Assessment of methyl methacrylate genotoxicity by the micronucleus test

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Abstract: The aim of this study was to evaluate the genotoxic potential of methyl methacrylate (MMA) vapor by simulating standard occupational exposure of 8 hours per day and using the micronucleus test. We used 32 adult male Wistar rats divided into three groups: A - 16 rats exposed to MMA for 8 hours a day, B - Eight rats receiving single subcutaneous doses of cyclophosphamide on the first day of the experiment (positive control), C - Eight rats receiving only water and food *ad libitum* (negative control). Eight rats from group A and all of the rats from groups B and C were sacrificed 24 hours after beginning the experiment (acute exposure in group A). The remaining animals in group A were sacrificed 5 days after the experiment began (repeated exposure assessment in group A, simulating occupational exposure 40 hours/week). Femoral bone marrow was collected from each rat at the time of sacrifice for use in the micronucleus test. Two slides were completed per animal and were stained with Giemsa staining. Two thousand polychromatic erythrocytes were counted per animal. The Kruskal-Wallis test followed by a multiple comparisons test (Dunn test) was used for statistical analysis. The median number of micronuclei was 7.00 in the group exposed to MMA for 1 day, 2.00 in the group exposed to MMA for 5 days, 9.00 in the group exposed to cyclophosphamide (positive control) and 0.756 in the negative control group ($p < 0.0001$). MMA was genotoxic when measured after 1 day of exposure but was not evidently genotoxic after 5 days.

Descriptors: Polymethyl Methacrylate; Genotoxicity; Mutagenicity Tests; Micronucleus Tests; Occupational Exposure.

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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Introduction

The widespread use of methyl methacrylate (MMA) as bone cement during orthopedic surgery and in dental braces and prostheses has raised interest in the potential toxicity of MMA. Experimental and clinical studies have shown that the components of MMA may cause a range of adverse effects.[1-3]

MMA is derived from methacrylic acid. It is transformed into a resin by light, heat, oxygen and oxygen compounds. Due to its volatility, occupational exposure occurs via inhalation, and MMA is first hydrolyzed in the nasal cavity by the enzyme carboxylesterase.[1]

Concern about the potential mutagenic and carcinogenic effects of genotoxic agents in populations exposed to MMA occupationally has grown, partially due to the possibility that mutagenic effects may occur

only after multiple years of exposure, increasing the incidence of cancer and characterizing so-called cumulative effects.[4]

Environmental mutagenesis (genetic toxicology) evaluates the potential genotoxicity of many substances that are considered to be crucial prerequisites for the development of adverse health effects such as cancer.[4]

Of all cancer cases, 80–90% are associated with environmental factors; some of these factors are well known, such as the correlation between smoking and lung cancer or between excessive sun exposure and skin cancer, but other factors are currently being evaluated, such as food components, and still other factors remain completely unknown.[4,5]

Genotoxicity tests are conducted via several methods, making them important for research, evaluating cell toxicity and identifying potential carcinogens and mutagens. Several techniques can be employed during testing, such as the determination of DNA/protein cross linking coefficients, mitochondrial enzyme activity, cell proliferation, repair of DNA breaks and mitotic index; the identification of damage, chromosomal aberrations and non-disjunctions; and the detection of apoptosis and necrosis.[4,5]

The term "micronucleus test" was first suggested by Boller and Schmid in 1970 and later by Heddle in 1977.[6] The micronucleus test detects mutagenic substances that break chromosomes (clastogenic substances) or that interfere with mitotic spindle formation, thus altering the equitable distribution of chromosomes during cell division.[4,7]

During the last decade, studies have investigated and identified the cytotoxicity and genotoxicity of certain methacrylates. Multiple dental resins contain a co-monomer such as triethylene glycol dimethacrylate (TEGDMA), which causes genetic mutations *in vitro*.[8]

To date, there are no studies in the literature that evaluate the genotoxic potential of MMA simulating occupational exposure. MMA is a substance that is widely used in dentistry and medicine, and studies that evaluate this substance simulating occupational exposure may contribute to a better understanding of the toxic effects of MMA on genes and the risk to the exposed workers.

The aim of this study was to investigate the genotoxic potential of MMA vapor by simulating an occupational exposure standard of 8 hours per day and using the micronucleus test.

Methodology

For this study we used 32 Wistar rats, all adult males weighing 200 to 250 g. The rats were separated into groups of four rats each in large rectangular cages measuring 49 × 34 × 16 cm, which were recommended for hosting five adult rats. The rats were placed in a biotherium controlled for temperature and humidity and subjected to a 12-hour dark-light cycle.

The animals were divided into 3 groups:

- A - 16 rats exposed to MMA for 8 hours daily to simulate occupational exposure, foregoing food and water during exposure to exclude the possibility of food and water contamination with the MMA vapors that could potentiate the action of MMA,[2,9]
- B - 8 rats receiving cyclophosphamide (Cytoxan, Baxter Oncology GmbH, Halle, Germany) in single subcutaneous doses (50 mg/kg) on the first day of the experiment (positive control);[5]
- C - 8 rats receiving water and food *ad libitum* (negative control).

The rats in groups B and C were housed in a biotherium separate from the rats in group A.

The plastic boxes in which the rats were housed contained an upper grid that covered three-quarters of the surface of the box with non-recycled white plastic, which restricted ventilation to simulate a working environment (Figure 1A). MMA exposure was controlled via amber glass bottles (Nadir Figueiredo Indústria e Comércio, São Paulo, Brazil) with a capacity of 100 mL, an opening of 2 cm in diameter and a perforated cover; 10 mL of MMA was added to each bottle (99.9% Classic - Indústria Brasileira Ltda., São Paulo, Brazil). The bottles were placed inside each box and fixed to the upper grid (Figure 1B). The evaporation rate of MMA was approximately 0.5 mL per day (estimated to be 150 ppm). The rats in the control group were also subjected to poor ventilation but were not exposed

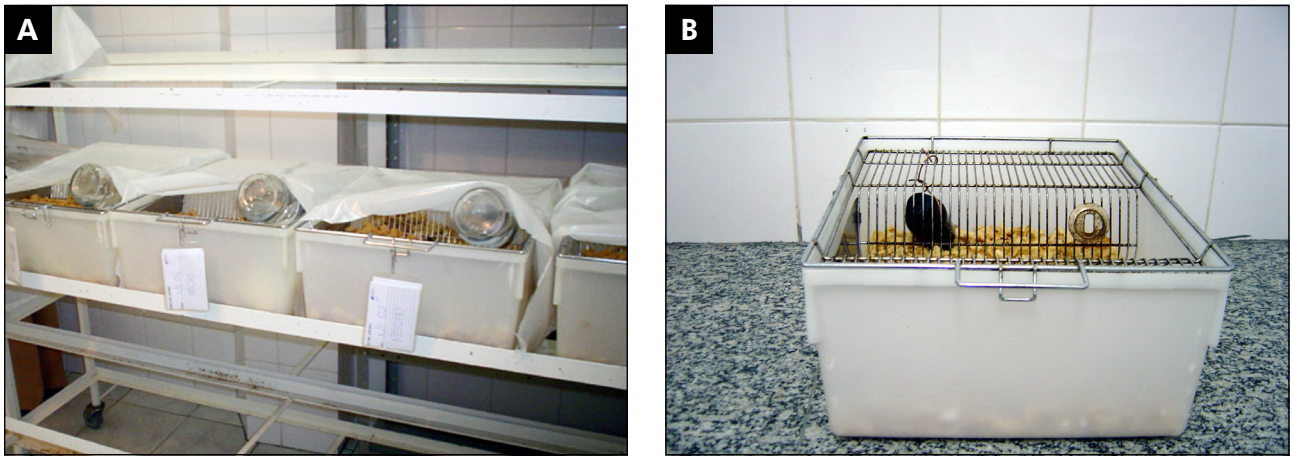

Figure 1 - A: Plastic boxes in which the rats were placed, each of which was covered with a non-recycled white plastic over three-quarters of its surface (restricting ventilation to simulate a working environment). **B**: Amber glass bottle containing MMA fixed in the upper grid.

to MMA.[2]

Eight rats from group A and all of the rats from groups B and C were sacrificed 24 hours after the experiment began (acute exposure in group A). The remaining animals in group A were sacrificed 5 days after the experiment began (repeated exposure assessment in group A, simulating occupational exposure of 40 hours/week). Euthanasia was performed with sodium pentobarbital (Syntec, Cotia, Brazil) by administering a dose of 100 mg/kg into the peritoneal cavity. Signs indicative of death included absence of breathing movements, absence of heartbeat and loss of reflexes.[10]

Bone marrow samples were collected from each rat's femur at the time of sacrifice, and two sample slides per animal were prepared.[5] The slides were stained with Giemsa staining (Dolles, São Paulo, Brazil). Two thousand polychromatic erythrocytes (1000 on each slide) were counted per animal using an optical microscope at 400× magnification to determine the number of micronucleated polychromatic erythrocytes.[5] Micronuclei were considered to be structures with suggestive halos surrounding their membranes that measured less than one-third of the diameter of the associated nuclei; the micronuclei were similar in staining intensity to the associated nuclei and were observed in the same focal plane during microscopy.[5] The analysis of the slides was blinded and performed by one observer (GAN) and reviewed by another observer (JLSP); both results were concordant.

Statistical analysis

The variable frequency of micronuclei showed no normality using the Kolmogorov-Smirnov test ($p = 0.075$), and the Levene test showed no homogeneity of variances ($p = 0.004$). Therefore, we used the nonparametric Kruskal-Wallis test followed by a multiple comparison of the ranks using the Dunn test. The statistical tests were performed at a significance level of 5%.

Approval by the Research Ethics Committee

This study was approved by the Ethics Committee on Animal Use of the Universidade do Oeste Paulista (CEUA – UNOESTE) (Protocol no. 703/11).

Results

The median number of micronuclei was 7.00 in the group exposed to MMA for 1 day, 2.00 in the group exposed to MMA for 5 days, 9.00 in the group exposed to cyclophosphamide (positive control) and 0.756 in the negative control group (Table 1, Figures 2 and 3).

The Kruskal-Wallis test showed a difference between the groups ($p < 0.0001$). The multiple comparisons test showed differences between the group

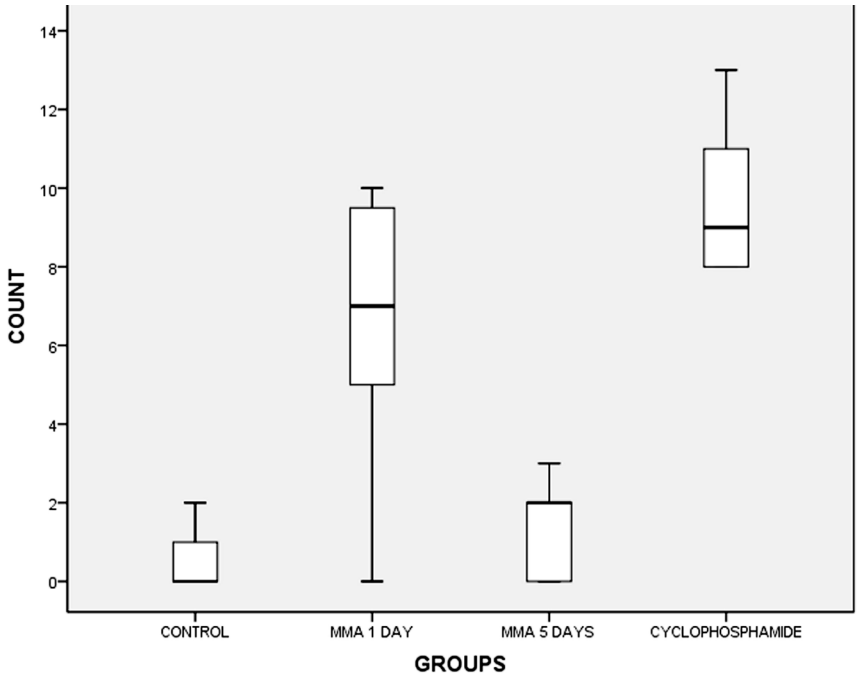

Figure 2 - Frequency of micronuclei per group studied (median and interquartile intervals) ($p < 0.0001$). Control: negative control; MMA: methyl methacrylate; cyclophosphamide: positive control.

Table 1 - Median and interquartile range of the micronucleus frequency in each group.

Group	Median	Interquartile range
MMA – 1 day	7.00[a]	5.00
MMA – 5 days	2.00[b]	2.00
Cyclophosphamide	9.00[a]	3.00
Negative control	0.75[b]	1.00

MMA: methyl methacrylate; Cyclophosphamide: positive control. Results with different superscripts differ significantly ($p < 0.0001$).

exposed to MMA for 1 day and the negative control group ($p < 0.05$) and between the group exposed to MMA for 1 day and the group exposed to MMA for 5 days ($p < 0.05$), but there were not differences between the group exposed to MMA for 1 day and the positive control group ($p > 0.05$). There was no difference in the frequency of micronuclei between the group exposed to MMA for 5 days and the negative control group ($p > 0.05$), but there was a difference between the group exposed to MMA for 5 days and the positive control group ($p < 0.001$).

Discussion

The micronucleus test is a widely used tool for assessing the safety of a substance, for classifying substances as carcinogenic or non-carcinogenic and for delivering results with strong statistical support.[11] The ease of implementing this test has lead to its widespread global adoption as a test for genotoxicity *in vitro* as well as a means of monitoring the human population.[4]

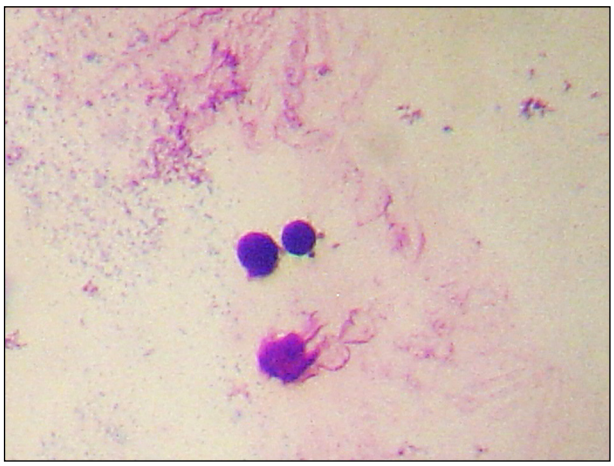

Figure 3 - Polychromatic erythrocyte with two micronuclei; animal exposed to MMA vapors for 1 day (Giemsa staining, 1000×).

The micronucleus test has been used extensively to test the genotoxicity of chemicals. The micronuclei in erythrocytes are easily viewed and are strong indicators for measuring chromosomal aberra-

tions.[4,7] These characteristics prompted us to choose the micronucleus test for evaluating MMA genotoxicity in this study.

The mechanisms of genetic toxicity and cytotoxicity of MMA monomers are still unclear.[8,12] Some studies have shown that these monomers reduce the levels of the glutathione radical (GSH), which protects cell structures from damage caused by reactive oxygen species (ROS), consequently contributing significantly to toxicity because a corresponding increase in ROS levels can activate pathways leading to apoptosis.[8,13] One study indicated that monomers induce toxic genetic events and that mitotic recombination is the main mechanism of action.[14]

The formation of micronuclei is indicative of chromosomal damage. The induction of DNA strand breaks was detected with monomers such as TEGDMA (triethylene glycol dimethacrylate) and HEMA (2-hydroxyethyl methacrylate).[8,12] Drozdz *et al*.[15] studied the toxicity of BisGMA (bisphenol A-glycidyl methacrylate) and found that this substance is genotoxic to human lymphocytes.

In this study, exposure to MMA vapors for 1 day resulted in the formation of micronuclei similar to those of the group exposed to cyclophosphamide. Cyclophosphamide in the doses used causes the formation of large numbers of micronuclei, which is why we used cyclophosphamide as a positive control in the micronucleus test.[11] However, the group exposed to MMA vapors for 5 days showed similar micronucleus formation to that of the negative control group. These findings show that only acute exposure to MMA vapors can trigger the formation of micronuclei, i.e., only acute exposure to MMA vapors has genotoxic action.

Depending on the monomer concentration, the mammalian cell cycle is delayed during G1 and G2/M as a consequence of DNA damage.[8,12] In this study, animals were exposed to 150 ppm of MMA in vapor form, which is only 1.5 times as much as the maximum recommended occupational exposure. Even this dose resulted in increased micronucleus formation after exposure for 1 day.

Acrylates and methacrylates act via clastogenic mechanisms to cause genotoxicity, and methacrylates require a higher dose to cause damage than do acrylates.[16-18] This fact, coupled with the method used for evaluating DNA damage, could be why some studies have not yet demonstrated the genotoxic effect of methacrylates.[19] This result might also explain why we observed a greater number of micronuclei in animals exposed to MMA for 1 day than in those exposed for 5 days. Perhaps DNA suffers from the initial impact of MMA aggression and then, after repeated doses, adapts to aggression and more effectively uses genetic repair mechanisms.

Conclusion

Although our findings show the potential for genetic damage only upon acute inhalation of MMA vapors, the use of personal and collective protection equipment, as well as limiting exposure to no more than 100 ppm of MMA for a maximum of 8 hours per day, as recommended by Occupational Safety and Health rules,[20] is recommended.

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