ORIGINAL RESEARCH

In Vitro Cytocompatibility of Dental Restorative Composite Resin Photopolymerized with a Novel Multifunctional Crosslinking Comonomer

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Abstract

Aim and objective: The present research aimed to assess the cytocompatibility of a novel dental restorative composite resin (DRC) copolymer containing dipentaerythritol penta-/hexa-acrylate (DPEPHA) as comonomer by tetrazolium assay.

Materials and methods: Twenty-seven photopolymerized specimens ($n = 9$ per group) were divided into a control G0 group (specimens without DPEPHA) and two trial groups [specimens with 20 wt% (G20) and 40 wt% (G40) DPEPHA]. Eluates from the specimens were extracted and filtered. L929 mouse fibroblasts were employed and an MTT assay was executed. Parametric tests and multiple comparison tests were utilized to analogize the average optical density (OD) and fibroblastic viability among and between the study groups, respectively.

Results: A significant difference was apparent ($p = 0.000$) when the means of OD and cell viability of the groups were compared. The assessed parameters were higher for the trial groups than the control. The novel copolymer P(GEU-Co-DPEPHA) (trial groups) possessed higher OD and fibroblastic viability than the P(GEU) (control).

Conclusion: The novel copolymer P(GEU-*Co*-DPEPHA) formed by the addition of DPEPHA in propriety DRC matrix was cytocompatible with L929 fibroblasts.

Clinical significance: P(GEU-*Co*-DPEPHA) is cytocompatible with the mammalian fibroblasts. Hence, the substitution of this crosslinking comonomer would improvise the physicomechanical properties of the DRCs without compromising biocompatibility.

Keywords: Cell viability, Comonomer, Cross-linker, Cytocompatibility, Cytotoxicity.

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Introduction

Materials employed in the field of dentistry ought to be innocuous to the oral tissues. Strictly, there should be no leachable or diffusible substances that would cause unfavorable local or systemic effects. Recent researches with technological enhancements have resulted in dental restorative composite resins (DRCs) being mechanically equivalent to amalgam restorations. These materials bond to enamel and dentin substructures without exorbitant removal of tooth structure for retaining the restoration.[1] The physical properties of DRCs are continuously improvised. Their complex chemical composition is attributable to myriad monomers and additives.[2] Primarily, a mixture of various methacrylate monomers, such as 2,2-*bis*[4-(2-hydroxy-3-methacryloxypropoxy) phenyl] propane (*bis*-GMA; G) and urethane dimethacrylate (UDMA; U) along with low viscosity co-monomers, such as triethyleneglycol dimethacrylate (TEGDMA; E), ethylene glycol dimethacrylate (EGDMA), or diethyleneglycol dimethacrylate (DEGDMA) is the composition of the organic matrix.[3–5] However, in the commercial resin matrix systems, the predominantly used comonomers are G, E, and U (GEU) in numerous combined ratios. Dental restorative composite resins also contain inorganic particulate ceramic reinforcing fillers that do not seem to play a vital role in the biocompatibility of the material, despite the organic component.[6]

Biocompatibility is the property of materials to function harmoniously within living tissues without inflicting any damage. Short- and long-term unfavorable tissue reactions ranging from postoperative sensitivity to irreversible pulp damage can result

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from malicious or cytotoxic restorative materials.[7] More than 12% of unfavorable reactions to dental biomaterials are contributed by the DRCs alone.[8] The quantity and quality of released organic components are responsible to determine the biocompatibility of the DRCs.[7] Deleterious consequences are caused by integrants leached out from the matrix due to insufficient photo-curing and/or resin deterioration during clinical service.[9–12]

Within the initial few days of photopolymerization, the majority of the unreacted components are released from the DRCs. Unreacted residual monomers infiltrate the human body through the mucosa and odontogenic complex.[13] The biological properties of the DRCs may be influenced by the integrants released from them. Comonomer E has been found as the principal compound released from polymerized resin composites into aqueous media in most of the studies. In addition, small quantities of G, U, and other comonomers may also be released from the DRCs. Comonomer E has cytotoxic potential and impedes cell growth.[14] Other unreacted comonomers including G, U, and EGDMA have been found to inflict cytotoxicity and mutagenicity on cells both *in vitro*[15,16] and *in vivo*.[17] Residual comonomers in the tissues induce estrogenicity,[18–20] genotoxicity,[21,22] and alteration of immune responses[23–25] although the clinical prevalence of these untoward events remains uncertain and controversial. Approximately 15–50% of the methacrylate groups do not participate in the incipient photopolymerization.[26] Under normal photopolymerization, DRCs create highly cross-linked networks and they accomplish approximately 45–70% of conversion.[27,28] As much as 25–50% of the methacrylate monomer double-bonds indeed linger to be inactive in the polymer.[29] The number of residual monomers in DRCs and the magnitude of the cytotoxicity effects is correlated.

The recent researches in the development of DRCs with new polymerization chemistries have been driven by the potential clinical liabilities of resin matrices that are based on methacrylate functionality. The evolutionary curve of DRCs has been abruptly sharp as novel compositions sprout with the motive of enhancing their wear resistance, esthetics, manipulation, and adhesion to dental hard tissues.[30] However, the biocompatibility of many newly developed materials is inexplicit, conspicuously when sought for long-term clinical usage. Therefore, cytotoxic effects elicited by the existing organic matrix comonomers are still an unresolved issue. This mandates the search for a novel organic matrix comonomer with improved biological and physicomechanical properties. Dipentaerythritol penta-/hexa-acrylate (DPEPHA) is a novel multifunctional hydrophobic monomer having high crosslinking potential, abrasion resistance, surface hardness, and good adhesive property. The phosphorylated form of DPEPHA (phosphate ester monomer) has been utilized as a bonding agent to lute zirconia crowns to the teeth.[31] However, no researches regarding neither DPEPHA addition in the conventional DRC resin matrix (GEU) nor the cytotoxic effects of the formed novel copolymer [P(GEU-*Co*-DPEPHA)] exist in the fraternity of dentistry. Hence, this research aims to study the cytocompatibility of a novel DRC copolymer containing novel comonomer DPEPHA at 20 wt% and 40 wt% concentrations by tetrazolium (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay. The null hypothesis of the research is that the novel P(GEU-*Co*-DPEPHA) would not adversely affect the cytocompatibility.

Materials and Methods

This *in vitro* cytocompatibility study was executed at Puducherry Centre for Biological Sciences, Puducherry. The institutional ethics committee (Reg. No. ECR/784/Inc/TN/2015; Rule 122DD; Drugs and Cosmetics Rule-1945) approved the research protocol (Approval No. VDCW/IEC/241/2021). The control and trial composite resin matrices were synthesized by following the steps described by Aydınoğlu and Yoruç.[32] The composition of the matrices was described in Table 1. The matrix-filler ratio was 30:70 wt% and the photoinitiator-amine ratio was 1:2. All the matrix monomers, camphorquinone (CQ), dimethylamino ethyl methacrylate (DMAEMA), and barium oxide (BaO) were acquired (Sigma-Aldrich Co., St Louis, MO, USA) and utilized without purifications. Barium fluoride (BaF_2; Sisco research laboratories Pvt. Ltd., Maharashtra, India) and zirconia nanoparticles (Nano Research Lab, Jamshedpur, Jharkhand, India) were also purchased.

Twenty-seven ($n = 9$ per group) disk-shaped specimens (diameter 22 mm; thickness 2 mm) were made out of laser-cut acrylic dies and silicone molds in dental crown flasks (Photosil Soft putty, DPI-Dental Products of India, Mumbai, India). The top surface of the flask was covered with a clear glass plate to obtain a flat surface. A polyester sheet was positioned over the dispensed matrix material and pressed with a glass tile onto the mold's surface to remove the superfluous material. The resin matrices were photopolymerized for 40 seconds with a light-curing unit (Guilin Woodpecker Medical Instrument Co., Ltd.; Guangxi, China; 420–480 nm, 650–800 mW cm^{-2}).

Eluate Preparation

The test specimens ($n = 3$ per group) were planted in 9 mL of culture medium [Dulbecco's modified Eagle medium (DMEM) + 5% fetal bovine serum + 100 IU/mL penicillin and 100 µg/mL streptomycin + 1% L-glutamine] contained in decontaminated cell-culture Petri-plates and incubated at room temperature for a day in 5% CO_2 atmosphere. For negative control (NC), culture medium without specimens, and for the positive control (PC), cell culture with distilled water were incubated. The eluates were sterilized using thin filter paper disks and transferred into decontaminated vials for refrigeration after proper sealing and labeling until further use. The specimens' surface area to volume of culture medium ratio was 3 cm^2/mL.[33]

Table 1: Composition of control and trial matrices

Matrix	*Composition*
G0	Monomeric ingredients: *Bis*-GMA (50 wt%), TEGDMA (20 wt%), DUDMA (30 wt%); GEU. Filler ingredients: BaO (30 wt%), BaF_2 (30 wt%), ZrO_2 (40 wt%). CQ-DMAEMA complex: 1 wt.%.
G20	Monomeric ingredients: *Bis*-GMA (40 wt%), TEGDMA (20 wt%), DUDMA (20 wt%), DPEPHA (20 wt%). Filler ingredients: BaO (30 wt%), BaF_2 (30 wt%), ZrO_2 (40 wt%). CQ-DMAEMA complex: 1 wt%.
G40	Monomeric ingredients: *Bis*-GMA (30 wt%),TEGDMA (20 wt%), DUDMA (10 wt%), DPEPHA (40 wt%). Filler ingredients: BaO (30 wt%), BaF_2 (30 wt%), ZrO_2 (40 wt%). CQ-DMAEMA complex: 1 wt%.

MTT Assay

L929 mouse fibroblasts were employed. In MTT assay, the enzymatic (succinate dehydrogenase) reduction of methylated salt of tetrazolium to insoluble crystalline formazan was analyzed and the chromatic saturation was established using a microplate reader (Alere AM2100). The chromatic saturation and mitochondrial activity are directly proportional to each other, which signifies fibroblastic viability. A brief stratagem was reported in Table 2. The enzymatic reduction activity can be directly quadrated with the formed crystalline formazan's quantity and interpreted as optical density (OD) at 570 nm. The cell viability compared to the blank was calculated by using the equation,

$$\text{Cell viability } \% = \left(\frac{OD570_e}{OD570_b}\right) \times 100$$

$OD570_e$ and $OD570_b$ represent the average of obtained OD values of the experimental eluates/extracts and blanks, respectively. When the fibroblastic viability of the specimen is decreased to 70% of the blank, it is deemed cytotoxic.[34]

Statistical Analysis

The acquired data were subjected to Kolmogorov–Smirnov normality test [Statistical Package for the Social Sciences (SPSS; version 21.0) Chicago, IL, USA]. Based on the results ($p > 0.05$), one-way analysis of variance (ANOVA) with *post hoc* Bonferroni multiple comparison tests were employed to compare the differences among and between the groups. $p < 0.05$ was considered to be statistically significant.

Results

With the NC, the study groups (G0, G20, and G40) were compared concerning the OD and cell viability. The mean values of the assessed parameters were presented in Table 3. A statistically significant difference existed among the groups ($p = 0.000$; Table 3). The highest OD value among the study groups was obtained for the G40 group and the least for G0. The highest cell viability among the study groups was obtained for the G40 group and the least for G0. Regarding the OD and cell viability, multiple comparison tests showed significant differences between the groups ($p = 0.000$; Table 4). Therefore, the novel copolymer P(GEU-*Co*-DPEPHA) is cytocompatible to the L929 mouse fibroblasts at both 20 wt% and 40 wt% concentrations of DPEPHA substituted in the propriety GEU resin matrix.

Table 2: MTT assay—step-wise procedure

Time [h]	*Procedure*
00:00	The fibroblasts were added to the culture medium to form a cell-medium mixture (1×10^4 cells/mL). A 96-well culture plate was inoculated with 100 µL/well of a cell-medium mixture (1,000 cells per well) followed by incubation.
24:00	The culture medium was removed after observing the cellular monolayer and rinsed with phosphate-buffered saline (PBS). 100 µL of eluates (e) were dispensed to pre-labeled wells followed by incubation. NC served as reagent blank (b).
48:00	Morphological aberrations were observed under the microscope. The eluates were pipetted out and the cells were treated with PBS. 50 µL of tetrazolium was dispensed into the wells and incubated in a dark environment for 3 hours.
51:00	After aspirating the MTT solution, 100 µL of dimethyl sulfoxide was added to each well and undulated for 30 minutes until blue-colored formazan crystals dissolve.
51:30	Finally, the culture plate was placed in a microplate reader to read the absorbance.

For reproducibility, the above-mentioned procedure was executed thrice in triplicate[34]

Discussion

In improvising the conversion rate and glass transition temperature of the DRC, integration of copolymerizable crosslinking comonomer to the resin matrix has been proved to be an efficacious way. Despite improved conversion rate, the monomer to polymer conversion is seldom complete and leads to the release of unpolymerized unreacted monomer that affects the cyto-/histocompatibility of surrounding tissues. It is essential to investigate the cytocompatibility of the photopolymerized DRC with DPEPHA cross-linker by studying the eluent's influence on cell growth.

In the present research, the resin matrix modification was executed by incorporating DPEPHA to GEU at 20 wt% and 40

Table 3: One-way ANOVA

Group	*Mean ± SD*	*F ratio*	*p value*
I. OD values [absorbance unit (au)]:			
G0	0.282 ± 0.002	3,116.284	0.000
G20	0.333 ± 0.001		
G40	0.363 ± 0.001		
NC	0.399 ± 0.012		
PC	0.112 ± 0.004		
II. Cell viability (%):			
G0	72.28 ± 1.00	23,528.133	0.000
G20	84.74 ± 0.43		
G40	92.00 ± 0.27		
NC	100.14 ± 0.17		
PC	28.55 ± 0.45		

Table 4: *Post hoc* Bonferroni multiple comparison tests

Group	*Compared group*	*Mean difference of OD values*	*Mean difference of cell viability*	*p value*
NC	G0	0.116222*	27.862222*	0.000
	G20	0.065222*	15.396667*	0.000
	G40	0.035667*	8.137778*	0.000
	PC	0.286667*	71.586667*	0.000
G0	G20	−0.051000*	−12.465556*	0.000
	G40	−0.080556*	−19.724444*	0.000
	PC	0.170444*	43.724444*	0.000
G20	G40	−0.029556*	−7.258889*	0.000
	PC	0.221444*	56.190000*	0.000
G40	PC	0.251000*	63.448889*	0.000

*The mean difference is significant at the 0.05 level

wt% concentrations. Intriguingly, the trial groups were more cytocompatible than G0. Hence, the null hypothesis was accepted. The reason ascribed for decreased cytotoxicity is the higher DC in the G20 and G40 groups than G0. Reactive moieties are responsible for the rate of conversion. For faster reaction kinetics, the acrylic moieties of multifunctional acrylates are used instead of the methacrylic moieties of propriety dimethacrylates. The second pendant methacrylate group is prognosticated to be 7.5–10 times less reactive after the first methacrylate group has reacted in dimethacrylates. As a result, the final conversion during polymerization is reduced.[35] The acrylate moieties with rapid reaction kinetics in DPEPHA, on the other hand, resulted in increased conversion, decreased residual monomer content, and eventually less cytotoxicity. This result obtained in the current research can also be corroborated with the research conducted by Ajay et al. which stated that increased DC would decrease the amount of residual unreacted monomer leaching out.[36] In addition, DPEPHA is classified under crosslinking acrylate monomers. The crosslinking property of this comonomer can be attributed to the reduction of residual monomer content upon copolymerization with increased DC. Wang et al. determined that the final conversion of double bonds decreased with increasing the amount of cross-linker percentage.[37] However, in the current study, the addition of DPEPHA to propriety resin matrix monomers decreased residual monomer content which is accredited to a positive conversion rate leading to higher OD values and cell viability than the control G0 group. Hence, from the results of the present and previous researches, it can be deduced that the DC and cross-linkers concentration are independent variables and the DC is dependent on the chemical nature of the cross-linker added. Howbeit, the cytotoxicity evaluation of the DRC employing DPEPHA comonomer is solitary research and therefore, appropriate literature corroboration of the result was less feasible.

With aged eluates, cell viability >100% was reported.[38] Solvents or polymer degradation are the major reasons for the elution of unbound monomers and/or additives from the DRC within the first hour after initial polymerization.[6] A common presumption is that the biomaterials' cytocompatibility might be correlated to the TEGDMA's quantity that was eluted out of the DRCs[39] and residual uncured monomer or oligomer.[40] Within 24 hours of initial polymerization, utmost leaching of the residual components from DRC is complete.[9] As a consequence, most toxic effects from DRCs manifest during the first 24 hours. However, the DRCs tend to release unreacted components beyond the initial 24 hour period, although the rate of release declines with time.[41] Hence, in the present research, the elution time was 24 hours rather than employing aged eluates.

It has been found that esterases were capable of cleaving some dimethacrylates rendering them inactive.[42] Similarly, residual comonomers leached from DRCs may be inactivated by binding to intracellular glutathione.[43] However, these cannot be assumed as reasons for the reduction in cytotoxicity.[44] Various studies have concluded that unpolymerized residual comonomers are noxious to gingival fibroblasts and oral keratinocytes of humans. The results of these *in vitro* studies stated that these monomers released from the final product were inherently cytotoxic and potentially harmful.[45] Nevertheless, the residual comonomers released into an aqueous locale for prolonged duration prospectively pave way for cytolysis and pulpitis.[46] The above findings emphasize the necessity for executing cytotoxicity tests for assessing the basic biocompatibility of the material. There are various methods in dental literature to evaluate cytotoxicity. In the present study, an MTT assay was employed. In the agar barrier test, agar is used to partition the specimens from the fibroblasts. The agar layer emulates the mucosal barrier. On the contrary, extracts/eluates used in the MTT assay simulate the released unpolymerized integrants in the saliva. Membrane integrity and mitochondrial succinyl dehydrogenase activities are the endpoints of the agar barrier test and MTT assay, respectively. The MTT assay differentiated the dilutions and the resin types. This distinction could not be discerned by the agar barrier test. Agar barrier test quantitatively reveals only the decolored areas and cytolytic index. Hence, cytotoxicity can be alternatively established qualitatively by MTT assay.[47] Simplicity, rapidity, precision, and needless radioisotopes are merits of the MTT assay. The chemical conversion of the tetrazolium salts by the live fibroblasts following extract/eluate treatment is the basis of this assay.[46] Hence, in the present research, an MTT assay was employed.

In the present research, ISO-approved L929 mouse fibroblasts were employed. Pulpal cells are predominantly fibroblasts which would be the victims of the residual comonomers after breaching the layer of odontoblasts.[48] The L929 fibroblasts were chosen over primary gingival fibroblasts because of their excellent reproducibility and higher susceptibility to the cytolysis by cytotoxins.[49] This provides high responsiveness to this method in determining cytocompatibility. Furthermore, L929 fibroblasts are effortlessly obtained with efficacious *in vitro* growth owing to their homologous shape and growth traits. Nonetheless, these fibroblasts are best suited for basic screening of the materials' cytocompatibility. Clinically pertinent human cell lines ought to be contemplated in future researches to confirm the validity of test materials' toxicity. This is because animal cell lines are deprived of human relevance metabolically and genetically.[50] For clinical relevance, strategies reproducing *in vivo* ambiances would be appropriate. Even though the MTT assay does not replicate the clinical scenario, it is cost-effective and readily accessible. Future researches replicating *in vivo* conditions would yield clinically apposite results.[47]

In the routine clinical procedure, DRCs are placed into the prepared cavity in an unpolymerized stage that triggers local responses. The surface of the photopolymerized DRC restoration is eventually finished and polished. This is executed to eliminate the superficial layer where the polymerization is profoundly inhibited by oxygen. Therefore, surface polishing dampens the cytolytic integrants' leaching from the material.[51] Hence, this mandates suitable specimen preparation methods to optimize the cytotoxicity assessment. The specimens with the inhibition layer had greater cytolysis than the polished specimens.[47] Therefore, the removal of the inhibition layer is a critical factor for good cytocompatibility. Since the removal of the inhibition layer increased the cytocompatibility, the assessment of the exact inherent cytotoxic potential of an unpolished novel resin material becomes mandatory. Hence, in the present study, the specimens were not subjected to polishing regimens.

Conclusion

After meticulously adhering to the investigational etiquettes and within the constraints of this research, it is deduced that the novel copolymer P(GEU-*Co*-DPEPHA) formed by the addition of DPEPHA in propriety DRC matrix was cytocompatible with murine fibroblasts.

Clinical Significance

DPEPHA is a novel multifunctional cross-linker which copolymerized with conventional composite resin comonomers. This novel copolymer P(GEU-*Co*-DPEPHA) is cytocompatible with the mammalian fibroblasts. Hence, the substitution of this crosslinking comonomer would improvise the physicomechanical properties of the DRCs without compromising biocompatibility.

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