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Original Article

Histocompatibility of Dental Restorative Composite Resin Photopolymerized with a Novel Multifunctional Comonomer: A Histopathological Analysis in Rats

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ABSTRACT

Background: Restorative dentistry is impossible without composite resins. Toxicity of these resins is common though possessing favorable properties. Modifications of the resin matrix are being executed to improve the properties of the material. Dipentaerythritol penta-/hexa-acrylate (DPEPHA) monomer has been recently identified as a cross-linking comonomer with the conventional resin matrix. **Purpose:** This study aimed to investigate the histocompatibility of DPEPHA comonomer in photopolymerized resin at 20 and 40 wt.% concentrations in rats. **Materials and Methods:** Eighteen male Wister rats were subjected to subcutaneous implantation of conventional resin specimens without DPEPHA (G0) and with DPEPHA at 20 wt.% (G20) and 40 wt.% (G40) concentrations. Then, the rats were subdivided according to the time of resection of the implantation sites at 1, 2, and 4 weeks (n = 6 rats). Tissue specimens were submitted to histopathological analysis. **Results:** Except for 4 weeks' time interval, there were significant differences ($P < 0.05$) in the inflammation among the groups at each time interval. The order of inflammation was NC <G40 ≤G20 <G0. Concerning the fibrous capsule thickness, significant difference existed among the groups at each time interval and within the groups at all 3 time intervals ($P < 0.05$). **Conclusion:** Composite resin specimens with DPEPHA comonomer exhibited good histocompatibility in rats at 40 wt.% concentration.

KEYWORDS: *Comonomer, composite resin, cross-link, histopathology, inflammation*

INTRODUCTION

In clinical dentistry, the use of direct restorative composite resin in the anterior esthetic zone is common and it is acquiring recognition as a posterior restoration material in the recent years. Photopolymerized composite resin particles may lodge within oral tissues as a result of contouring or removing these restorations. After trauma, fractured composite resin pieces have been found lodged in the lip,[1] and gingival afflictions in sites prepared hitherto for Class V restorations have been related to small composite particles resulting from finishing the restorations.[2] These findings suggest that composite resins can play a role in tissue pathosis.

Biocompatibility is one of the most important facets of dental biomaterials. In the dental literature, numerous modus operandi for assessing the biocompatibility have been described. The most basic *in vitro* cytotoxicity experiments are those executed on cell or tissue cultures, as well as *in vivo* methods concerning subcutaneous connective tissue or bone

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implantation of the trial materials in experimental animals.[3]

Animal implantation experiments were used to determine the toxicity of composite resins, and the findings were more quantifiable. The findings indicated that neutrophil infiltration occurred after implantation, accompanied by fibrous tissue encapsulation and chronic lymphocyte and macrophage infiltration at different times after implantation.[4-7] As a result, the existence of fibrous tissue and its distribution surrounding the implants are indicators of tissue response. Therefore, a material's biocompatibility is inversely proportional to the amount of inflammation that occurs around it.[8]

Enhanced physicomechanical properties and clinical performance of resin composite restorative materials are directly dependent on the monomer to polymer conversion. Cytotoxicity can be resulted from the release of unpolymerized residual monomers or oligomers. Even in completely set materials, significant quantities of short polymeric chains remain unbound. As a result, leachable toxic components are likely to elute into the pulp. Inadequate polymerization results in a number of adverse effects, including poor physical properties, oral solubility, and increased microleakage, which contributes to recurrent decay and pulpal irritation. There is also a correlation between the unpolymerized leachable resin quantity in the composite and the magnitude of cytotoxicity.[9]

Biocompatibility can also be affected by direct contacts at the interface between a restoration and the oral tissues. Plaque and gingival indices, as well as probing depths, were found to be substantially higher in the vicinity of 5–6-year-old direct composite restorations relative to nonrestored sites.[10] Part segregation of components from resinous dental materials can be caused by one of two mechanisms. Unbound monomers and additives are extracted by solvents, such as saliva and/or dietary solvents, after polymerization, particularly during the first 24 h. As a consequence, monomer-to-polymer conversion is a crucial feature of a resin restoration's biocompatibility. However, leachable substances may be created over time as a result of erosion and degradation.[11-13] Photo, thermal, mechanical, and chemical factors may all lead to resin degradation and erosion. Salivary esterases, for example, have been found to dissolve the surfaces of composite resins, potentially releasing methacrylic substances.[14]

Multifunctional methacrylate monomers are homo-/copolymerized to create strongly cross-linked polymer networks with many desirable physicomechanical properties.[15] Typical final double-bond conversions vary from 55% to 75% during the polymerization of multifunctional methacrylate monomers for dental restorations.[16-18] The high cross-linking density in the system limits the mobility of reacting species, resulting in low final conversions.[15]

As compared to their methacrylate analogs, multifunctional cross-linking acrylate monomers have higher reactivity.[19] The acrylate cross-linking multifunctional comonomer dipentaerythritol penta-/hexa-acrylate (DPEPHA) is not encountered in the dental literature concerning biocompatibility issues. Hence, the present study aims to investigate the *in vivo* histocompatibility of restorative composite resin photopolymerized with DPEPHA comonomer upon implanted in the subcutaneous connective tissue of rats. The null hypothesis of the research is that the incorporation of DPEPHA comonomer in resin matrix of dental composite would not affect the histocompatibility.

Materials and Methods

Bisphenol-A-glycerolate dimethacrylate (*bis*-GMA), triethylene glycol dimethacrylate (TEGDMA), 2-diurethane dimethacrylate (DUDMA), camphorquinone (CQ), dimethylaminoethyl methacrylate (DMAEMA), DPEPHA, and barium oxide (BaO) were procured from Sigma (Sigma-Aldrich Co., St Louis, MO, USA) and used as received. Barium fluoride (BaF_2) was purchased from Sisco Research Laboratories Pvt. Ltd. Maharashtra, India. Zirconia nanoparticles (ZrO_2) were purchased from Nano Research Laboratory, Jamshedpur, Jharkhand, India. Institutional Animal Ethics Committee (IAEC; Reg. No. 889/PO/Re/S/05/CPCSEA; January 30, 2018) approved the protocol of the study (Approval No. SVCP/IAEC/PhD/2/02/2020).

Formulation of control and trial composite matrices and photopolymerization

In the control group G0 matrix, monomeric ingredients include *bis*-GMA (G; 50 wt.%), TEGDMA (E; 20 wt.%), and DUDMA (U; 30 wt.%), which is collectively denoted as GEU. Filler ingredients include BaO (30 wt.%), BaF_2 (30 wt.%), and ZrO_2 (40 wt.%). The total matrix: filler ratio was 30:70 wt%. The CQ: DMAEMA (1:2) is 1 wt.%. The trial groups G20 and G40 matrices have an additional comonomer DPEPHA at 20 wt.% and 40 wt.%, respectively.

In order to polymerize the resin matrices of all groups, rubbery washer-like molds (4 mm diameter, 2 mm thick) were used. A clear cellophane sheet was placed on a glass tile with the mold secured on it. The resin matrix was stowed and condensed within the mold. The other side was also covered and pressed with another cellophane

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sheet to achieve even surface of resin composite after curing with light-curing unit (Guilin Woodpecker Medical Instrument Co., Ltd.; Guangxi, China; absorbs light in the 420–480 nm, 650–800 $mWcm^{-2}$) for 40 s.[9] Polymerized control and trial groups are denoted as P(GEU) and P(GEU-*Co*-DPEPHA), respectively, based on the matrix formulation.

Animal model

A total of 18 mature, 3–4-month-old male Wister Albino rats weighing 150–200 g were used in this experimental study. The instructions of the Animal Research: Reporting of *in vivo* Experiments were followed when caring for the animals. The rats were housed in individual polypropylene cages at 23°C ± 2°C with 55% ± 10% relative humidity. For acclimatization, 12 h of alternative bright–dark cycles was followed. They were fed with water *ad libitum* and rodent pellet diet (Krishi Cattle and Lab Animal Feeds, Bengaluru, India). Cage husk bedding was changed thrice a week.[20]

Ketamine hydrochloride (80 mg/kg-Ketajet®50; Sterkem Pharma Pvt. Ltd; Mumbai, India) and xylazine (10 mg/kg-Xylaxin®, Indian Immunological Ltd; Telangana, India) were given intraperitoneally to the rats to achieve deep anesthesia. This anesthetic technique offered an operatory period of around 30 min. On the dorsum of the rats, four sites with maximum interdistance were selected. Using surgical scissors and hemostat forceps, four 1 cm long and 15 mm deep incisions were made after shaving and disinfection. For negative control (NC), incision made in the left anterior quadrant was sutured without implantation. The G0, G20, and G40 specimens were implanted inside the right anterior, right posterior, and left posterior incisions, respectively. The skin was closed with 3/0 silk sutures.[9]

The rats were anesthetized using the above protocol at 1, 2, and 4 weeks[21] after implantation. The implanted sites with 1 cm margin of subcutaneous tissue were excised and the sites were sutured back. 10% formalin was used to fix the tissues. Under a light microscope (Leica DMD108), the histopathology of hematoxylin and eosin-stained slides was examined. A magnification of ×40 was used to identify the cell types, collagen forms, and fibrous tissues. The amount of tissue inflammation and the inflammation process were graded using 4 numbers (Grade 0: no inflammation to Grade III: severe inflammatory response).[9] The presence of fibroblastic cells, as well as the consistency of fibrous tissue and collagen, was used to measure and scale the amount of tissue healing. The criteria for the inflammatory grades are tabulated in Table 1. Fibrous capsule thickness measurements were performed using Image-Pro Premier software (Version 9.0 [9.1.5262.28]; Maryland, USA).[21] From every slide stained, two random sections were selected. In each section, images of five random valuation sites were imaged at × 10 magnification. A total of 10 measurements were made in each of the valuation sites and averaged. The mean of five valuation sites per section was reckoned yielding two mean values of two sections of each animal.

Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS Inc., Chicago, IL, USA; version 21.0). To compare between the groups and between the time intervals concerning the grade of inflammation, Chi-square test and Friedman test were employed (α =0.05), respectively. Concerning the fibrous capsule thickness, Kolmogorov–Smirnov test of normality showed normal distribution of the data. One-way analysis of variance test followed by Bonferroni *post hoc* multiple pairwise comparisons was performed (α = 0.05).

Results

The inflammation grades and the mean (standard deviation) fibrous capsule thickness of the groups at 3 time intervals are tabulated in Table 2. After 1 week, G0 and G20 exhibited no (12.5% and 12.5%) to moderate (60.0% and 40.0%) inflammation, whereas in G40, the inflammation ranged from no (12.5%) to mild (45.5%) (P = 0.024). After 2 weeks, G0 exhibited moderate (33.3%) to severe (75.0%) inflammation, whereas G20 exhibited mild (22.2%) to severe (25.0%) inflammation and G40 exhibited mild (33.3%) to moderate (33.3%) inflammation (P = 0.026). After 4 weeks, the inflammation in G0 ranged between mild (30.0%) and severe (100%), whereas the inflammation in G20 and G40 ranged between no (12.5% and 25.0%) and moderate (40.0% and 20.0%) (P = 0.145). At all the time intervals, NC exhibited no-to-mild inflammatory response and there were significant differences (P < 0.05) in the inflammation within the groups. The order of inflammation from minimum to maximum was NC <G40 ≤G20 <G0. Concerning the fibrous capsule thickness, significant difference existed among the groups at each time interval and within the groups at all 3 time intervals (P < 0.05). Histopathological findings of the control and trial groups are tabulated in Table 3. Figures 1-3 show the histopathological changes of the groups associated with the corresponding resin materials at different time intervals.

Discussion

In the present research, the histological examinations were assessed after 1, 2, and 4 weeks in accordance with

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Table 1: Criteria for the inflammation grades

No inflammation (Grade 0)	Mild inflammation (Grade I)	Moderate inflammation (Grade II)	Severe inflammation (Grade III)
No inflammatory cells	Presence of macrophages and/or plasma cells	Presence of macrophages and/or plasma cells	High density of inflammatory cells
Fibroblasts>30	Inflammatory cells <30	Accumulations of granulocytes and/or lymphocytes. ≥30 inflammatory cells <60	Inflammatory cells ≥60
Mature fibrous tissue with many collagen fibers	>10 fibroblasts <30. Immature fibrous tissue with few collagen fibers	>5 fibroblasts <9	Local necrosis areas. >1 fibroblasts <4

Table 2: The inflammation grades and the mean±standard deviation fibrous capsule thickness

	1 week					2 weeks					4 weeks				
	Inflammation grade				Mean±SD fibrous capsule thickness (µm)	Inflammation grade				Mean±SD fibrous capsule thickness (µm)	Inflammation grade				Mean±SD fibrous capsule thickness (µm)
	0	I	II	III		0	I	II	III		0	I	II	III	
G0	1	2	3	0	458.90±13.81[a, b]	0	0	3	3	342.07±20.18[a, b]	0	3	2	1	225.66±8.32[a, b]
G20	1	3	2	0	413.96±31.26[a, b]	0	2	3	1	289.19±17.38[a, b]	1	3	2	0	179.67±8.34[a, b]
G40	1	5	0	0	262.44±15.55[a, b]	0	3	3	0	198.11±17.62[a, b]	2	3	1	0	114.49±8.80[a, b]
NC	5	1	0	0	None	2	4	0	0	None	5	1	0	0	None

Fibrous capsule thickness: [a]Significant difference among the groups at each time interval (P<0.05); [b]Significant difference within the groups at different time intervals (P<0.05). NC: Negative control, SD: Standard deviation

Table 3: Histopathological descriptions of the groups

	G0	Trial groups G20 and G40
1 week [Figure 1]	Thick inflammatory fibrous capsule along with tissue necrosis as well as marked fibrosis was observed. Inflammatory response showed neutrophils and tissue macrophages infiltration	Mild fibrinous inflammatory response with a conspicuous granulation tissue formation was perceived. Mild inflammatory cells infiltration comprising lymphocytes, tissue macrophages and few PMNs was noticed
2 weeks [Figure 2]	Moderate to severe inflammatory reaction with intense thick fibrous capsule containing PMNs and multinuclear giant cells infiltration. Active angiogenetic sites were observed	Marked reduction in inflammation along with regression of inflammatory cells. The fibrous capsule was lined with a layer of active fibroblasts with less inflammatory cells infiltration comprised of macrophages and lymphocytes
4 weeks [Figure 3]	Mild to severe inflammatory reaction with PMNs and lymphocytic infiltrations with slight angiogenesis. Marked thickening of the fibrous capsule was still observable	Gradual healing with abrogation of inflammatory cells infiltrations. Well-formed fibrous capsule with multiple parallel collagen fibers. Commendable reduction of lymphocytes and macrophages infiltration suggesting decreased inflammatory reactions

NC: In all the 3 time intervals, no to mild inflammatory changes were observed without necrosis. No fibrosis or fibrous capsule observed [Figures 1-3]. PMNs: Polymorphonuclear, NC: Negative control

the American National Standards Institute specifications, with the exception of 8 weeks, to determine the short- and long-term tissue reactions of the control and trial composite resin material.[22] Photopolymerization of resinous materials would result in the formation of a solid phase, reducing the unreacted residual monomer content and thus in turn decreasing the potential harmful stimuli. Although total photopolymerization can lessen the harmful stimulants, cured resins on no account photopolymerize completely and tend to degrade eventually. In cell cultures, leaching of unpolymerized residual monomers such as TEGDMA, 2-hydroxyethylmethacrylate (HEMA), or *bis*-GMA may cause adverse reactions.[23-26] According to the studies, the cytotoxic potential of basic monomers was found to be highest for *bis*-GMA followed by UDMA, TEGDMA, and HEMA.[27] *Bis*-GMA has been shown to elicit synthetic estrogenicity and thus cytotoxic.[28,29] Because of its lipophilic nature, TEGDMA can penetrate the cytosol and membrane lipid compartments of mammalian cells.[30]

At the end of the 1st week, control and trial composite specimens elicited mild-to-moderate inflammation which progressed to moderate–severe inflammatory reactions in the 2nd week. This is attributable to the residual monomers being leached over time, causing adverse inflammatory responses in the 2nd week. It is also be surmised that the rats' immune system might react inactively against the foreign bodies.[9] This could be the prospective explanation for a moderate

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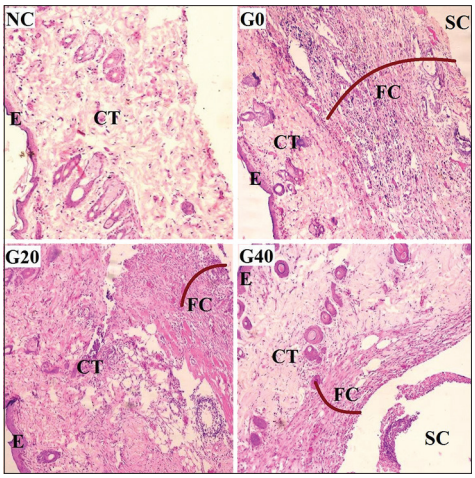


Figure 1: Histopathological sections of the groups at 1st week. E: Epithelium, CT: Connective tissue, FC: Fibrous capsule, SC: Specimen cavity

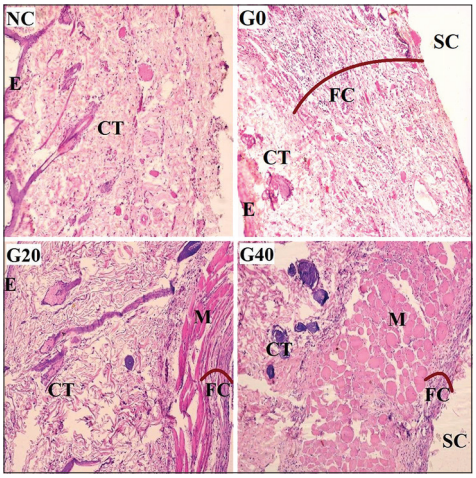


Figure 2: Histopathological sections of the groups at 2nd week. E: Epithelium, CT: Connective tissue, FC: Fibrous capsule, SC: Specimen cavity

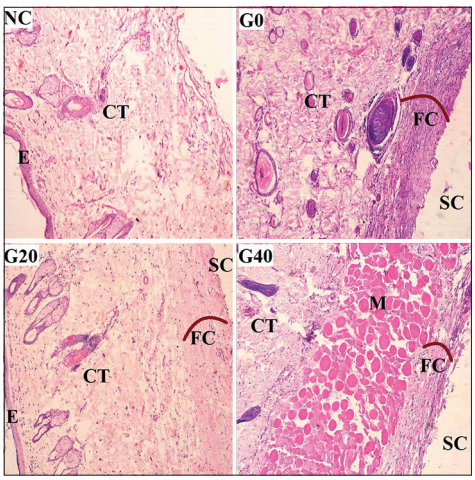


Figure 3: Histopathological sections of the groups at 4th week. E: Epithelium, CT: Connective tissue, FC: Fibrous capsule, SC: Specimen cavity

or severe reaction. Due to immune cell elimination of the superficial free monomers, inflammatory responses diminished to moderate and mild levels after 4 weeks. The G40 group had the least inflammation and the G0 exhibited the maximum. The well tissue tolerance was explained by the formation of fibrous capsule or fibrosis around the resin composite specimens.[31,32] Subcutaneous surgical placement of G40 specimens exhibited accelerated healing with a well-defined thin fibrous capsule after 4 weeks post-implantation. Meanwhile, with G0 specimens, extensive fibrosis was observed which was evident until the 4th week with thick remodeling fibrous capsule. G20 specimen implants exemplified similar tissue response, yet with significantly lesser degree and a medium fibrous capsular formation than that observed with G0 specimens. Biocompatibility of restorative composite resin is highly influenced by the volume and chemical nature of its percolated elements. Therefore, the desirable result of G40 is attributable to the presence of acrylate moieties in DPEPHA and ultimately leads to the acceptance of the null hypothesis. Greater extent of polymerization (conversion rate) and cross-linking, lesser residual monomer, and pendant double bonds are the characteristics of multifunctional acrylates when compared to multifunctional methacrylates. Nevertheless, the acrylates form highly reactive secondary radicals in contrast to the stable tertiary radicals of methacrylates.[19] Therefore, from the above context, it is inferred that the trial groups have had released less or negligible unreacted residual monomer that could stimulate inflammatory reactions.

With the exception of peak inflammatory response, the results of the present research were consistent with the study conducted by Ozbas *et al*.[3] However, peak inflammatory response was spotted after 2 weeks in the present research, which was in accordance with Feiz *et al*.[9] The degree of monomer–polymer conversion, composition of resin composite, dimensions of tested materials, and implantation techniques could all be factors in this discrepancy.[9] Ortengren *et al*. stated that the maximum elution (85%–100%) of monomers occurred within 7 days.[33] As a result, the elution of monomers after polymerization was checked in the current study from day 7 onward.

Infiltration of polymorphonuclear cells (PMNs), lymphocytes, and the presence of fibroblasts and epithelioid cells were observed in all groups after the resin specimens inserted in the subcutaneous tissues of rats. Eventually, the fibroblasts and epithelioid cells increased and lymphocytic infiltration associated with the implantation of all specimens decreased. Four weeks after implantation, a granulomatous inflammatory

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response was still observed in G0. These observations led to the suggestion that composite resin particles entrapped in oral tissues have the potential to induce long-term inflammation.[34]

Implantation of biomaterials subcutaneously in laboratory animals has been shown to be the reliable way to determine the histocompatibility of dental materials. Toxicity and inflammatory responses to dental biomaterials that are subcutaneously implanted exhibit distinct reactions. The dimensions of implanted materials have been shown to affect tissue reactions in histopathological studies.[3] In some researches, the test specimens were secured directly into subcutaneous connective tissues.[8] In other relevant studies, the test resin specimens were contained within inert tubes and implanted.[3,35] Nevertheless, the physical form of test specimens also affects the histocompatibility. Cylindrically shaped resins[6,7,36] or polyethylene tubes containing fresh resin specimens[4,7] were implanted in soft connective tissues of rats, in previous researches of histocompatibility to composite resins. Tissue reactions in those studies commenced with the infiltration of inflammatory cells and then progressed to fibrous encapsulation around composite resin specimens accompanied with a mild chronic inflammatory infiltrate.[34] In this present research, 2 mm thick resin composite specimens were implanted to simulate clinical scenario.

Conclusion

Thus, in accordance with the results of this research, it can be concluded that photopolymerizing restorative composite resin with DPEPHA comonomer (up to 40 wt.%) showed good histocompatibility in rats.

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Conflicts of interest

There are no conflicts of interest.

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