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Assessment of genetic damage by methyl methacrylate employing in vitro mammalian test system

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Abstract

Methyl methacrylate (MMA) is a volatile liquid widely used in the manufacture of acrylic polymers. In modern dentistry, MMA is the mainstream material in denture bases. MMA has been implicated as primary irritant and sensitizer, which can cause allergic eczematous reaction on the oral mucosa as well as skin. To date, there is growing concern that MMA may produce genetic damage by inducing mutation. In this study, colony forming efficiency, DNA synthesis, and cytogenetic assays were performed to investigate the adverse effects of MMA in cultured CHO cells. MMA was found to decrease colony formation in a dose- and time-dependent manner ($P<0.05$). MMA also inhibited DNA synthesis in a dose-dependent manner ($P<0.05$). The chromosome aberrations induced by MMA were the chromatid-type aberrations in the treated cultures. Moreover, the gaps and breaks were the most common type of aberrations observed. The sister-chromatid exchange frequencies were found to increase in the concentration of MMA. In this study, MMA was found to be not only a cytotoxic agent but also a genotoxic agent. The effects observed following treatment with low dose for longer duration is of relevance to the condition of the oral mucosa of the denture wears. Denture base resin could constantly release MMA extended periods, possibly causing moderate toxic reactions and possibly contributing to adverse effects on the mucosa.

Keywords: Methyl methacrylate; Cytotoxicity; DNA synthesis; Chromosome aberration; Sister-chromatid exchange

1. Introduction

Methyl methacrylate (MMA) is a clear, transparent liquid at room temperature with the following physical properties: boiling point of 100.8°C, density of 0.945 g/ml at 20°C and heat of polymerization of 12.9 kcal/mol [1]. It exhibits a high vapor pressure and is an excellent organic solvent. MMA can be readily polymerized by light, heat, oxygen, ionizing radiation and catalysts. It is used widely in dentistry in the manufacture of dental prostheses [1] and in orthopedic surgery as bone cement to fill spaces in bone [2].

In modern dentistry, MMA is the mainstream material in denture bases. Denture base resins are usually composed of prepolymerized polymethylmethacrylate powder particles, which are mixed with monomers of MMA and a crosslinking agent [3,4]. Despite the various methods used to initiate the polymerization of denture base resin, the conversion of monomers to polymers is not complete, and some unreacted monomers MMA are left in the denture base that are leachable into water as well as saliva [5–8].

MMA has been implicated as primary irritant and sensitizer, which can cause allergic eczematous reaction on the oral mucosa as well as skin [9]. Studies have shown that MMA is a cytotoxic agent [6,7,10,11]. In addition, MMA was found to induce papilloma and fibroma in terms of sequential histopathological changes on hamster cheek pouches [12]. However, the potential cytogenetic implications of MMA remain to be elucidated.

Introduction of chemicals in the working environment requires the assessment of their harmful effects. There is growing concern that MMA may produce genetic damage by inducing mutation. It has been suggested that in genotoxicity testing, the in vitro cytogenetic tests should take a central role in test battery [13]. Viable

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DNA lesions from the initial step in carcinogenesis, and short-term assays like chromosome aberration (CA) and sister-chromatid exchange (SCE) have come to play a central role in testing genotoxic/mutagenic and carcinogenic potential of chemicals.

The aim of this study was to examine the cytogenetic effects of MMA by mammalian test system. In this study, colony forming efficiency and DNA synthesis assays were used to evaluate cytotoxicity of MMA. Furthermore, CA and SCE assays were used to assess cytotoxic and genotoxic effects of MMA in cultured CHO cells.

2. Materials and methods

All tissue culture biologicals were purchased from Gibco Laboratories (Grand Island, NY). 5-bromo-2-deoxyuridine (BrdU) and colchicine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [methyl-^{3}H]thymidine was obtained from Amersham International PLc. (Amersham, Buckinghamshire, UK). MMA was obtained from Fluka (Switzerland). MMA were prepared in dimethyl sulfoxide (DMSO) and the final concentration of DMSO in the medium did not exceed 0.25% (v/v). DMSO at these concentrations was not cytotoxic to CHO cells. The final concentration of MMA used in this study was 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} (v/v), corresponding to 9.33×10^{-1}, 9.33×10^{-2}, 9.33×10^{-3} and 9.33×10^{-4} μg/ml, respectively.

2.1. Cell culture

The CHO cells, derived from Chinese hamster ovary, were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in McCoy's 5A medium with 10% fetal calf serum, 100 unit/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured. The cells were subcultured at 1:4 splits every 3rd day.

2.2. Colony forming efficiency

The ability to form colonies after MMA treatment was taken as a survival assay. Cells were at the end of the 2, 6, 12, and 24 h treatment, trypsinized, counted (hemacytometer) and plated at 100 cells/60 mm dish. All dishes were incubated in complete McCoy's 5A medium for 7 days, and then colonies were stained with 10% Giemsa dye and counted. Results were expressed as percentage of controls.

2.3. DNA synthesis inhibition assay

Exponentially growing cells were diluted in complete medium and seeded in 24-well plate (1×10^5 cells) according to Chang et al. [14] with minor modification. Before seeding, sterilized coverslips were put onto the plate. After treatment with different doses of MMA for 2 h, the fresh medium supplemented with 1 μCi/ml/well [methyl-^{3}H]thymidine was added for 1 h. The radioactive medium was then discarded and cells treated twice with 4°C 0.9% NaCl and fixed with 4°C 5% trichloroacetic acid solution three times. Finally, cells were washed twice with 4°C distilled water. The coverslips were taken up and put into the scintillation vials. Liquid scintillation cocktail was added to each vial and ^{3}H-activity was counted in a scintillation counter (Aloka LSC-900, Tokyo, Japan).

2.4. Cytogenetic assays

The possible genotoxic effects were studied in exponentially growing cells. For all the experiments, an untreated culture as a control was kept simultaneously. After subcultivation, various concentrations of MMA were added to cells for 24 h treatment. The cells were recovered in MMA-free growth medium after treatment with different concentrations of MMA. After the treatment, the cells were thoroughly washed with prewarmed phosphate-buffered saline and were supplemented with fresh medium containing 10 μg/ml BrdU. The cells were allowed to undergo only one cell cycle for the CA assay, while for SCE frequency studies were carried out after two cell cycles in BrdU-containing medium. Colchicine (0.3 μg/ml) was added to the cultures during the last 2-h of incubation. During all the above-mentioned steps, the cultures were protected from the direct light to prevent photolysis of BrdU-containing DNA [15]. Following a 15 min hypotonic treatment, the cultures were terminated with chilled acetomethanol (1:3) fixative and air-dried preparations were made on clean slides. The staining methods were prepared as described early [16]. The identification of CAs was carried out of following the criteria recommended by WHO [17]. Metaphases were identified in M1, M2 and M3 of the cell cycle and a minimum of 40 cells in M2 were scored for SCE assay and 70 cells in M1 for the assay of CA.

2.5. Statistical analysis

Assays were repeated three times to ensure reproducibility. Student's *t*-test was applied for the statistical analysis of the results from cytotoxicity assays. Fisher's exact test (two-tail) was applied for the statistical analysis of the results from cytogenetic assays.

3. Results

Clonogenic survival was studied in cells treated with different doses of MMA. MMA has a clear negative effect on colony-forming ability as compared to that of control culture (Fig. 1). Cells treated with MMA resulted in a dose- and time-dependent decline in the colony count ($P<0.05$).

In the DNA synthesis inhibition assay, we studied the effect of MMA of [methyl-^{3}H]thymidine incorporation in CHO cells. The different doses of MMA reduced the rate of [methyl-^{3}H]thymidine incorporation (%) in a dose-dependent manner ($P<0.05$) (Fig. 2). MMA at the concentration level of 10^{-4} (v/v) inhibited the DNA synthesis to only 63% of that in the untreated control ($P<0.05$).

The data presented in Table 1 describe the number and type of CAs induced by MMA. As shown in Fig. 3, the CAs induced by MMA were the chromatid-type aberrations in the treated cultures. Moreover, the gaps and breaks were the most common type of aberrations observed (Table 1). However, rings were found to appear in the MMA concentration up to 10^{-3} (v/v) (Fig. 4).

The details of SCE frequencies observed following the treatment with MMA are provided in Table 2. The SCE

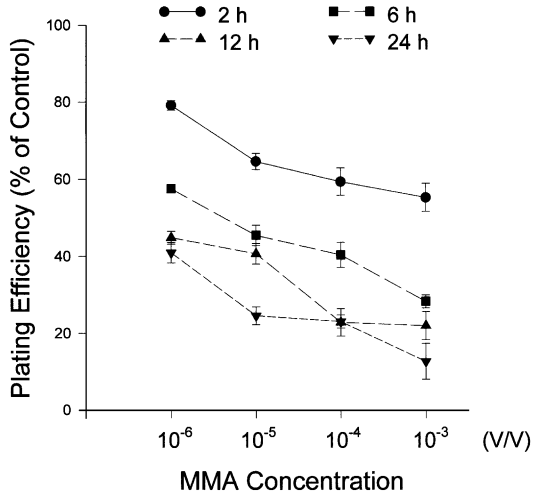

Fig. 1. Clonogenic survival of CHO cells treated with different doses of MMA for 2, 6, 12, and 24 h. The data represent the average of mean ± SD.

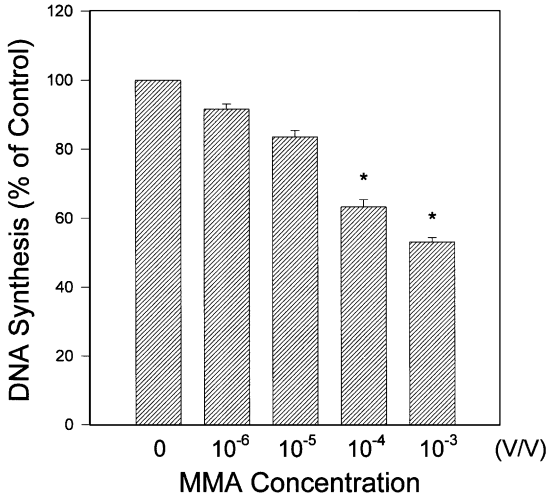

Fig. 2. Effects of various concentrations of MMA on DNA synthesis of CHO cells are expressed as percentages of ^{3}H-thymidine incorporation relative to untreated control. * denotes significant differences from control values with $P<0.05$.

Table 1
CAs observed after MMA treatment

Concentration (v/v)	Break	Gap	Ring
0	5	5	—
10^{-6}	9	5	—
10^{-5}	15	6	—
10^{-4}	10	4	—
10^{-3}	5	4	4

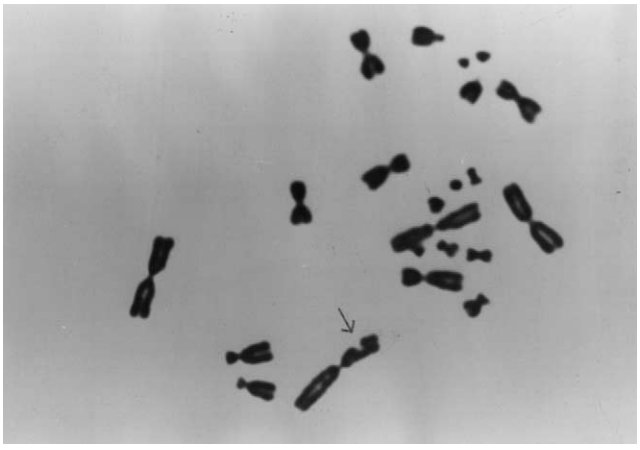

Fig. 3. Photomicrography of CA, the arrow indicates the break of chromatid.

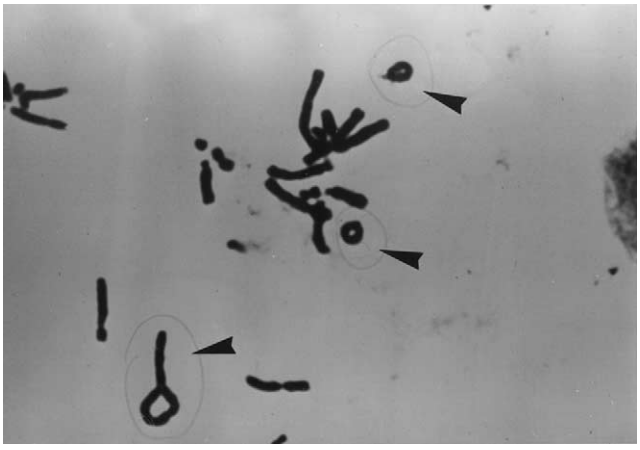

Fig. 4. Photomicrography of CA, the arrows indicate the ring formation.

Table 2
SCE frequencies observed after MMA treatment

Concentration (v/v)	Total SCE number	SCE/cell
0	169	4.23±0.53
10^{-6}	268	6.70±0.84
10^{-5}	333	8.33±1.27
10^{-4}	305	7.63±1.03
10^{-3}	330	8.25±0.97

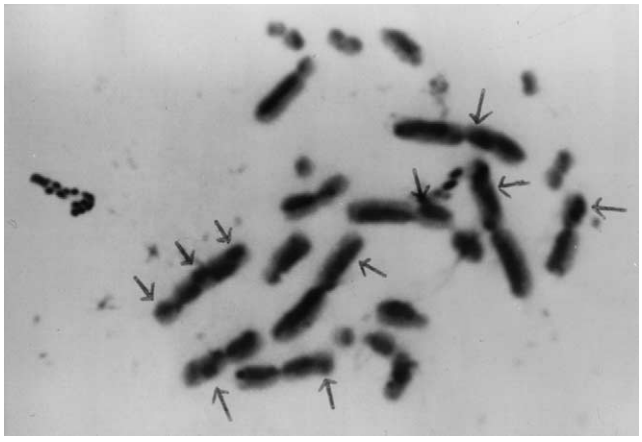

Fig. 5. Photomicrography of SCE frequencies, the arrow indicates the areas of SCE.

was shown in Fig. 5. The SCE elevation in MMA exposed cultures were statistical significant compared with 4.23 SCE/cell value for control cultures ($P<0.05$).

4. Discussion

In vitro cytogenetic tests have come to play a central role in testing for genotoxic/mutagenic and carcinogenic potential of chemicals in a majority of the countries. Our choice of cell line and use of cell in continuous culture permits an accurate evaluation of the changes, independently from factors such as age, metabolic and hormonal states of the donor that may influence the cells in primary culture. CHO cells have a small number of relatively large chromosomes; they grow fast; reproducible results can be obtained from the same cell source if cells are stocked in a frozen state.

In the present study, MMA was found to inhibit cell growth in a dose- and time-dependent manner ($P<0.05$). Our results confirm previous studies that MMA is a cytotoxic agent to cultured cells [7,8,10,11]. However, the actual mechanism of the cytotoxicity of MMA is not well known. To the best of our knowledge, this is the first study to report MMA was capable of inhibiting DNA synthesis. Alternation of lipid metabolism [18], cytokine production [19], and inhibition of cell viability via mitochondrial activity [20] may account for the cytotoxicity. Moreover, our data showed that the inhibition of DNA synthesis might be another mechanism of MMA-induced cytotoxicity.

CA assay has been used as an effective screen for evaluating the possible genotoxic potential. MMA might induce several types of DNA lesions and the unrepaired or misrepaired lesions lead to CA. SCE is a cytological revelation of DNA breakage and misrepair, and represents an efficient system for the detection of genotoxic substances. The mechanisms involved in CA and SCE formation are different and a combination of both the assays proves more effective.

A critical factor in assays for structural damage is the interval between treatment of the cells with the test chemical and harvesting of the cells for analysis. The length of this interval is dependent on the cycle time of the cell type being used. Ideally, chromosome should be observed at the metaphase following the first full cell cycle after treatment, as many aberrant cells fail to survive the first mitosis. Observations made earlier than this, or after the second mitosis, tend to underestimate the frequency of aberrations [21].

SCE were first demonstrated in ring chromosomes and subsequently by autoradiography in rod chromosomes. When the mammalian cells are cultured through a single replication cycle in the presence of the thymidine analogue, BrdU, one DNA strand in each daughter chromatid is substituted with BrdU. After a second cycle of substitution, one chromatid contains one substituted DNA strand while both strands of its sister chromatid are substituted. At this stage the chromatids can be differentiated using BrdU by fluorescence microscopy [21].

In this study, following the treatment with MMA, the quantitative analysis showed a dose-dependent in number of CAs. The CAs induced by MMA were the chromatid-type aberrations in the treated cultures. This indicated that MMA induced CAs in late S or G2 phase. Furthermore, the gaps and breaks were the most common type of aberrations observed (Table 1).

The inhibitors of DNA synthesis usually induce a very high frequency of gaps when the cells are treated in late S or G2 phase [13]. A dose-dependent increase in the number of SCEs, observed after the treatment of MMA, suggests that it is a potent inducer of SCEs. The chromatid aberrations are predominantly induced by DNA-crossing agents. It was proposed that intrastrand cross-links give rise to CAs and interstrand cross-links give rise to SCEs [22]. In agreement with our study, MMA can inhibit DNA synthesis. The increases in CAs and SCEs suggest that MMA could induce intrastrand as well as interstrand cross-links in DNA.

It is generally accepted correlation between mutagenic and carcinogenic effects of a variety of chemicals, the genetic activity of MMA has been investigated previously. However, these results remained very controversial. In this study, MMA was found to increase CAs

and SCEs frequencies in CHO cells. Our data were in agreement with that MMA was a mutagen by using *Salmonella tpyhimurium* system [23], and significantly induce chromosome damage resulting in micronuclei formation in bone marrow polychromatic erythrocytes [24]. Moreover, workers occupationally exposure to MMA have higher SCE number in their peripheral lymphocytes [25]. In contrast, the Ames test was negative without rat liver enzyme metabolizing system [26]. Chan et al. [27] have also showed that inhalation exposure of MMA for 102 weeks did not induce any increased incidence of neoplasms in rat or mice. These differences are not clear. Whether the induction of genotoxicity by MMA, however, is less clear from our present study. Our data showed that the inhibition of DNA synthesis might be one of the mechanisms underlying MMA cytotoxicity. It could be conceivable that MMA induced-chromosome damage could affect DNA synthesis. However, the detailed mechanism of DNA inhibition remains to be further defined.

A previous study showed that MMA released into saliva was detected with a maximum concentration of 45 μg/ml in whole saliva or 180 μg/ml in the salivary film on the fitting surface, and MMA could be detected up to one week after insertion of an autopolymerized appliance with the lower detection limit of their analysis being 1 μg/ml [5]. In this study, it was found that the concentration level of MMA (10^{-6} (v/v) corresponding to 9.33×10^{-4} μg/ml) could easily reach the effective cytotoxic and cytogenetic level. In the oral cavity, it is possible that the amount of MMA released into oral mucosa may be higher than the concentrations tested in this study that could easily reach the effective toxic level, especially in the area under the denture.

Although, we still do not know whether the damage of denture to the oral mucosa is a reversible or irreversible reaction. Supposedly, it will depend on the severity of the insult by the amount of MMA release. Moreover, the toxic effects of denture on adjacent tissues need further clarification, because of possible protection by the presence of neutralizing factors such as saliva. We suggest that thorough soaking of denture before insertion may be beneficial. In addition, implantation of acrylic cement in human beings has repeatedly been reported to be associated with fatal cardiorespiratory collapse [28–30]. It should be sufficient to remove residual MMA and an optimum polymerization, therefore, is necessary for denture as well as bone cement.

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