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Effect of Methyl Methacrylate and Methacrylic Acid on ROS Production and Cellular Antioxidants.

Ganesh Kumar A[1], Jagdish SK[2]; Nandagopal S[3*] and Joshua Daniel Egan L.

[1]Department of Microbiology, Hindustan College of Arts and Science, Padur, Chennai-603 103, Tamil Nadu, India.
[2]Department of Prosthodontics, SRM Dental College, SRM University, Ramapuram, Chennai-600 089, Tamil Nadu, India.
[3]Department of Biotechnology, Sathyabama University, Chennai-600 119, Tamil Nadu, India.
[4] Complete Learning Solution, Chennai-600 063, Tamil Nadu, India.

ABSTRACT

To explore the oxidative stress induced by methacrylic acid and methyl methacrylate on 3T3 cell lines. In the present study, in-vitro studies were done to determine the effect of the test compounds on cell lines by performing dye exclusion assay, intracellular glutathione, superoxide dismutase and catalase assay. Results revealed that by treatment with both the test compounds the viability of cells decreased by dye exclusion assay whereas the levels of glutathione, SOD and CAT increased. It could be inferred that further study has to be done, to either control the release of these monomers or substance that can replace these monomers in the preparation of dentures.

Keywords: Methacrylic acid, Methyl methacrylate, Glutathione, Superoxide dismutase, Catalase

Corresponding author

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INTRODUCTION

Dentures, also known as false teeth, are prosthetic devices constructed to replace missing teeth; they are supported by the surrounding soft and hard tissues of the oral cavity. Conventional dentures are removable. However, there are many different denture designs, some which rely on bonding or clasping onto teeth or dental implants. Modern dentures are most often fabricated in a commercial dental laboratory or by a denturist using a combination of tissue shaded powders polymethylmethacrylate acrylic (PMMA). Methacrylate based resins are being used extensively in dentistry, numerous studies have demonstrated adverse reactions and cytotoxicity to these resins. They have been shown to cause irritation, inflammation and allergic responses in patients and laboratory technicians and its clinical symptoms include erythema, erosion and burning sensation of the oral mucosa [1, 2].

Chair side reliners are materials that are used to increase the life of a denture. They have isobutyl methacrylate and 1, 6-hexanediol methacrylate as primary components. The monomer methyl methacrylate and a degradation by product methacrylic acid (MA) released from chair side hard reliners might be cytotoxic. Methacrylic acid, abbreviated MA, is an organic compound. This colourless, viscous liquid is a carboxylic acid with an acrid unpleasant odor. It is soluble in warm water and miscible with most organic solvents. Methacrylic acid is produced industrially on a large scale as a precursor to its esters, especially methyl methacrylate (MMA) and poly (methyl methacrylate) (PMMA). The methacrylates have numerous uses, most notably in the manufacture of polymers with trade names such as Lucite and Plexiglas. MAA occurs naturally in small amounts in the oil of Roman chamomile. More than 3 million tons of methyl methacrylate (MMA) are produced annually [3]. MMA is a raw material for the manufacture of other methacrylates. These derivatives include ethyl methacrylate (EMA), butyl methacrylate (BMA) and 2-ethyl hexyl methacrylate (2-EHMA). Methacrylic acid (MAA) is used as a chemical intermediate as well as in the manufacture of coating polymers, construction chemicals and textile applications [4].

It is proposed that an increase in the liberation of the reactive oxygen species (ROS) and free radicals formation in the cells could cause cytotoxicity and cell death. Numerous studies have demonstrated ROS induced apoptosis in cell cultures which are exposed to the resin monomers. Thus, the basic trigger to the toxicity seems to be excessive production of ROS in cells exposed to these monomers [5-7]. In this study 3T3 cell a fibroblast is used to determine the ROS production and levels of cellular antioxidants.

Detoxification of the free radicals is done by the cellular antioxidant enzymes such as glutathione, Catalase (CAT) and Superoxide dismutase (SOD). An increase in the ROS production is normally mitigated by these cellular antioxidant enzymes. Cell death occurs if the toxic insult is beyond the repairing capacity of these antioxidant enzymes or in case of malfunctioning of these enzymatic antioxidants [6, 7]. Hence, this study was carried out with the objective to evaluate the cell viability and oxidative stress induced by methacrylic acid and methyl methacrylate in 3T3 cell line, this information would throw light on the possible mechanism of toxicity of these compounds.

MATERIALS AND METHODS

The cell culture studies and the analytical aspects of various enzyme activities were performed in Pondicherry Centre for Biological Sciences, Puducherry.

Chemicals

Methacrylic acid (MA) and methyl methacrylate (MMA) were purchased from Sigma-Aldrich Chemicals, MO, USA. All antioxidant enzyme related chemicals used in this study were purchased from Cayman Chemicals, Ann Arbor, USA. Dulbecco's Modified Eagle Medium (DMEM), trypsin, penicillin, streptomycin, dimethyl sulphoxide (DMSO), foetal 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.

Cell Culture

The 3T3 cell line used in this study was obtained from the CIC Culture Collection of the University of Granada (Spain). Cells were placed under sterile conditions in 75cm^2 flasks that contained 30 ml of culture medium consisting of Dulbecco modified Eagle's medium (DMEM) + 2mM of glutamine + 10% bovine calf serum. Flasks were kept at 37°C in an atmosphere of 5% CO2 and 95% humidity until cells reached confluence. The cells were cultured and maintained for a minimum of two passages and the third passage cells were used for this study.

Treatment of cells with test compounds

Cells were plated at a density of 1 X 10^6 cells per well in 96-well tissue culture plates. The test compounds methacrylic acid (MA) and methyl methacrylate (MMA) were dissolved in DMSO and serially diluted with culture medium. The maximum concentration of 0.5% DMSO was used. At this concentration, DMSO is not cytotoxic. These compounds were immiscible in the culture media and hence, were dissolved in 0.5% DMSO. The test concentrations are based on the amount of compounds leaching out from the reline resins at various time intervals as reported in previous studies by HPLC in artificial saliva at different time durations [8]. Based on this report, the concentrations of methacrylic acid (MA) and methyl methacrylate (MMA) at 10, 20, 40, 80 & 160 µg/ml were used as test doses and were exposed to the cell cultures. Time duration for treatment were 6hours, 24 hours and 48 hours.

Dye Exclusion Assay

This assay was performed in 96 well tissue culture plate. Various concentration of test compounds (10, 20, 40, 80 and 160 µg/ml) were added. After addition the cultures were incubated in an incubator with 95% air, 5% CO2 and humidified atmosphere at 37°C for 6 hours, 28hours and 48 hours. After the incubation period a suitable volume of a cell suspension (20-200µl) was taken in an appropriate tube and an equal volume of 0.4% tryphan blue was added and gently mixed. It was allowed to stand for 5 minutes at room temperature. 10µl of stained cells were place in a haemocytometer and the number of viable (unstained) and dead (stained) cells were counted. The percentage of viable cells is the number of viable cells divided by the number of dead and viable cells. Morphology of the cells were also assessed by microscopic methods to determine the cytotoxicity of the cells after treatment.

Determination of Total Intracellular amount of Glutathione

Treated cells were seeded and grown in cell flasks under the identical conditions which have already been described. At 80% confluence, cells were trypsinized, centrifuged (1700 rpm for 5 minutes at room temperature), resuspended in fresh medium, and plated in six-well dishes (10^6 cells/well). After attachment, the medium was replaced and cells were incubated in fresh serum-free medium containing the fly ash 12.5 to 1000 microgram/ml for 24 hours at 37◦C. The nontreated cells served as the control. After washing, the cells were scraped into 1mL PBS. To extract cellular GSH, the cells were then dispersed using a sonicator by two 20 sec bursts. An aliquot of sonicate was taken for protein determination [9, 10]**.** The remainder of sonicate was immediately acidified with 5% sulfosalicylic acid (2:1 v/v) to prevent spontaneous oxidation of GSH. After standing for 10m on ice, the sonicate was centrifuged at 10000 rpm for 10 min at 4◦C to remove the denaturated proteins. The resultant supernatants were transferred into 1.7mL plastic tubes and the acidified samples were frozen for further use. GSH levels were determined using the DTNB-GSSG reductase recycling assay with minor modifications [11]. Before measurement of GSH, the sample was thawed and back titrated to pH 7.0 with 0.2N NaOH. The increments in absorbance at 412nm were converted to GSH.

DTNB-GSSG reductase recycling assay

Glutathione determination is based on an enzymatic recycling assay of the 5, 5-dithio-bis (2-nitrobezoic acid)-glutathione disulfide reductase (DTNB-GSSG reductase). The DTNB-GSSG reductase recycling assay is a specific and sensitive procedure [12].Anderson, 1985). As indicated in reaction (1) the reduced form of glutathione (GSH) is oxidized by 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) to give glutathione disulfide (GSSG) with formation of 5-thio-Z-nitrobenzoic acid (TNB). GSSG is reduced to GSH by the action of highly

specific DTNB-GSSG reductase and NADPH. The rate of TNB formation is followed at 412 nm and is proportional to GSH.

$$2\ GSH + DTNB \longrightarrow GSSG + TNB \quad (1)$$

$$GSSG + NADPH + H^{+} \longrightarrow 2GSH + NADP^{+} \quad (2)$$

Superoxide radical scavenging activity

Measurement of superoxide anion scavenging activity was performed based on the method described by Fridivich 1989. The assay mixture contained 25µl cell supernatant with 0.05 ml of L- methionine (200mM), 0.05 ml of Nitro blue tetrazolium (1.5 mM NBT) solution. The reaction mixture was illuminated for 30 min and the absorbance at 560 nm was measured against the control samples. All the tests were performed in triplicate and the results averaged.

Catalase Assay

The 3T3 cells were plated in 48 well plate and 500 µl of DMEM media contain CFA-NPs 100 ug/well was added and incubated for 60 min. The spent media was discarded and add different concentration of quercetin-PLA particles (5-50 µg/ml) was added with fresh DMEM media. Incubate the treated wells for 20 hour and catalase levels were calculated after treatment units per ml = dilution factor / time. Cell alone wells were considered as negative control and CFA-NPs alone wells were considered as positive control.

Statistical Analysis

Statistical analysis was done using EXCEL (MegaStat) for determining ANOVA and Pearson's correlation matrix.

RESULTS AND DISCUSSION

Dye exclusion method revealed that when Methyl methacrylate was tested for its activity against 3T3 cells, the viability of cells decreased when the concentration and the time duration of exposure were increased. In case of Methacrylic acid also the number of viable cells decreased upon increase of concentration and time duration. Both the monomers were found to be toxic for the cells when morphological observed (Table 1 and Table 2). Chaves et al., reported cytotoxic effects of IBMA, degradation product MA and 1,6-hexanediol dimethylacrylate (1,6-HDMA) in L 929 fibroblast cell lines of mouse. In the present study both the test compounds decreased the viability of the cells. These observations are in agreement with the above report [8]. A dose-dependent cytotoxicity in human primary cell cultures exposed to Methyl Methacrylate and Methacrylic acid was observed in this study. The cause of cytotoxicity in both the above monomers could not be established clearly. However, based on previous reports, it could only be speculated that the cell viability and cytotoxicity are the consequences of enhanced ROS formation induced by these monomers [13, 14]. Glutathione level was found to increase gradually upon exposure of Methyl methacrylate on 3T3 cells at different concentration for a time duration of 6 hours. Similarly when the time duration was further increased to 24 hours and 48 hours the level of glutathione increased. Statistical analysis for increase in glutathione level based on time interval gave a p value of 0.506. A study conducted on the depletion of cellular GSH as the probable cause for the toxicity of TEGDMA, HEMA and UDMA and suggested that this could have left the human gingival fibroblast cell cultures with no substrate for the enzyme GPx to detoxify the ROS produced by the toxins derived from the above monomers [15]. When Methyl methacrylate was tested on 3T3 cells for the alteration in CAT level, it was found that the level increased based on the concentration and exposure similarly the SOD levels were also increased when 3T3 cells were exposed to different concentrations of Methyl methacrylate for different time intervals. p value of 0.990 was found for CAT and for SOD it was 0.079 by ANOVA. Methaacrylic acid when tested for the level of glutathione it was found to increase on the cells exposed to various concentrations, CAT and SOD was also found to increase which was similar to Methyl methacrylate but with variations in the increase. Statistical analysis for glutathione level and SOD by treatment with methacrylic acid for different time intervals was found to be statistically significant which showed a p value of 0.000, whereas CAT results were not found to be statistically significant. On the contrary, Martin et al showed that even with the supplementation of exogenous supply of glutathione, TEGDMA was cytotoxic in *in vitro* cultures and hence, these authors proposed that decrease in intracellular GSH by TEGDMA is not major cause for cytotoxicity and many more complex mechanisms may be involved [16]. SOD is the "first line"

enzymic 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 reactive oxygen species (O_2) to H_2O_2 and O_2 [17]. The rate of SOD catalyzed O_2 dismutation plays pivotal role in quenching the reactive oxygen species [18, 19]. CAT is the heme enzyme that requires NADPH for its activity and it plays crucial role in full functioning of this enzyme. CAT promotes the conversion of H_2O_2 into O_2 [20] and it regulates intracellular H_2O_2 by its destruction in the cell [17]. The Pearson's correlation matrix for both chemicals Methyl methacrylate (MMA) and Methacrylic acid (MA) was tabulated in with significance of 0.05 and 0.01 level. GSH-MMA recorded with positive correlation with GSH-MA, SOD-MMA, SOD-MA and DEM-MMA recorded with positive correlation with DEM-MA respectively. It showed high level of correlation is recorded between GSH-MA and SOD-MA of 0.962 followed by DEM-MMA and DEM-MA. GSH-MMA negatively correlated with DEM-MMA and DEM-MA of 0.930 and 0.922 respectively (Table 3).

The results of our study thus showed that at the tested concentrations, the monomers caused a concentration dependent increase in the activity of cellular antioxidants, Viz glutathione, superoxide dismutase and catalase. The increase in the activity was evident as early as 6 hours after exposure. This shows that both the monomers were capable of inducing oxidative stress in cells through reactive oxygen species production (ROS) within a short span of time. The oxidative stress was evident even at 24-48 hours after recorded exposure. Thus the possible mechanism of toxicity of these monomers is induction of oxidative stress in cells. Further study has to be done, to either control the release of these monomers or substance that can replace these monomers in the preparation of dentures. In the present investigation, the Methyl methacrylate and Methacrylic acid caused a dose-dependent fall in the activities of SOD, CAT and GPx and this effect could be due to the overutilization of these antioxidant enzymes towards suppression of ROS and free radicals generated due to the test compounds in the fibroblast cell cultures. The fall in GPx activity could probably be due to the lipid peroxidation of the cellular membrane, which would have occurred due to reduction of GSH to GSSG during the catalysis of H_2O_2 to H_2O, as reported by Lautterberg et al [21]. Consequently, the cellular damage would have occurred due to peroxidation of lipids on the cellular membrane and this could be the reason for the cytotoxicity and loss of cell viability observed in the 3T3 cell cultures exposed to the toxic monomers Methyl methacrylate and Methacrylic acid. It is likely that some other mechanisms also would have contributed for the cytotoxicity of both these monomers. An in depth study is, however, warranted to establish this contention.

Table 1: Antioxidants comparison after treatment with Methyl methacrylate on 3T3 cells and its effect by Dye Exclusion Method.

Hours	Statistical Analysis	GSH-MMA	CAT-MMA	SOD-MMA	MMA-DEM
6hrs	Mean	17.97	215.75	12.33	764.17
	Standard Deviation	8.35	79.34	1.72	199.09
	Min	7.93	111.75	9.31	450
	Max	26.98	290.38	14.16	950
	Range	19.05	178.63	4.85	500
24hrs	Mean	20.93	218.99	15.15	692.33
	Standard Deviation	8.79	78.94	2.24	194.48
	Min	10.28	115.33	11.3	420
	Max	30.58	297.34	17.35	940
	Range	20.3	182.01	6.05	520
48hrs	Mean	23.98	223.62	20.28	587.67
	Standard Deviation	9.01	79.62	2.67	209.63
	Min	12.97	120.46	15.69	360
	Max	33.78	300.56	22.85	930
	Range	20.81	180.01	7.16	570

Methyl methacrylate (MMA), Glutathione (GSH), Catalase (CAT), Superoxide dismutase (SOD) Dye Exclusion Method (DEM).

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Table 2: Antioxidants comparison after treatment with Methacrylic acid on 3T3 cells and its effect by Dye Exclusion Method.

Chemical Parameters					
Hours	Statistical Analysis	GSH-MA	CAT-MA	SOD-MA	MA-DEM
6hrs	Mean	9.64	179.09	11.42	724.33
	Standard Deviation	2.21	56.83	3.52	215.54
	Min	7.55	105.73	7.43	456
	Max	12.36	251.55	15.17	990
	Range	4.81	145.82	7.74	534
24hrs	Mean	13.57	194.44	17.73	626.67
	Standard Deviation	2.5	57.88	6	206.75
	Min	10.9	119.66	11.62	400
	Max	16.21	260.33	25.08	950
	Range	5.31	148.67	13.46	550
48hrs	Mean	16.67	207.89	23.82	585.33
	Standard Deviation	2.02	58.57	6.09	233.83
	Min	14.36	132.19	17.76	378
	Max	18.66	282.71	31.72	919
	Range	4.31	150.52	13.96	541

Methacrylic acid (MA), Glutathione (GSH), Catalase (CAT), Superoxide dismutase (SOD) Dye Exclusion Method (DEM).

Table 3: Correlation Matrix

Chemical Parameters	GSH-MMA	GSH-MA	SOD-MMA	SOD-MA	DEM-MMA	DEM-MA
GSH-MMA	1.000					
GSH-MA	.794	1.000				
SOD-MMA	.597	.864	1.000			
SOD-MA	.840	.962	.859	1.000		
DEM-MMA	-.930	-.792	-.698	-.843	1.000	
DEM-MA	-.922	-.711	-.685	-.792	.931	1.000
18		sample size				
± .468		critical value .05 (two-tail)				
± .590		critical value .01 (two-tail)				

Methacrylic acid (MA), Methyl methacrylate (MMA), Glutathione (GSH), Superoxide dismutase (SOD), Dye Exclusion Method (DEM).

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