ORIGINAL RESEARCH

Evaluation of *In Vitro* Cytotoxicity of Heat-cure Denture Base Resin Processed with a Dual-reactive Cycloaliphatic Monomer

Ranganthan Ajay[1], Karthigeyan Suma[2], Murugesan SreeVarun[3], Kandasamy Balu[4], Veeramalai Devaki[5], Natesan Devi[6]

Abstract

Aim: The aim of this study is to evaluate cytotoxicity of tricyclodecane dimethanol diacrylate (TCDDMDA) when added to conventional heat-cure methyl methacrylate (MMA) monomer at 10% and 20% (v/v) concentrations.

Materials and methods: Twenty seven disk-shaped processed specimens were divided into control group ($n = 9$; comprises specimens made without substituting TCDDMDA in MMA) and two experimental groups ($n = 9$ each; specimens prepared by substituting TCDDMDA in MMA at 10% and 20% (v/v) concentration). Eluates were prepared by placing three specimens of each group into 9 mL of culture medium and then incubated at 37°C for 24 hours. Continuous cells lines of L929 mouse fibroblast cells were used and MTT assay was employed to assess cytotoxicity. One-way analysis of variance (ANOVA) with *post hoc* Tukey's honestly significant difference (HSD) test was used to compare the mean optical density (OD) values and cell viability among the groups.

Results: A statistically significant difference was obtained ($p = 0.000$) when the mean and standard deviation of OD and cell viability (%) of the groups were compared. Highest OD value and cell viability was obtained with E20 group followed by E10 group.

Conclusion: Addition of TCDDMDA in MMA of heat-cure denture base resin has no cytotoxic effect on L929 mouse fibroblasts.

Clinical significance: Dual-reactive TCDDMDA is a crosslinking monomer which has no cytotoxic effects on mammalian cell cultures. Hence, incorporation of TCDDMDA to MMA can be extrapolated and projected for fabricating dentures without compromising biocompatibility.

Keywords: Cell viability, Cycloaliphatic monomer, Cytotoxicity, Modified monomer.

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Introduction

A biocompatible material is one which has the quality of being nondestructive in oral environment. Almost all dental materials have the characteristic feature of releasing substances into the oral environment to a varying degree, where denture base resins are not an exception.[1] Denture base acrylic resins were introduced to dentistry in the late 1930s. Since then, there have been reports of reactions to prostheses fabricated out of these materials. Descriptions of oral reactions to denture base acrylic resins often include symptoms like stomatodynia, glossodynia, rubor, and mucosal erosion.[2] In free-radical polymerization, the monomer to polymer conversion is not complete and the unreacted residual monomer released from the denture base may cause irritation or allergic oral reactions when in contact with the oral mucosa.[3] Cytotoxic effects of denture base resins are attributed to polymer-to-monomer ratio, storage time, water immersion, polymerization method, and cycle. It must not be inferred that by following heat-polymerizing method, release of residual monomer is completely eradicated.[4]

Residual monomer (MMA) along with numerous other toxic chemicals such as formaldehyde, methacrylic acid, benzoic acid, dibutyl phthalate, phenyl benzoate, and phenyl salicylate are produced on the denture base during incomplete polymerization process. The cytotoxicity test is performed with eluates of acrylic resin specimens to assess the material's biocompatibility indirectly on cell cultures. Cytotoxicity tests can also evaluate the toxic concentrations of the specimens, cell morphology and growth, degree of cellular damage, and enzymatic activities.[5] The cytotoxicity methods were elucidated and regulated by ISO standard 10993-5.[6] MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) analyzes the mitochondrial succinate dehydrogenase enzyme activity (cellular respiratory activity) and hence, this is an excellent marker of cell survival.[7]

Cytotoxic effects of several substances such as *N*-acetyl cysteine (NAC),[8–10] silver nanoparticles[11] and montmorillonite nanocomposite[12] were evaluated by incorporating them in the denture base acrylic resin. Carbon-graphite fiber[13] and associations between MMA and glycidyl methacrylate with elemental iodine[14]

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were also evaluated for cytotoxicity. Regis et al.[15] concluded that methacryloyloxy undecylpyridinium bromide is more cytotoxic than MMA monomer. Cochis et al.[16] concluded that the biosurfactants that prevent *Candida albicans* biofilm formation on prosthetic materials were noncytotoxic.

Tricyclo decanedimethanol diacrylate (TCDDMDA; Fig. 1) is a new dual-reactive cycloaliphatic acrylic monomer. This monomer possesses easily polymerizable carbon–carbon double bonds and a secondary reactive group. Few monomers have been researched with heat-cure polymethyl methacrylate (HC-PMMA) denture base resins pertaining to cytotoxicity. However, the effect of TCDDMDA with MMA of HC-PMMA has not been encountered yet in the dental literature. Hence, the aim of this present *in vitro* study is to evaluate cytotoxicity of TCDDMDA when added to conventional heat-cure MMA monomer at 10% and 20% (v/v) concentrations.

Materials and Methods

This study was conducted at Puducherry Centre for Biological Sciences, Puducherry. Heat-cure denture base acrylic resin (DPI, Mumbai, India) and cycloaliphatic monomer, TCDDMDA, (Sigma-Aldrich, Germany; CAS Number 42594-17-2) were used. Eluation method and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay were executed. Control group: E0 comprises specimens made without substituting TCDDMDA in MMA (100% MMA). Experimental groups: E10 and E20 have specimens each prepared by substituting TCDDMDA in MMA at 10% and 20% (v/v) concentration, respectively. The substituted monomers were stored in three individual identical dark glass containers and labeled with appropriate concentrations. Blinding of author was accomplished by concealing the labels with opaque stickers with random numbers from one to three (M1, M2, and M3) to avoid expectation bias.

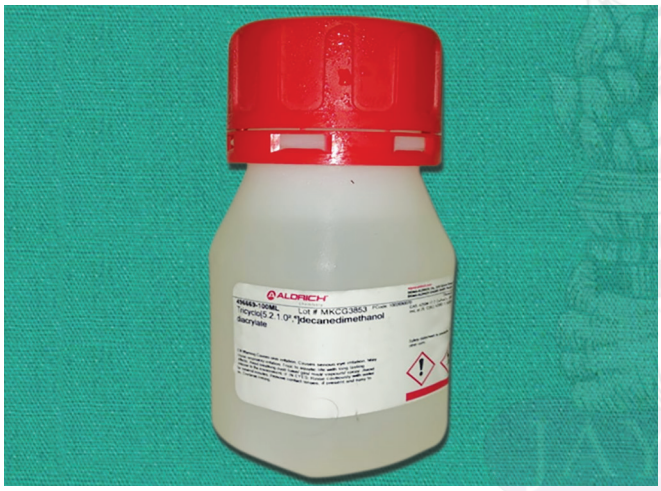


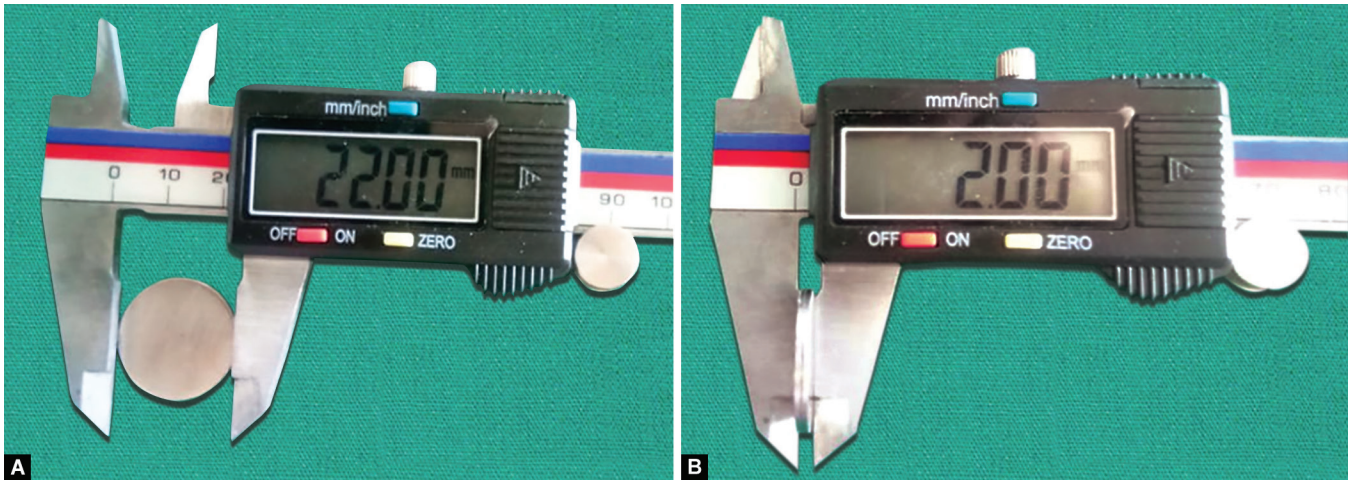
Fig. 1: Tricyclodecane dimethanol diacrylate

Specimen Preparation

Nine HC-PMMA specimens of each group were fabricated under aseptic conditions from disk shaped steel dies (22 mm diameter; 2 mm thick: Fig. 2), invested in the dental flask to obtain mold space. According to the manufacturer's instruction, the polymer and monomer were proportionated at 3:1 ratio. The dough forming time was 8 minutes for group E0 and 15 minutes for the groups E10 and E20. The mold spaces were then trial-packed with the dough at a packing pressure of 3500 psiin mechanical press (Sirio Dental Srl, Meldola FC, Italy) for 10 minutes. Heat-cure cycle was 74°C for 8 hours followed by terminal boiling treatment at 100°C for 1 hour in an acrylizer (Unident Instruments India Pvt. Ltd.). After half-an-hour bench cooling, the processed specimens were finished and smoothened on both sides with 600 grit silicon carbide papers.[17] All the polymeric specimens were prepared by single investigator. The final thickness of the specimens was re-assured with the help of digital Vernier caliper (ISO 9001:2000) to 2.0 ± 0.1 mm. This specimen configuration was selected because it is approximately the minimum thickness in a complete denture or removable partial prosthesis. Hence, it fit the experimental model by allowing the culture medium to completely cover both sides of specimens.[17] Prior to cytotoxicity testing, specimen disks were exposed to ultraviolet light for 30 minutes to kill microorganisms that may have contaminated the specimens during fabrication.

Eluate Preparation

Eluates were prepared by placing three specimens of each group into sterilized glass petri dishes with 9 mL of Dulbecco's modified Eagle medium (DMEM), supplemented with 5% fetal bovine serum, antibacterial and antimycotic solutions (usually 100 IU/mL penicillin, 100 µg/mL streptomycin), and 1% L-glutamine,[18] and then incubated at 37°C for 24 hours.[3] The Petri dishes were also allocated with the same random number found on the monomer container. Thereby,



Figs 2A and B: (A) Diameter of the metallic die; (B) Thickness of the metallic die

the monomer's concentration in the polymerized specimens was blinded. A petri dish with only 9 mL of culture medium without disks was incubated to serve as negative control (NC).[17,19] For the positive control (PC) group, distilled water was added to the cell culture. Distilled water lacks ions and hence, it disturbs the intracellular osmotic balance resulting in cell lysis.[20] The experiment was performed thrice in triplicate for reproducibility.

The eluates were then sterilized by filtering through 0.22-µm cellulose acetate filters into sterile glass vials. The eluate vials were then sealed, labeled with corresponding random numbers, and stored in refrigerator. The ratio of total surface area of the specimens [$2\pi r(r + h)$ cm^2] to the volume (9 mL) of elution medium was 3 cm^2/mL,[21] as recommended by ISO 10993-12.[22]

Cell line, Culture Medium and Reagents

Continuous cell lines of L929 mouse fibroblast cells[23] (Puducherry Centre for Biological Sciences, Puducherry) were propagated in DMEM supplemented with antibacterial and antimycotic solutions and 5% v/v fetal bovine serum. The culture was maintained at 37°C in an atmosphere of 5% CO_2/95% air.[24,25] The tetrazolium salt, MTT (Fig. 3), was dissolved in phosphate-buffered saline solution (PBS, pH = 7.4) at a concentration of 5 mg/mL and stored at 4°C immediately before use.[12] Figure 4 shows the culture medium, cell line, and other reagents used in this study.

MTT Assay

For cytotoxicity analysis, MTT assay was employed. By this technique, the methyl tetrazolium salt is incorporated into cell culture. The succinate dehydrogenase (SDH) enzyme from the viable cells reduces the tetrazolium salt into insoluble violet to blue colored formazan. The relative value of color intensity was determined in spectrophotometer with a wavelength of 570 nm. Mitochondrial activity is directly proportional to the color intensity and therefore, greater the viable cells. A brief step-wise procedure was elucidated in Table 1.

Determination of Cytotoxicity

A decrease in the number of living cells indicates a decrease in the metabolic activity of the cells. This decrease directly correlates with the amount of formazan formed, as monitored by the optical density (OD) at 570 nm in absorbance unit (au). The cytotoxicity evaluator was blinded from the concentration of test monomer in the specimens. To calculate the cell viability compared to the blank, the equation, cell viability% = $(OD_{570e}/OD_{570b}) \times 100$ was used. OD_{570e} and OD_{570b} denote mean values of the measured optical density of the 100% extracts of the test sample and blanks, respectively. The lower the viability% value, the higher the cytotoxic potential of the test specimen. If viability is reduced to <70 % of the blank, the test specimen has a cytotoxic potential.[6]

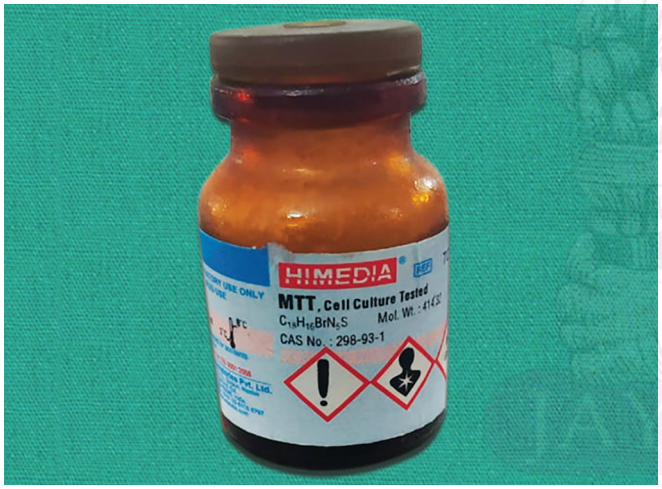


Fig. 3: MTT reagent

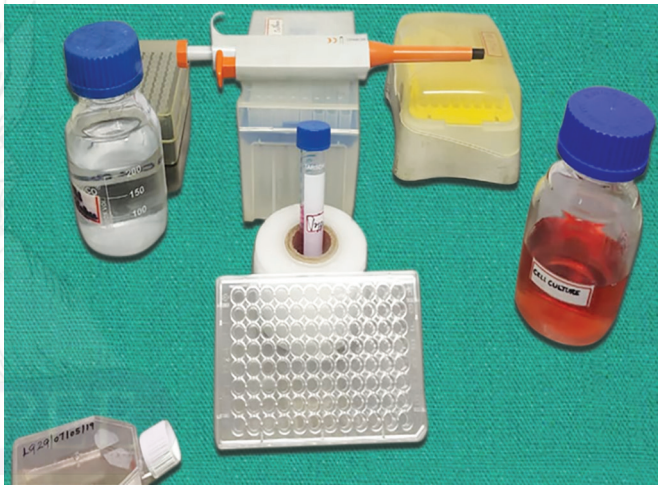


Fig. 4: L929 mouse fibroblast cell line, 96-well culture plate, culture medium, and reagents

Table 1: MTT assay—step-wise procedure

Time (hour)	*Procedure*
00:00	L929 mouse fibroblast cells in DMEM culture medium (cell suspension) were prepared at a concentration of 1×10^4 cells/mL. 100 µL/well of cell suspension was inoculated in 96-well cluster cell culture plates for a final concentration of 1×10^3 cells/well and incubated at 37°C in an atmosphere of 5% CO_2 for 24 hours
24:00	When the cellular monolayer was attained, the culture medium was removed, rinsed with PBS,[14] and 100 µL of eluates were added to appropriate prelabeled wells containing cells. Cells with medium alone served as NC. Cells with 100 µL distilled water served as PC. The culture plate was incubated at 37°C in an atmosphere of 5% CO_2 for 24 hours. NC was used as reagent blank
48:00	Microscopic evaluation of morphological alterations was done. The eluates will be removed and the cells will be washed with PBS.[16] 50 µL of MTT solution will be added to each well, and the culture plate will be incubated at 37°C in an atmosphere of 5% CO_2 for 3 hours in a dark environment[6]
51:00	The MTT solution will be aspirated and 100 µL of dimethyl sulfoxide will be added to each well and swayed to dissolve the blue-colored formazan crystals formed for 30 minutes
51:30	Subsequently, the culture plate will be transferred to a microplate reader, usually a UV-visible spectrophotometer equipped with a 570-nm filter, to read the absorbance (reference wavelength of 650 nm)

The experiment was performed thrice in triplicate for reproducibility[6]

Statistical Analysis

The obtained data were analyzed through SPSS, version 18.0 (SPSS, Chicago, IL). Statistician was blinded by concealing the test monomer concentration in each group. Shapiro–Wilks test was used to test normality of the obtained data. Based on the distribution, one-way analysis of variance (ANOVA) with *post hoc* Tukey's honestly significant difference (HSD) test was used to compare the mean OD values and cell viability between the groups. The obtained data were considered to be statistically significant when *p* value was less than 0.05.

Results

The OD values and cell viability percentage of the experimental groups (E0, E10 and E20) were evaluated and compared with the NC. Table 2 describes the mean and standard deviation of both OD values and cell viability. Figures 5 and 6 depict the mean and standard error of the OD values and cell viability. According to one-way ANOVA (Tables 3 and 4), statistically significant difference existed among the groups ($p = 0.000$). The mean OD and standard deviation of NC was 0.64 au ± 0.001. Highest OD value was obtained for E20 group (1.34 au ± 0.005) and the least for PC (0.23 au ± 0.005). The mean OD and standard deviation for E0 and E10 groups were 0.71 au ± 0.004 and 0.95 au ± 0.014, respectively. The mean cell viability (%) and standard deviation of NC was 100.21 ± 0.270. The highest cell viability was obtained for E20 group (209.70 ± 1.037) and the least for PC (35.52 ± 0.722). The mean cell viability and standard deviation for E0 and E10 groups were 110.74 ± 0.751 and 149.70 ± 2.396, respectively. Table 5 summarizes a statistically significant interaction between the groups in all the compared combinations ($p = 0.000$) through *post hoc* Tukey's HSD test. Greater the OD value and cell viability%, greater the cytocompatibility. Therefore, addition of TCDDMDA to MMA at both 10% and 20% (v/v) concentrations was considered to be noncytotoxic to the L929 fibroblasts.

Table 2: Mean and standard deviation of OD values and cell viability

	Mean ± SD	
Groups	*OD (au)*	*Cell viability (%)*
E0	0.71 ± 0.004	110.74 ± 0.751
E10	0.95 ± 0.014	149.70 ± 2.396
E20	1.34 ± 0.005	209.70 ± 1.037
PC	0.23 ± 0.005	35.52 ± 0.722
NC	0.64 ± 0.001	100.21 ± 0.270

Table 3: One-way ANOVA for OD values

Source	*Sum of squares*	*Mean square*	*F ratio*	*p value*
Between groups	6.051	1.513	2.893	0.000
Within groups	0.002	0.000		

Table 4: One-way ANOVA for cell viability %

Source	*Sum of squares*	*Mean square*	*F ratio*	*p value*
Between groups	148816.684	37204.171	2.332	0.000
Within groups	63.809	1.595		

Discussion

Biocompatibility experiments are mandatory to evaluate and assess the biological behavior of various dental materials. Numerous researches have been executed to test the cytotoxicity of denture base acrylic resins.[3] In this present study, the monomeric modification was executed by adding TCDDMDA to MMA at 10% and 20%. This modification was tested for cytotoxicity by MTT assay

Table 5: *Post hoc* Tukey's HSD test

Group	*Compared group*	*Mean difference of OD values*	*Mean difference of cell viability %*	*p value*
E0	E10	−0.248*	−38.960*	0.000
	E20	−0.631*	−98.956*	
	PC	0.480*	75.221*	
	NC	0.067*	10.536*	
E10	E20	−0.383*	−59.996*	0.000
	PC	0.728*	114.181*	
	NC	0.316*	49.496*	
E20	PC	1.111*	174.177*	0.000
	NC	0.698*	109.491*	
PC	NC	−0.412*	−64.686*	0.000

*The mean difference is significant at the 0.05 level

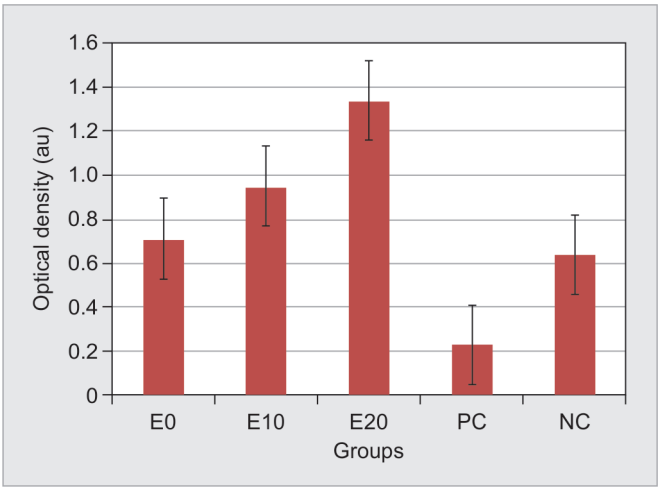


Fig. 5: Mean OD values and standard errors

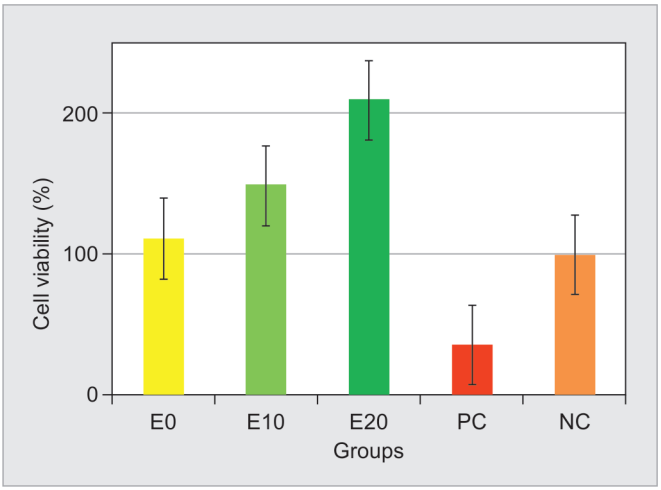


Fig. 6: Mean cell viability (%) and standard errors

using L929 mouse fibroblasts. Interestingly, both experimental groups (E10 and E20) were cytocompatible with cell viability greater than 100%. Lefebvre et al.[26] showed cell viability greater than 100% with aged eluates of photo-polymerized resin. Likewise, monomeric modification by adding *N*-acetyl cysteine[8–10] and silver nanoparticles[11] ameliorated the mammalian cell viability. However, on the contrary, MUPB[15] and polyoxymethylene[17] (an acetal resin) deteriorated the cell viability. Cell viability values greater than 100% suggest stimulation in response compared with the control and values less than 100% suggest inhibition. A rise in OD value indicates an increase in the number of viable cells and hence, increased reduction of MTT into insoluble blue formazan and *vise versa*. In this study, control and experimental groups had higher OD values than NC.

Denture base acrylic resins have elicited various degrees of *in vitro* cytotoxicity and *in vivo* allergic reactions which are probably caused by unreacted residual monomer (C═C) present after the polymerization.[17] Ajay et al. chemically characterized heat-cure denture by adding TCDDMDA to MMA and observed the disappearance of carbon–carbon double-bond (C═C) peak which indicated a clear reduction in the residual monomer.[27] The tricyclodecane (TCD) tri-ring central group of TCDDMDA offers steric hindrance effect that slows the rate of polymerization and facilitates the monomeric conversion to polymer, thereby reducing the residual unpolymerized monomer (C═C double bond) content in the final polymerized specimens. Also, TCD tri-ring monomers are classified under cross-linking monomers. Cross-linking monomers upon polymerization reduces the residual monomer content. Ethylene glycol dimethacrylate (EGDMA), a commercially used cross-linking monomer, does not possess the steric-hindrance property which very well unique in the TCD tri-ring monomers (TCDDMDA). Horie et al.[28] found the final conversion of MMA with EGDMA decreased with increasing content of the cross-linking agent. This is owing to the fact that rigid polymer structure hinders the conversion of MMA monomers peculiarly at curing temperature lower than glass transition (T_g) temperature. Therefore, once T_g is reached, further conversion of MMA does not happen, thereby limiting the conversion of residual monomer with no effect of heat thereafter. Moharram et al.[29] concluded that the addition of 12% or 17% of triethylene glycol dimethacrylate (TEGDMA) or tetra hydro furfuryl methacrylate (THFMA) cross-linkers to the denture base resins reduced the amount of residual monomer. Viljanen et al.[30–32] concluded that addition of dendritic cross-linker (dendrimer) to denture base resin resulted not only better mechanical properties but also a high degree of polymerization and low residual monomer. Hence, in this present study, the addition of TCDDMDA to MMA (E10 and E20 groups) decreased residual monomer content and attributed to greater OD values and cell viability percentage when compared to E0 and NC groups.

Another plausible reason for high cell viability percentage and OD values in experimental groups can be attributed to polymerization temperature and time. Based on these factors, different amounts of residual monomer remain unreacted and thus resulting in various degrees of cytotoxicity.[33,34] In long curing cycle, the T_g of the matrix phase (97–100°C) is higher than the temperature used for the polymerization (74°C for 8 hours).[35] Because of lower molecular chain motions and immobilization of monomer in the glassy polymer, the monomer elicit poor ability to polymerize.[36] It has been recommended that the curing cycle of heat-cured acrylic resins should always include a terminal boiling treatment for at least 1 hour to achieve maximum monomer conversion to polymer.[33] The long curing cycle (74°C for 8 hours) that does not include a terminal boiling treatment possibly results in higher residual monomer levels and, consequently, increased cytotoxicity. This presumption was confirmed by Urban et al.,[37] who concluded that the short curing cycle with terminal boil promoted lower amount of residual monomer (0.08%) when compared with the long curing cycle without terminal boil (0.24%). Hence, in this current study, long curing cycle followed by 1-hour terminal boil has been executed to reduce the cytotoxicity by residual monomer.[38]

Huang et al.[39] stated that, compared to light-cured and heat-cured resins, auto-polymerized acrylic resins showed a higher cytotoxic effect for fibroblasts and epithelial cell lines. Rose et al.[40] evaluated heat-cured, light-cured, and autopolymerized resins and concluded that heat-cured resin was not considered cytotoxic and light-cured resins were considered to be of low cytotoxicity. Autopolymerized resin was considered the most cytotoxic because of its monomer, urethane dimethacrylate, which caused greater inhibition of cellular growth.[17] Hence, in the present study, heat-cure denture base resin was used.

In patients, dental materials contact different target cells possessing specific functions. Fibroblasts are the dominant cells in the oral mucosal connective tissue. Since acrylic resin is in close contact with keratinized or nonkeratinized epithelium, molecules lower than 100 kilo dalton can infiltrate into the underlying connective tissue, providing an entrance for the residual monomer to reach the connective tissue fibroblasts.[41] In *in vitro* cytotoxicity tests, permanent cell lines, or primary cells are commonly used. Schmalz[42] showed that the use of these primary cells offers no decisive advantage. Furthermore, primary cells are mainly ill-defined and rare in availability. Therefore, in the present study, permanent cell lines, L929 mouse fibroblasts, were used to evaluate the cytotoxicity of the specimens.

Generally, dental resin materials contact oral tissues both directly and indirectly. Direct tissue-material contact occurs in tissues like exposed dental pulp, oral mucosa, and blood cells. Indirect resin–tissue contact occurs when the tissue is exposed to exudates released from the acrylic resins into the local environment, such as when oral mucosa is exposed to chemicals released into the saliva.[24] In the present study, eluates of the test specimens (E0, E10, and E20) were prepared by placing three specimens of each group, immediately after sample fabrication, into a sterile glass petri dish with culture medium. In *in vitro* researches, it is important for the materials to be tested immediately after processing to avoid the loss of toxic substances released from the specimens at initial stage.[17]

In this present *in vitro* cytotoxicity research, substituting concentrations of TCDDMDA in MMA was 10% and 20%. In the majority of *in vitro* studies regarding the monomer modification, the substituting concentrations of experimental monomer in proprietary monomer were 10% and 20% with an average range of 2.5–50% v/v.[43] Various *in vitro* assays have been executed to evaluate the cytotoxicity of different dental biomaterials. ^{3}H-Thymidine assay is a precise method where the number of DNA synthesizing cells is counted. However, because of the exorbitant cost, advanced technique, and associated radioactive wastes, this test is seldom used in experiments.[20] Therefore, owing to wide range applicability and ease of access, MTT assay was implemented and executed in this study.[44,45]

Before commencing the cytotoxicity assay, in many researches, specimens were subjected to ultrasonic cleansing with distilled

water for 30 minutes as a step of decontamination, though this is not usual clinical procedure. While ultrasonic cleansing, potential toxic components from the specimens may have partially eluated into the distilled water bath and thus reducing the amounts of toxic components in the culture medium during cytotoxicity assay.[46] Hence, ultrasonic cleansing was avoided in the present study which might affect the cytotoxic potential of the specimens.

The present research is a triple-blinded study. The author, the cytotoxicity laboratory personnel, and the statistician were blinded by concealing the concentration of TCDDMDA substituted in MMA to avoid expectation bias that might creep into the result. Sample size of 9 specimens per group was arrived from the previous researches evaluating the cytotoxicity of denture base resins.

The results of the present *in vitro* research may not be directly and completely applied to *in vivo* conditions. However, considering *in vitro* researches as a simple means of evaluation and with confounding factors eliminated, they are often regarded as sources of proof in assessing the cytotoxicity of denture base resins.[47] Limitations in the simulation of *in vivo* environment should be taken into consideration while generalizing the results of *in vitro* experiments to clinical practice.[20] Experimenting denture base resins by cell culture methods is relatively simple to execute, reproducible, controllable, and cost-effective.[48] The materials used as dentures are being subjected to changes in the moist oral environments. Therefore, the results of *in vitro* experiments cannot be extended and extrapolated to the clinical scenario.[4] Hence, it mandates further *in vivo* researches to identify and distinguish the leaching chemicals or their derivatives from denture base resins with TCDDMDA in a moist environment. The correlation between leaching chemical and its effects on different cellular physiology may be enthralling topics of future investigations.

Conclusion

Following the experimental protocol and within the limitations of this *in vitro* research, it is concluded that the addition of TCDDMDA (in both 10% and 20% concentrations) in MMA of heat-cure denture base resin has no cytotoxic effect on L929 mouse fibroblasts.

Clinical Significance

Dual-reactive TCDDMDA is a cross-linking monomer which has no cytotoxic effects on mammalian cell cultures. Hence, incorporation of TCDDMDA to MMA can be extrapolated and projected for fabricating dentures without compromising biocompatibility.

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