Evaluation of Cytocompatibility of Thermopolymerized Denture Base Copolymer Containing a Novel Ring-opening Oxaspiro Comonomer

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ABSTRACT

Aim and objective: To evaluate the cytocompatibility of a novel denture base copolymer processed with 3,9-dimethylene-1,5,7,11-tetraoxaspiro[5,5]undecane (DMTOSU) comonomer using human keratinocytes (HKCs) and gingival fibroblasts (HGFs) by tetrazolium assay.

Materials and methods: The specimens were grouped based on the composition of the resultant polymer and curing regimen employed. Nine disk-shaped specimens per group were polymerized by one of the following curing regimens. G_{CW} : plain poly (methyl methacrylate) [P(MMA)] polymerized by short curing cycle in water-bath without DMTOSU; G_{TW} : P(MMA-Co-DMTOSU)^W copolymer polymerized with 20 wt% DMTOSU at 70°C for 2 h followed by short curing cycle in water bath; G_{TA} : P(MMA-Co-DMTOSU)^A copolymer polymerized with 20 wt% DMTOSU at 60°C for 45 min followed by 130°C for 20 min in an autoclave. Human keratinocytes and HGFs were employed to evaluate cell viability (CV%) by elution method through tetrazolium assay.

Results: A statistically significant difference was obtained ($p < 0.05$) among the groups with both the cell types. The ascending order of cytocompatibility is $G_{CW} < G_{TW} < G_{TA}$ with the CV% > 70%.

Conclusion: The novel P(MMA-Co-DMTOSU) denture copolymer is found to be more cytocompatible with HKC and HGF than the P(MMA).

Clinical significance: The novel P(MMA-Co-DMTOSU) denture base copolymer cytocompatible to HKCs and HGFs might bypass polymerization shrinkage and food accumulation at denture–tissue interface. Therefore, this copolymer is also anticipated to prevent oral malodor and stomatitis due to good tissue adaptability and dimensional accuracy.

Keywords: Cell viability, Copolymer, Cytocompatibility, Cytotoxicity, Ring-opening

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INTRODUCTION

Thermopolymerized denture base resin (TP-DBR) owing to its incomplete degree of conversion (DC) yields methylmethacrylate (MMA) as unreacted residual monomer (URM).¹ Each ingredient of the liquid monomer is a known contact/respiratory sensitizer/irritant.^{2,3} The toxicity of the monomers can be due to direct contact or leached out URM and its metabolites or in combination. Methylmethacrylate is known to get absorbed through the skin affecting the nerve myelination and conduction velocity in the fingers along with numbness.⁴ Sensory-motor peripheral neuropathy has been reported among dental technicians fabricating dental prostheses over 30 years.^{5,6} Methylmethacrylate also can be absorbed through gloves (latex, vinyl, nitrile, and butadiene–styrene) that can enhance the skin reactions by the trapped monomer.⁷

Contact dermatitis and respiratory hypersensitivity owing to monomeric vapors and trimmed particulates in the dental laboratories among the dental technicians or personnel have been reported.^{8,9} Also, during the practical handling/manipulation of DBR by the dental under/postgraduates, such health hazards could be discerned.¹⁰ Eczematous symptoms in the hands/skin owing to monomeric contact allergens of acrylic resin materials has been observed in 2 to 7% of dental practitioners.^{11,12} Formaldehyde (HCHO) is an oxidative metabolite of the residual MMA and leaches out from the denture bases.^{13,14} The allergic inflammatory oral mucosal reactions inflicted by the HCHO in denture users

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mandate to evaluate its release from the DBRs.¹⁵ Other inimical URM metabolites (methacrylic acid, benzoic acid, phenyl benzoate, and phenyl salicylate) and integrant that do not participate in

polymerization (dibutyl phthalate) also get surfaced on the denture base owing to incomplete DC.¹⁶

The polymerization reaction of acrylic resins is seldom complete paving way to noxious URM release. Its concentration varies based on the polymerization techniques and conditions.¹⁷⁻²⁰ The URM eventually leach into the aqueous ambient of oral cavity.²¹ Leached URM and metabolites are blameworthy for numerous *in vitro* cytotoxic reactions^{22,23} and *in vivo* allergic retaliations.^{24,25} Nevertheless, polymerization temperature and time determine the amount of URM responsible for various cytotoxic degrees.^{26,27} Commonly, elution/extract method was employed to evaluate the *in vitro* cytotoxicity on cell cultures by assessing enzymatic activity, which indirectly estimate the cytocompatibility of DBRs.²⁸

Myriad denture base monomeric modifications have been executed.²⁹ In our previous research, 3,9-dimethylene-1,5,7,11-tetraoxaspiro[5.5]undecane (DMTOSU), a ring-opening anti-shrinking comonomer, was incorporated in the TP-DBR in an attempt to develop a new copolymer with no polymerization shrinkage. This comonomer successfully copolymerized yielding novel P(MMA-Co-DMTOSU) copolymer with superior DC than the neat poly(methyl methacrylate) [P(MMA)].^{30,31} However, the cytocompatibility of this novel copolymer has not been reported yet in the dental literature. Hence, the present research aims to evaluate the cytocompatibility of the novel P(MMA-Co-DMTOSU) denture base copolymer employing human keratinocytes (HKCs) and human gingival fibroblasts (HGFs). The null hypothesis was that the P(MMA-Co-DMTOSU) denture base copolymer would not be cytotoxic to the cells.

MATERIALS AND METHODS

The present cytocompatibility research was conducted at Puducherry Center for Biological Sciences, Puducherry. The chemicals used for DMTOSU synthesis and the chemical ingredients of TP-DBR were purchased from Aldrich Co. (Sigma-Aldrich, St. Louis, MO, USA) and used as acquired without further purifications (Table 1). The synthesis of DMTOSU and proportionating the integrants of TP-DBR were executed by following the procedures elucidated in our previous investigations.^{30,31} The polymerized specimen disks were categorized into three groups ($n=9$ per group) based on the composition of the polymer/copolymer and curing regimen executed. The groups, their compositions, and curing regimens are tabulated in Table 2. The mold spaces needed for the fabrication of specimen disks were achieved by following the modulus operandi of a previous research.¹⁶ All the specimen disks (22 mm diameter; 2.0 ± 0.1 mm thick) were prepared by single investigator. The specimen disks were ultraviolet radiation treated for half an

hour to prevent the contamination of the cell lines and culture medium. The cells employed in this research were human cervical keratinocytes (HeLa cell line; HKC) and HGF. Preparation of the eluates and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium reduction assay was executed by strictly adhering ISO 10993-5 guidelines. Cells in culture medium without eluate treatment served as negative control (NC) or blank. Specimens' surface area [$2\pi r(r+h)$ cm²] to elution medium's volume (9 mL) ratio was 3 cm²/mL as recommended by ISO 10993-12 guidelines. A step-wise MTT assay procedure is elucidated in Table 3. Cell viability (CV%) is directly proportional to amount of formazan formed, as detected by the optical density (OD) at 570 nm absorbance in a microplate reader (Alere AM2100). To quantify the

CV%, the equation, $CV\% = \left(\frac{OD\ 570e}{OD\ 570b} \right) \times 100$ was used, where OD

570e and OD 570b represent the average of the obtained OD of eluates and blanks, respectively. $CV\% < 70\%$ of the blank indicates that the tested material is apparently cytotoxic.

Statistical analysis was performed by Statistical Package for the Social Sciences (SPSS) version 21.0 software program (SPSS Inc., Chicago, IL, USA). Test of normality by Kolmogorov-Smirnov test exhibited a statistically significant difference within the distribution of data among each group concerning HKC. Hence, the continuous data were furnished as median and quartiles [interquartile range (IQR)]. Kruskal-Wallis test and *post hoc* Dunn's test were employed to recognize differences in the CV% among and between the groups. Concerning HGF, statistically insignificant difference within the distribution of data among each group was found by the normality test. The continuous data were furnished as mean and standard deviation (SD). One-way analysis of variance (ANOVA) and *post hoc* Bonferroni tests were used to ascertain the differences in the CV% among and between the groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

Concerning the HKC, the median (IQR) CV% of G_{CW} , G_{TW} , and G_{TA} were 80.23 (0.7), 86.37 (0.21), and 92.10 (1.1), respectively (Fig. 1). There exists a statistically significant difference among the groups. In the *post hoc* multiple comparisons, except for NC- G_{CW} , NC- G_{TW} , and G_{CW} - G_{TA} comparisons, all other comparisons showed statistically insignificant differences ($p = 0.406$). The significant difference of G_{CW} - G_{TA} and insignificant difference of NC- G_{TA} comparisons were suggestive of cytocompatibility of G_{TA} . Concerning then HGF, the mean (SD) CV% of G_{CW} , G_{TW} , and G_{TA} were 74.77 (0.48), 88.88 (0.44), and 89.66 (0.84), respectively (Fig. 2). There is a significant difference

Table 1: Chemicals used in the research

Chemicals for DMTOSU synthesis	TP-DBR ingredients and function	
	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
1,3-dichloro-1,1,3,3-tetrabutyl distannoxane (Otera's catalyst)		Boron trifluoride diethyl etherate (BFDE) as initiator in cationic ring-opening polymerization
		Di-tert-butyl peroxide (DTBP) as initiator

Table 2: Groups, composition, and curing regimen

Group	Composition	Curing regimen
G _{CW}	Powder (75 wt.%): P(MMA) pre-polymeric powder with 2 wt.% DBPO. Liquid (25 wt.%): MMA + 10% TCDDMDA.	Thermo-polymerized at 74°C for 2 h followed by 100°C for 1 h 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.	Thermo-polymerized at 70°C for 2 h 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.	Thermo-polymerized at 60°C for 45 min followed by 130°C for 20 min in digital vertical autoclave. Resultant copolymer: P(MMA-Co-DMTOSU)^A.

Table 3: MTT assay—step-wise procedure

Time [h]	Procedure
00:00	HKC and HGF were plated separately in 48 well plates at a concentration of 2×10^4 cells/well in DMEM (Dulbecco's modified Eagle medium) culture medium with antibiotic solution and 10% fetal bovine serum and incubated at 37°C in 5% CO ₂ atmosphere for 24 h. Three specimens were suspended in 9 mL of culture medium and incubated as above. The resultant medium was used as eluates for treating cells.
24:00	Once the monolayer of the cells was achieved, the culture medium was aspirated and the cells were rinsed with PBS (phosphate-buffered saline). 200 µL of eluates were added to cells and the plates were incubated as mentioned above. Cells with no eluate treatment served as NC. NC served as blank.
48:00	The eluates were aspirated and the cells were washed with PBS. 100 µL of MTT solution (0.5 mg/mL) was incorporated to each well and incubated as above for 4 hours in a dark environment.
51:00	The MTT solution was discarded from the cells and washed with 200 µL of PBS. The reduced violet-blue formazan crystals were dissolved with 100 µL of di-methyl sulfoxide and swayed for 30 min.
51:30	Subsequently, the culture plates were transferred to a microplate reader and the color intensity was measured at 570 nm absorbance.

The experiment was performed thrice in triplicate for reproducibility⁶

among and between the groups ($p < 0.05$). Concerning both HKC and HGF, G_{CW} possessed greater cytotoxicity than the other groups and G_{TA} exhibited the least cytotoxicity. The ascending order of cytocompatibility is $G_{CW} < G_{TW} < G_{TA}$ with the CV% $> 70\%$. Hence, the novel copolymer P(MMA-Co-DMTOSU) is cytocompatible with both HKC and HGF.

Fig. 1: Human keratinocytes and cell viability percentage **Fig. 2:** Human gingival fibroblasts and cell viability percentage

DISCUSSION

In this present research, the TP-DBR was modified by adding 20 wt.% DMTOSU to the powder-liquid mixture. This modification yielded novel P(MMA-Co-DMTOSU)^W copolymer of G_{TW} and P(MMA-Co-DMTOSU)^A copolymer of G_{TA}. The resultant copolymers were subjected to *in vitro* cytotoxicity test by MTT assay employing HKCs and HGFs. Intriguingly, both G_{TW} and G_{TA} were cytocompatible with CV% greater than 85%. Likewise, modifying DBR with N-acetyl cysteine (NAC)³² and silver nanoparticles³³ did not affect the mammalian CV%. Ajay et al.¹⁶ concluded that modifying the DBR polymer with a cycloaliphatic comonomer resulted in higher OD values and CV% than NC. Addition of carbon-graphite fibers³⁴ and conglomerates of MMA-elemental iodine³⁵ in the DBR were found to be noncytotoxic. Cochis et al.³⁶ concluded that inclusion of biosurfactants in prosthetic materials to prohibit biofilm formation were cytocompatible. Conversely, methacryloyloxyundecyl pyridinium bromide (MUPB)³⁷ and polyoxymethylene³⁸ in the DBR jeopardized the CV%. An increased CV% can be inferred as an increased number of viable cells owing to the increased reduction of soluble MTT into

insoluble violet–blue formazan and *vice versa*. Hence, from the above context, it is apparent that the type of comonomer added determines the cytocompatibility of the DBR.

Denture base resins elude varying degrees of *in vitro* cytotoxicity and *in vivo* hypersensitive reactions that are attributable to the URM (C=C; carbon–carbon double bond) owing to incomplete DC. Furthermore, curing regimen and postcure thermal treatments influence the DC and URM release.^{17,31} In the current research, though G_{CW} [P(MMA)] exhibited cytocompatibility with both HKC and HGF, CV% were lesser than the G_{TW} and G_{TA} . This can be ascribed to the URM leach-out from the polymerized specimens. The URM ensued due to the incomplete polymerization or low DC. In our previous research, P(MMA-Co-DMTOSU)^A manifested lower DC than the P(MMA).³¹ This result concerning GCW is congruent with the previous studies, where DBR polymer without cycloaliphatic comonomer demonstrated lesser DC and greater cytotoxicity than the copolymer containing the same comonomer.^{16,39}

The cytotoxicity of G_{TW} was in-between the G_{CW} and G_{TA} . The higher CV% of G_{TW} than G_{CW} can be ascribed to the curing regimen employed and the composition. The polymerization of GTW exploited dual hybrid initiations videlicet free radical (R^o) initiation [Dibenzoyl peroxide (DBPO) and Di-tert-butyl peroxide (DTBP)] and cationic (H⁺) initiation Boron trifluoride diethyl etherate (BFDE). The context of using H⁺ initiation was to achieve ring opening of the DMTOSU comonomer and expansion. Bailey and Endo⁴⁰ reported 4.3% expansion of DMTOSU while processing with 1 mol% BFDE at 37°C and 7% expansion at 70°C. Therefore, in G_{TW} , the initial thermopolymerizing temperatures were held at 70°C for cationic initiation by BFDE. Thus, P(MMA-Co-DMTOSU)^W had a ring-opened poly (ether carbonate) (PEC) chain containing pendant C=C bond (unsaturated carbon–carbon bond) of methylene moiety resulting in lower DC than the P(MMA) in our antecedent study.³¹ This may be misinterpreted as high URM content that would decrease the CV% of P(MMA-Co-DMTOSU) W copolymer. On the contrary, in the present research, P(MMA-Co-DMTOSU)W possessed greater CV% than the P(MMA). This may be because the unsaturated bond in the ring-opened PEC chain is noncytotoxic. However, the unsaturated bond in the MMA polymeric chain is well-known for its cytotoxicity.

The CV% of the G_{TA} was greater than the G_{CW} and G_{TW} , which can be attributable to the DC. In our previous research, P(MMA-Co-DMTOSU)^A possessed the highest DC owing to hybrid dual R^o-H⁺ initiations.³¹ Higher the DC, lesser the URM, and higher the CV%. Nonetheless, G_{TA} was polymerized in the autoclave resulting in highly cross-linked P(MMA-Co-DMTOSU)^A. The crosslinking site was the pendant C=C site of the ring-opened PEC chain which led to high DC, negligible URM, and resulted in cytocompatible crosslinked denture copolymer. Hence, DMTOSU can act as a crosslinker when cured in the autoclave. Therefore, there is an inverse relation between DC and URM content/cytotoxicity. Also, the URM content and cytotoxicity are directly proportional.

The type and crosslinker's concentration also determine the cytotoxicity of the DBR. The commonly employed crosslinker is ethylene glycol dimethacrylate (EGDMA). Horie et al.⁴¹ reported a decreased DC with increased EGDMA concentration. Moharram et al.⁴² found that incorporating 12 or 17% of triethylene glycol dimethacrylate (TEGDMA) or tetrahydrofurfuryl methacrylate (THFMA) crosslinkers to the DBR decreased the URM content. Viljanen et al.⁴³ concluded that addition of dendritic cross-linker (dendrimer) to DBR increased the DC and

lowered the URM. Hence, in this present study, the incorporation of 20 wt.% of DMTOSU in TP-DBR produced P(MMA-Co-DMTOSU)^W and P(MMA-Co-DMTOSU)^A copolymers with higher CV% than the P(MMA). Therefore, the null hypothesis was accepted.

Denture base resin contacts disparate oral cells with conspicuous functions. Fibroblasts are the dominant cells in the oral mucosal connective tissue. However, the first layer of contact occurs on the keratin layer and keratinocytes. HKC was selected due to the potential direct apposition of the DBR on to the keratinocytes. Since DBR remains in close approximation with keratinized/nonkeratinized epithelium, leached eluates from the denture less than 100 kDa can percolate through the epithelium to reach underlying connective tissue/fibroblasts.⁴⁴ Various *in vitro* cytotoxicity tests have been performed with mouse fibroblasts.²⁸ Although mouse fibroblasts are mammalian in origin, use of human cells would be appropriate to simulate and correlate *in vivo* response more accurately. Human fibroblasts are exposed after epithelial ulcerations caused by traumatic dentures and exhibit amplified clinical relevance when compared to other mammalian fibroblasts. Studies with human cells that simulate some clinical perspectives are scarce in the dental literature. Hence, in the current research HKC and HGF were employed which is in accordance to Japanese guidelines to evaluate the biological effects of dental materials.⁴⁵

Predominantly, oral tissues are in contact with resinous polymerized dental biomaterials directly and indirectly. Oral mucosa, dental pulp, periodontal ligament cells, bone, and blood cells come into direct tissue–material contact. Indirect polymeric material–tissue contact occurs through the exudates leach out from the DBR.⁴⁶ In the present research, eluates of the test specimens were acquired promptly following specimens' polymerization. It is mandatory to test the specimens swiftly after curing to prevent the deprivation of noxious URM leached from the specimens at an early stage and avoid false-negative results.

The results of the present research may not be projected on *in vivo* scenario. However, with negating factors extinguished, the *in vitro* studies are frequently considered as relevant corroboration in evaluating the cytocompatibility of DBRs.⁴⁷ The DBRs experience drastic mechanical and thermal changes in the oral environments while in service. Simulating the *in vivo* oral aqueous ambience is the prime limitation to be considered while extrapolating the outcomes of *in vitro* studies to actual clinical conditions.⁴⁸ Nevertheless, evaluating the cytotoxicity of DBRs by MTT assay is comparatively easy to execute, reproducible, reliable, and inexpensive. Therefore, it warrants *in vivo* studies withal to recognize the tissue changes caused by the P(MMA-Co-DMTOSU) copolymer.

CONCLUSION

Adhering the experimental etiquette and considering the limitations, it can be concluded that the novel P(MMA-Co-DMTOSU) copolymer is cytocompatible with HKC and HGF.

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