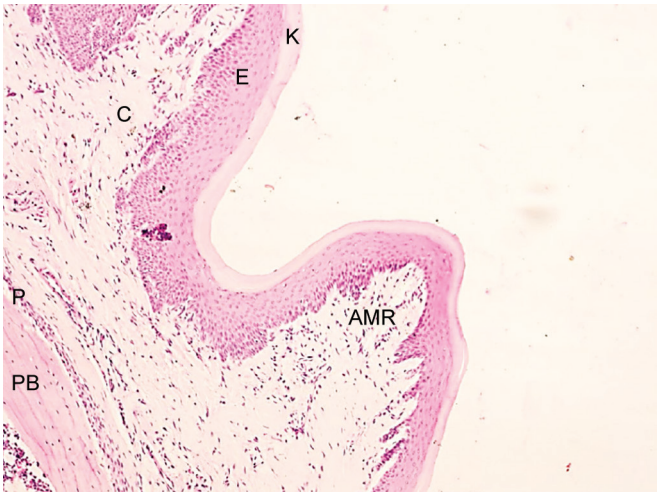


Fig. 5: Negative control histological section in hematoxylin and eosin staining (100 magnification). K, keratin layer; E, epithelium; C, connective tissue; P, periosteum; PB, palatine bone; AMR, antemolar rugae

>100°C. In a previous research, heating DTBP (3 mol%) at 120–130°C resulted in polymerization of DMTOSU that was cross-linked.[42] Hence, in this current research, cross-linking in the G_{TA} at the pendant C=C site of the ring-opened poly(ether carbonate) chain led to high DC, negligible URM and resulted in histocompatible DBA. Therefore, both P(MMA-*Co*-DMTOSU)^{W} and P(MMA-*Co*-DMTOSU)^{A} DBAs exhibited palatable tissue compatibility without jeopardizing the palatal tissue morphology, and thus, the null hypothesis was accepted.

There are also human trials concerning the tissue reactions under complete or partial dentures. Jani and Bhargava[43] found a general increase in the palatal ET associated with increased KT with no change in stratum granulosum and corneum. Increased epithelial volume and hyperkeratotic changes were also reported.[44] Turck[45] observed thicker stratum corneum under dentures. On the contrary, Ostlund[36] showed thinner or absent stratum corneum under complete dentures with parakeratosis. Hence, from the above literature evidence, the histomorphometric changes under the denture are considered debatable. The plausible reason could be the differences in the composition and type of prosthetic appliances used by the subjects, polymerization regimens, and masticatory loads.

The most common method to assess the biocompatibility of the biomaterials in animals was through subcutaneous implantation that does not simulate the oral tissue contact by the material. DBR remains in close contact with oral tissues. Erstwhile, studies determined the biological responses of DBR directly on rats with the DBA.[33,41,46–48] Hence, in the present research, the DBAs were fabricated to simulate palatal tissue contact by the appliance. Also, the DBA was retained on the molars with self-adhesive resin cement. Meister et al.[49] found that composite resin without acid-etching was efficacious in retaining the appliance. Jorge et al.[21,27] luted the DBA with autopolymerizing acrylic resin. The heat emanated during polymerization and URM from the material could cause histological vagary. Barclay et al.[50] faced ineffective retention with self-cure resin cement, which was eventually superseded with zinc oxide–eugenol cement. These cements could cause histological variations jeopardizing the results.

The results of the present research may not be completely appertained to the clinical scenario. This is owing to the 2 weeks of continuous DBA-palatine tissue contact, which in actuality is ill-advised to a denture patient. Conventionally, the denture patients are strongly recommended denture removal at night aiming to provide rest and allowing the denture-bearing tissues to revive from the masticatory load compressions since morning. Nevertheless, the DBAs were not able to be regularly removed and cleansed in order to maintain denture hygiene as done routinely by the patients. All the above-mentioned factors might affect the outcome of the present research. Anesthetizing the rats each time during insertion and removal of DBA is impractical due to ethical constraints. Therefore, formal human trials should be considered in the future to ascertain and adjudicate the biocompatibility of the novel denture copolymer.

Conclusion

Hence, from the results of the current research, it can be concluded that the novel TP denture copolymer P(MMA-*Co*-DMTOSU) containing 20 wt.% of DMTOSU comonomer exhibited a better histocompatibility in rats than the P(MMA).

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