Original Article

Effect of isobutyl methacrylate and methacrylic acid eluted from chairside denture hard reliners on enzymatic cellular antioxidants: An *in vitro* study in human primary buccal mucosal fibroblasts

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Abstract

Aim: This study was conducted with the objective to evaluate the cytotoxicity of monomers isobutyl methacrylate (IBMA) and methacrylic acid (MA) in human buccal mucosal fibroblast primary cell culture and to study their effect on cellular enzymatic antioxidants-glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT).

Materials and Methods: The tissue for fibroblast cell culture was harvested from oral buccal mucosa of a healthy donor. Fibroblast cells were plated at a density of 1×10^4 cells per well in 96-well tissue culture plates. Cells were exposed to various concentrations of IBMA and MA. The cell viability and various enzyme activities were evaluated 24 h after exposure to the above treatments. All tests were done in triplicate. Cell viability was assessed by trypan blue dye exclusion assay and all enzyme activities were done using assay kits from Cayman Chemicals, Ann Arbor, USA.

Results: At all concentrations tested a statistically significant decrease in viability was observed in IBMA- and MA-treated cells. Around 42% cells were viable at the highest test concentration of IBMA (80 μmol/L) and only 20% cells were viable at the highest dose (144 μmol/L) of MA exposure ($P < 0.05$). Dose-dependent decrease in the GPx and SOD activities was observed in cells treated with IBMA and MA ($P < 0.05$). CAT activity was not detectable in the controls. However, a fall in CAT activity was detected in cells exposed to IBMA and MA at all concentrations tested ($P < 0.05$).

Conclusion: IBMA and MA leaching out from the chairside denture hard reliners are cytotoxic on human buccal fibroblast primary cell cultures. This could be due to the oxidative stress caused by the generation of reactive oxygen species which is evidenced by the fall in activities of antioxidant enzymes (GPx, SOD, and CAT) and cytotoxicity.

Key Words: Chairside reliners, cytotoxicity, enzymatic antioxidants, free radical, oxidative stress

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INTRODUCTION

Relining is done to increase the service of a denture and to compensate for tissue changes in the residual ridges. Relining can be accomplished either in laboratory or chairside.[1] These chairside hard reliners are composed of monomers such as isobutyl methacrylate (IBMA) and 1,6 hexanediol dimethacrylate. Apart from these monomers many other compounds such as dibutyl phthalate (plasticizer), degradation by products such as methacrylic acid (MA) and benzoic acid also leach out from reline resins.[2-6] Toxicity of denture base resins has been attributed to the residual monomers leaching out from these resins.[6] The residual monomer content of chairside hard reline resins has been found to be more than that of heat polymerized denture base resins.[2] Due to higher residual monomer content, chairside reline resins have the potential to be more cytotoxic than heat polymerized resins. Traumatic stomatitis following intraoral relining[7] and chronic urticaria from an acrylic resin prosthesis have been reported.[8] *In vitro* studies have also confirmed toxicity of chairside hard reliners.[2,4,5]

Interactions of these monomers with human cells and the mechanisms by which these monomers induce toxicity are still unclear. Precise understanding of the mechanisms of toxicity is essential to modify these compounds chemically or structurally so that their toxicity can be minimized or eliminated. Hence, studies which throw light on interactions of the monomers with various cellular components provide the foundation knowledge for future production of safe and biocompatible monomers. It should be mentioned here that such studies are already underway for monomers such as triethylene glycol dimethacrylate (TEGDMA), urethane dimethacrylate, 2-hydroxylethyl methacrylate (HEMA), and bisphenol A-glycidyl methacrylate, which are part of restorative dental resins.[9-12] There has been less focus on monomers eluted from denture base resins and reline resins in this aspect. Our study is initial step toward understanding of the mechanism of toxicity of chairside reline resins. One resin monomer IBMA and one degradation by-product MA were used in this study. The toxicity of these two monomers has already been demonstrated in *in vitro* studies using various cell lines.[2,4,5] It has been proposed that the toxicity of monomers is due to increase in reactive oxygen species (ROS) or free radical generation.[12] We have hypothesized that this increased ROS production could be due interaction of the monomers with enzymatic antioxidants of the cells.

Detoxification of free radicals is done by various enzymatic and nonenzymatic antioxidant mechanisms. The enzymatic antioxidants in cells include glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD). An increase in the ROS production is normally mitigated by these cellular antioxidants.[13,14] Cell death or apoptosis occurs due to damage to DNA, proteins, and lipids or by disruption of cellular signaling pathways if the toxic insult caused by ROS is beyond the repairing capacity of these cellular antioxidants.[15] It is likely that an imbalance in the oxidant-antioxidant defense mechanism induced by these compounds could result in oxidative stress in the cells leading to decreased cell viability and cytotoxicity. Hence, this study was conducted with the objective to evaluate the cytotoxicity of IBMA and MA in human buccal mucosal fibroblast primary cell culture and to study their effect on cellular enzymatic antioxidants.

MATERIALS AND METHODS

Cell culture studies were conducted at Chennai Dental Research Foundation, Mylapore, Chennai, with assistance from the Department of Pharmacology and Toxicology, Dr. ALM PG Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai, after getting necessary approval from these institutions. Ethical clearance was obtained from the Institutional Review Board.

Chemicals

IBMA (MA - 99% with 250 ppm MEHQ as inhibitor, No: 155721) and MA (IBMA - 97%, No: 169919) were purchased from Sigma-Aldrich Chemicals, MO, USA. All antioxidant enzyme kits used in this study, such as GPx assay kit (Item No. 703102), CAT assay kit (Item No. 707002) and SOD assay kit (Item No. 706002), were purchased from Cayman Chemicals, Ann Arbor, USA. Dulbecco's Modified Eagle Medium (DMEM), trypsin, penicillin, streptomycin, dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), and phosphate buffered saline (PBS) were purchased from Hi Media, Mumbai, India. All the other chemicals used in this study were purchased locally and were analytical grade.

Fibroblast culture

The tissue for fibroblast cell culture (submucosa)[16] was harvested from oral buccal mucosa of a healthy donor after getting informed and written consent. Before isolating the tissue, oral rinse with 0.2% chlorhexidine was done for 1 min to prevent any chance of contamination. Tissue was excised by incisional biopsy under local anesthesia. The tissue fragments were immediately placed in DMEM for at least 1 h rinsed thrice in PBS and minced into small tissue pieces. The fibroblast cells were collected by brief trypsin-EDTA application (0.02%) and cultured in DMEM containing crude collagenase, 10% FBS and 100 μg/ml

of streptomycin. Cultures were incubated at 37°C in humidified atmosphere of 5% CO_2. The cells were cultured and maintained for a minimum of two passages and the third passage cells were used for this study.[16]

Treatment of fibroblast cells with test compounds

Fibroblast cells were plated at a density of 1×10^4 cells per well in 96-well tissue culture plates. The test compounds IBMA and MA were dissolved in DMSO and serially diluted with culture medium. The maximum concentration of 0.5% DMSO was used. The test compounds were immiscible in the culture media and hence, were dissolved in 0.5% DMSO. At this concentration, DMSO has been shown to be noncytotoxic.[4,5] The test concentrations are based on the amount of IBMA and MA eluted from reline resins at various time intervals as quantified in previous studies by HPLC analysis in artificial saliva.[4,5] Test concentrations of IBMA and MA are given in Table 1. DMSO (0.5%) treated cells served as vehicle control. The test and the control samples were maintained for 24 h.[4,5] Cell viability and various enzyme activities were evaluated 24 h after exposure to the above treatments. All tests were done in triplicate.[17,18] Cell viability was assessed by trypan blue dye exclusion assay.

Trypan blue dye exclusion assay

The dye exclusion test[19] is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes and are not stained by dyes such as trypan blue, eosin, or propidium, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. After treatment with the testing reagents, cells from each plate and from the control (10 µl) were mixed with equal volume of 0.4% trypan blue, mixed gently and allowed to stand for 5 min in room temperature. 10 µl of stained cells was placed in hemocytometer, the number of viable cells (unstained) and dead (stained) cells were counted. Cell viability was expressed as a percentage of controls.

Determination of antioxidant enzymes (glutathione peroxidise, catalase, and superoxide dismutase) in fibroblast culture

The enzyme assays were performed using kits as per the manufacturer's instructions. The principles of each assay and their coefficient of variance are given below:

Table 1: Concentrations of test monomers IBMA and MA used in the study

Monomer	Concentrations tested (µmol/L)
IBMA	5, 10, 20, 40, 80
MA	9, 18, 36, 72, 144

The table indicates the various concentrations of monomers (µmol/L) used in our study. IBMA:Isobutyl Methacrylate, MA:Methacrylic Acid

The GPx activity was measured indirectly based on a coupled reaction with the enzyme glutathione reductase (GR). This kit could be used to measure all the types of glutathione (GSH) dependent peroxidases in cell lysates. Briefly, in the presence of NADPH, the oxidized glutathione (GSSG) formed after the reduction of hydroperoxide by GPx is recycled to its reduced state (GSH) by GR. The oxidation of NADPH is accompanied by a decrease in absorbance at 340 nm which is proportional to the GPx activity in the sample.[20] One unit of GPx was defined as the amount of enzyme that catalyzes the oxidation of 1 nmole of NADPH per minute at 25°C. The coefficient of variation for the test ranges from 5.7% to 7.20%.

In the presence of hydrogen peroxide (H_2O_2), CAT reacts with methanol to produce formaldehyde which reacts with the chromogen, i.e., 4-amino 3-hydrazino-5-mercapto-1, 2, 4-triazole (purparald) to form purple color on oxidation, whose color intensity was quantified at 540 nm.[21] The coefficient of variation for this assay ranges from 3.8% to 9.9%.

The superoxide radicals generated by the reaction of xanthine oxidase and hypoxanthine were detected by formation of tetrazolium salt which was detected by monitoring the absorbance of the samples at 440–460 nm.[22] One unit of SOD was the amount of enzyme needed to produce 50% dismutation of superoxide radical. All the three types of SOD (CU/Zn, Mn, and FeSOD) were measured using the assay kit. The coefficient of variation for this test ranges from 3.20% to 3.7%.

Statistical analysis

Data were analyzed using IBM SPSS Statistics for Windows (Version 19.0, IBM Corp., Armonk, NY, USA). Descriptive statistics were employed to obtain the mean and standard deviation for the activities of the various concentrations of both the monomers. On performing Kolmogorov–Smirnov test, the data were found to have non-normal distribution, and hence, a nonparametric test was used. Kruskal–Wallis ANOVA followed by Mann–Whitney's U-test (as a *post hoc* test) was used to compare the GPx, SOD, CAT activities and cytotoxicity for IBMA and MA monomers individually.

RESULTS

The cytotoxicity and cell viability data of IBMA and MA exposed fibroblast cell cultures are presented as a percentage of controls [Table 2 and Figures 1, 2]. At all concentrations tested, a statistically significant decrease in cell viability was observed in IBMA-treated cells and

only around 42% cells were viable at the highest test concentration (80 μmol/L) of IBMA ($P < 0.05$). Almost similar pattern of dose-dependent fall in cell viability was observed in cells exposed to MA. The cell mortality was as high as 39.5% even in the lowest dose (9 μmol/L), and only 20% cells were viable at the highest dose (144 μmol/L) of MA exposure ($P < 0.05$).

Dose-dependent decrease in the GPx and SOD activities was observed in cells treated with IBMA and MA [Tables 3 and 4]. There was no statistically significant difference ($P > 0.05$) in GPx and SOD activities between the control group and the lowest test concentrations of IBMA (5 μmol/L) and MA (9 μmol/L). However, at all other test concentrations of IBMA and MA a statistically significant fall in activities of GPx and SOD was seen ($P < 0.05$) compared to control. CAT activity was not detectable in the controls. However, CAT activity was detected in cells exposed to IBMA and MA [Tables 3 and 4]. With the increase in test concentration of IBMA and MA, a significant decrease in activity of CAT was observed ($P < 0.05$) when compared to the lowest concentration of monomer tested.

DISCUSSION

The present study was undertaken with the hypothesis that toxicity of IBMA and MA could be due to increase in ROS production in cells caused by an interaction of the monomers with enzymatic antioxidants. Both the monomers were tested on human primary buccal mucosal fibroblast cells. A primary cell culture involves harvesting of cells from a healthy donor and then culturing it in tissue culture plates until uniform confluency of cells is

Table 2: Viability of cells (% of control) exposed to various concentrations of IBMA and MA

Concentration of IBMA (μmol/L)	Cell viability as percentage of control (Mean±1S.D.)	Concentration of MA (μmol/L)	Cell viability as percentage of control (Mean±1S.D.)
Control	100	Control	100
5	71.43±1.01*	9	60.43±2.05*
10	63.63±1.50*	18	52.43±1.97*
20	55.56±2.10*	36	42.57±1.90*
40	47.70±1.97*	72	26.87±1.32*
80	42.60±2.0*	144	20.17±1.43*
χ^2	16.648	χ^2	16.648
P	0.005	P	0.005

Values presented are Mean±1S.D (Standard Deviation) for three replicates in each test concentration. Cells treated with 0.5% DMSO were used as control. *P<0.05 indicates significant differences compared to control. IBMA:Isobutyl Methacrylate, GPx:Glutathione Peroxidase, CAT:Catalase, SOD:Superoxide Dismutase, DMSO:Dimethyl Sulfoxide

Table 3: Effect of IBMA on GPx, CAT and SOD activity in primary human buccal mucosal fibroblasts

Concentration of IBMA (μmol/L)	GPx Activity in nmol/min/ml (Mean±1S.D.)	CAT Activity in nmol/min/ml (Mean±1S.D.)	SOD Activity in U/ml (Mean±1S.D.)
Control	52.25±3.81	-	0.25±0.01
5	50.93±5.10	113.97±1.9	0.25±0.02
10	42.45±2.94*	108.58±2.5$^+$	0.20±0.01*
20	30.56±5.10*	100.66±3.15$^+$	0.17±0.01*
40	30.59±2.51*	93.58±2.5$^+$	0.14±0.02*
80	27.16±2.94*	90.30±1.84$^+$	0.10±0.01*
χ^2	14.67	16.46	16.16
P	0.012	0.006	0.006

Values presented are Mean±1S.D (Standard Deviation) for three replicates in each test concentration. Cells treated with 0.5% DMSO were used as control. *P<0.05 indicates significant differences compared to control. ^+P<0.05 indicates significant differences compared to lowest test concentration of IBMA (5μmol/L). IBMA:Isobutyl Methacrylate, GPx:Glutathione Peroxidase, CAT:Catalase, SOD:Superoxide Dismutase, DMSO:Dimethyl Sulfoxide

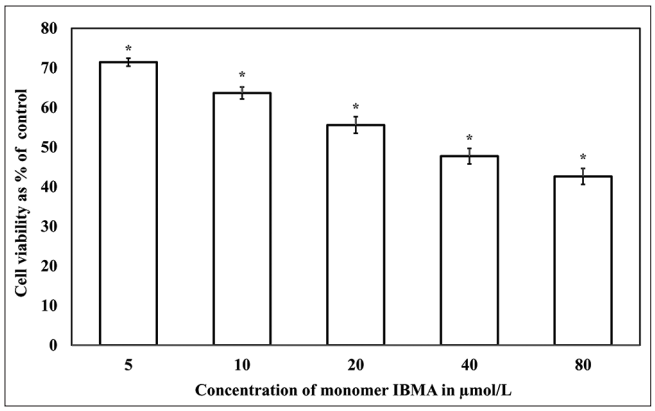

Figure 1: Viability of human buccal mucosal fibroblast cells exposed to various concentrations of IBMA for 24 h. The values presented are mean of three observations in each test concentration expressed as a percentage of viable cells compared to control. The error bars indicate standard deviations. *P < 0.05 indicates significant differences compared to control. IBMA: Isobutyl methacrylate

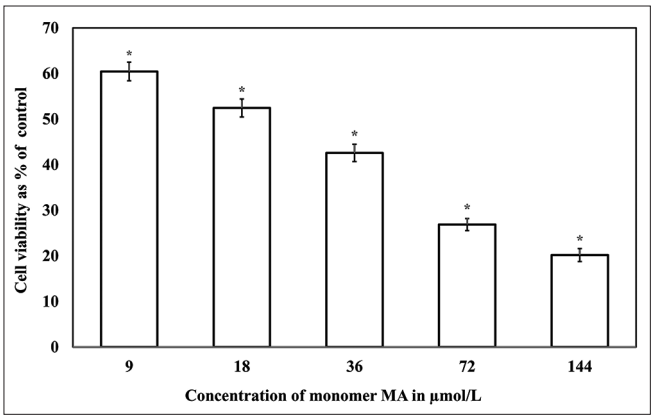

Figure 2: Viability of human buccal mucosal fibroblast cells exposed to various concentrations of MA for 24 h. The values presented are mean of three observations in each test concentration expressed as percentage of viable cells compared to control. The error bars indicate standard deviations. *P < 0.05 indicates significant differences compared to control. MA: Methacrylic acid

Table 4: Effect of MA on GPx, CAT and SOD activity in primary human buccal mucosal fibroblasts

Concentration of MA (μmol/L)	GPx Activity in nmol/min/ml (Mean±1S.D.)	CAT Activity in nmol/min/ml (Mean±1S.D.)	SOD Activity in U/ml (Mean±1S.D.)
Control	56.39±1.36	-	0.22±0.02
9	57.73±2.94	44.0±3.15	0.20±0.02
18	49.23±2.94*	32.75±1.91^{+}	0.17±0.01*
36	35.65±5.10*	26.08±2.50^{+}	0.14±0.01*
72	28.01±2.55*	19.0±2.60^{+}	0.12±0.01*
144	22.07±2.94*	8.58±2.50^{+}	0.08±0.01*
χ^2	16.09	16.65	16.03
P	0.007	0.005	0.006

Values presented are Mean±1S.D (Standard Deviation) for three replicates in each test concentration. Cells treated with 0.5% DMSO were used as control. *$P<0.05$ indicates significant differences compared to control. $^{+}P<0.05$ indicates significant differences compared to lowest test concentration of MA (9μmol/L). MA:Methacrylic Acid, GPx:Glutathione Peroxidase, CAT:Catalase, SOD:Superoxide Dismutase, DMSO:Dimethyl Sulfoxide

obtained.[16] On the other hand, secondary cell cultures use cell lines from cancer cells that have unlimited growth potential and can be maintained for a long time. Most of the cytotoxicity tests performed previously have tested the monomers on secondary cell lines.[4,5] There are very few studies which have used primary cells for cytotoxicity testing. We chose to perform this study on primary cells as it closely simulates clinical conditions compared to secondary cultures.[16]

It was observed that at all the concentrations tested, both IBMA and MA were cytotoxic. It was also seen that cell death was more pronounced (up to 80% at a concentration of 144 μmol/L of MA) in cells treated with MA than in cells treated with IBMA [Table 2]. This difference could be due to the higher concentrations of monomer MA tested. These test concentrations are based on the amount of IBMA and MA eluted from chairside reliners as quantified in the previous studies.[2,4,5] These results coincide with the findings from previous reports.[4,5] It was also seen that there was a dose-dependent fall in activities of GPx and SOD in cells treated with both the test compounds. A similar dose-dependent decrease in expression of GPx and SOD was observed in mouse macrophages exposed to HEMA.[23] CAT activity was not detectable in the control cells. However, a decrease in CAT activity was observed in the cells exposed to IBMA and MA. These findings suggest that cell death has occurred even in the presence of cell's antioxidant mechanisms to combat increased ROS production. This type of cell death observed in monomer exposed cells could be due to increased oxidative stress and formation of ROS which exceed the capabilities of intracellular antioxidant mechanisms.[15,24]

The cell may produce ROS in response to various endogenous or exogenous signals.[13] SOD is the "first line" of enzymatic antioxidant, and it protects against oxidative damage mediated by superoxide radicals. Three isomeric forms of SODs have been identified, and all of them are metalloproteins, and they catalyze the dismutation of highly ROS ($O^{\bullet-}$) to H_2O_2 and oxygen. The rate of SOD catalyzed $O^{\bullet-}$ dismutation plays pivotal role in quenching the ROS.[25] GPx is a selenium-dependent tetrameric peroxidase enzyme, which employs reduced GSH as cofactor. This enzyme catalyzes the metabolism of H_2O_2 to water (H_2O) involving the concomitant conversion of GSH to its GSSG.[26] CAT is a heme-based enzyme usually located in the peroxisomes. CAT has a high substrate turnover rate and scavenges nearly 6 million molecules of H_2O_2 per minute.[27] Thus, a fall in activities of all these antioxidants observed in our study suggests that H_2O_2 could be the primary ROS generated in cells exposed to the monomers. The observed decrease in activity of the enzymes could be due to their overutilization in the process of detoxification. The absence of CAT activity in the control groups suggests that ROS production in cells not exposed to the monomers was below the threshold to induce CAT expression.[28] Makino *et al.* suggested that low levels of H_2O_2 were detoxified by GPx while CAT was induced by higher levels of H_2O_2.[29] These findings suggest that exposure of cells to IBMA- and MA-induced the formation of ROS even at their lowest concentration tested. The expression of GPx and SOD in the control cells is their activity seen in the normal physiologic state.

The most important nonenzymatic antioxidant is a tripeptide, γ-l-glutamyl-l-cysteinyl-glycine also known as GSH. The cysteine residue of the molecule has a sulfhydryl (thiol) group (-SH) that contributes to its antioxidant activity.[30,31] In its reduced form the thiol group in the cysteine residue of GSH detoxifies ROS directly by donating reducing equivalents. It also functions as a substrate for the enzymatic antioxidant GPx indirectly. During these processes, GSH is converted to its oxidized form (GSSH) by coupling with another molecule of GSH. GSH is recycled back to its reduced form by the enzyme GR.[32] When intracellular GSH is decreased, an increase in ROS is expected. Decrease in intracellular GSH indirectly affects the activity of the enzymatic antioxidant GPx. Decrease in GSH may occur due to the formation of GSH adducts with methacrylate-based resins. Such GSH adduct formation has been reported in monomers such as TEGDMA.[33,34] The carbon-carbon double bond in the methacrylate resins carry a positive charge due to the electron withdrawing nature of the adjacent carbonyl moieties. These carbon atoms with the positive charge react with nucleophiles such as amino groups (in DNA) or thiol groups (-SH) via a Michael addition reaction.

Thus, the thiol groups of GSH react with the positively charged carbon atoms of TEGDMA resulting in adduct formation. A similar reaction may be possible with the monomers tested in our study. In this study, the intracellular concentration of GSH was not quantified. Further studies on the effects of IBMA and MA on intracellular GSH could throw more light on this aspect. This also does not rule out the possibilities of other mechanisms leading to apoptosis and cell death which need further investigation.

In our study, it is shown that the cell death increases with increase in the concentration of the monomer eluted. Thus decreasing the leaching out of monomers from the chair reliners is indispensible to reduce toxicity due to these resins. *In vivo* scenario might actually be different from *in vitro* studies, like a dilution of monomers due to saliva. However, still an exposure of the tissues to high concentrations of monomers cannot be neglected. Practical means of reducing the amount of residual monomer contents in resins is the need of the hour. Water storage of dentures for 1–2 days before its delivery or subjecting denture to postpolymerization water bath treatment at 55°C for 10 min could reduce the release of residual monomers from dentures.[5,35]

CONCLUSION

It is proposed that the IBMA and MA leaching out from the chairside denture hard reliners are cytotoxic on human buccal fibroblast primary cell cultures. This could be due to the oxidative stress caused by the generation of ROS, and it is evidenced by the fall in activities of antioxidant enzymes (GPx, SOD, and CAT) and cytotoxicity. An in depth evaluation of mechanism of toxicity is, however, warranted.

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Conflicts of interest
There are no conflicts of interest.

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