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Research Article

Heat-Cured Methyl Methacrylate Induces Increased Expression of HSP70 and iNOS in the Liver

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ABSTRACT
Introduction: Exposure to methyl methacrylate (MMA), which is a widely used monomer in dental and medical fields, has been shown to be associated with adverse health effects on liver. Aim: This study aims to evaluate the mechanism toxic effect of MMA on liver, via investigating alterations in the expression levels of inducible nitric oxide synthase (iNOS) and heat shock protein70 (HSP70) in the liver, subsequent to the oral administration of MMA in rats. Methods: 20 Sprague-Dawley female rats were randomly selected and divided into two groups: control group, and experimental group. The experimental group was orally administered 120 mg/kg of MMA daily 5 times per week for four weeks. Changes in the expression of HSP70 and iNOS in the liver tissue was investigated using Immunohistochemistry technique. Results: Exp group had statistcaly higher hepatic expression of iNOS ($p < 0.01$) in comparison to the control group, by approximately three folds. Similarly, rat hepatocytes in the experimental group showed statistically significant ($p < 0.01$) increase, by approximately 15 folds, in the expression of HSP70 compared to that in the control group. Conclusion: The presented data suggest that exposure to MMA might cause liver injury as indicated by the prominanat elevation of the oxidative stress biomarker iNOS and the biological stress biomarker HSP70.

Keywords: methyl methacrylate, iNOS, HSP70, liver injury, immunohistochemistry.

INTRODUCTION

Methyl methacrylate (MMA) is the most important ester of methacrylic acid that is broadly used in dental, medical and industrial applications[1-4]. For instance, in medicine, MMA has been used as bone cement for orthopedic surgery[5,6] due to its outstanding biocompatibility and haemocompatibility[7,8], and in manufacturing hard contact lenses, rigid intraocular lens implants[9], maxillofacial prostheses[10], leaded acrylic radiation shields, and vascular corrosion casting[11]. In dentistry, MMA is a main component of many dental restorative and prosthetic resins appliances intraorally or in the dental laboratory[12]. However, exposure to MMA monomers has been reported to cause a wide range of adverse health effects[13]. Occupational exposure to MMA, which has been considered as the primary source of exposure[14], may occur in chemical industry during manufacturing and further processing of MMA and its polymers[15]. Besides, orthopedic surgeons, surgical nurses, and other operating room staff may be exposed to MMA upon direct contact with the MMA monomer[16]. In addition, dental technicians become exposed to MMA during making, grinding and finishing acrylic dental prostheses[17]. Moreover, patients who wear acrylic dental prostheses or have an orthopaedic surgery may be exposed to the residual monomer, which is an unreacted MMA monomer released from the appliances consequent to incomplete conversion of MMA monomer to polymer[18-21].

Heat shock protein70 (HSP70) is a member of heat shock proteins (HSPs) family, whose expression is low in cells under normal conditions, and is significantly increased in cells exposed to biological stress such as heat, high pressure, and toxic compounds[22]. Such stresses act to damage proteins, causing their unfolding or aggregation[23]. The HSP70 plays a role in proteins folding by preventing their aggregation, promoting their folding, and refolding the aggregated proteins[24-26].

Inducible Nitric Oxide Synthase (iNOS) is a calcium-independent enzyme and its production is induced by cytokines[27-29]. Subsequently, higher levels of iNOS produce large amount of nitric oxide (NO) leading to the increased formation of free radicals and the consequent oxidative stress, which cause serious harm to all cellular components[30-33].

Although MMA has been shown to be toxic to the liver[34]. However, the mechanism by which MMA exerts such toxic effects has never been studied before. This study aimed to evaluate the toxic effect of MMA on the liver, via investigating any alterations in the expression levels of heat shock protein70 (HSP70) and inducible nitric oxide

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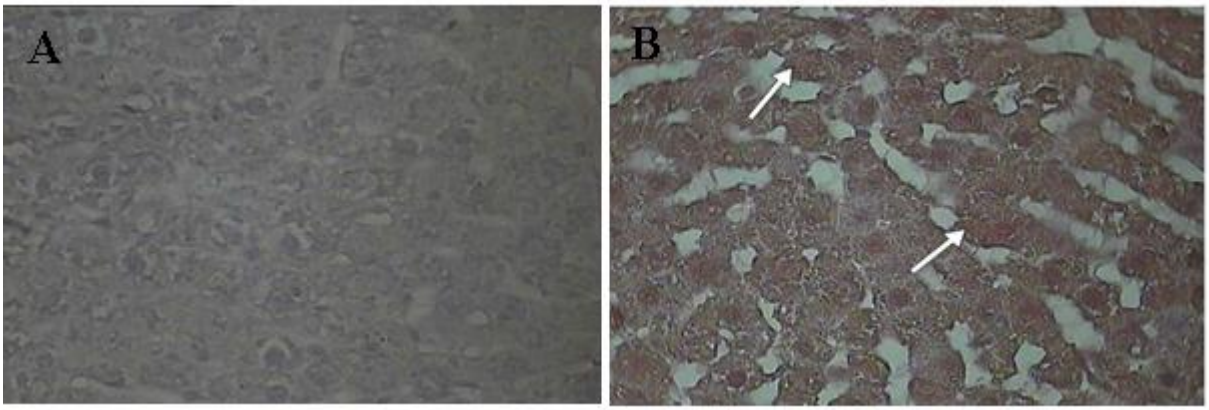

Figure 1: Immunohistochemical staining of HSP70 in 5µm thick paraffin-embedded liver sections from control (A) and experimental (B) rats. A: HSP70 immunostaining is hardly observed in the liver sections from control rats. B: HSP70 immunostaining is very strong in the liver (at the tip of the arrows) following the administration of MMA in the experimental rats.

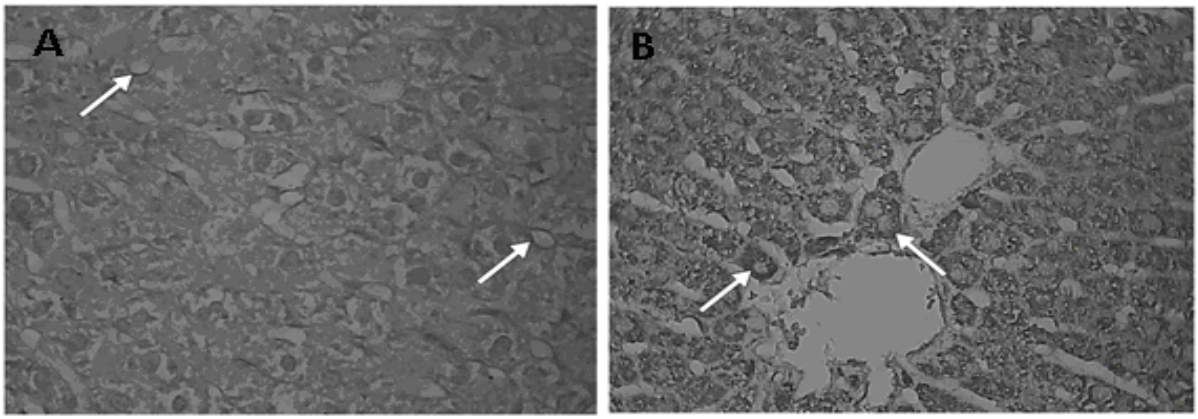

Figure 2: Immunohistochemical staining of iNOS in 5µm thick paraffin-embedded liver sections from control (A) and experimental (B) rats. A: iNOS immunoreactivity is hardly observed except for endothelial demarcation of the endothelial cells in the control rats (at the tip of the arrows). B: iNOS immunoreactivity can be observed very visible in the liver (at the tip of the arrows) following the administration of MMA in the experimental rats.

synthase (iNOS) in the liver, subsequent to the oral administration of MMA in rats. The results may shed light on the mechanism by which MMA induces toxicity to the liver.

METRIALS AND METHODS

Animals

The experimental procedures were approved by the Animal Casre and Use Committee (ACUC) of Jordan University of Science and Technology that follow the National Institute of Health Guide for the use of laboratory animals (NIH No 8023).

Female Sprague-Dawley rats weighteing 230-280g were purchased from the Animal Care Unit at Jordan University of Science and Technology (Iirbid, Jordan).The rats were maintained in a 12:12 light/dark cycle and at standerd temprature and air moisture. The animals were insupated for 10 days prior to starting the experiment. Rats were given access to clean water and standard rodent food (Sahil-Huran Animal Food Company, Ramtha, Jordan). The rats (n=20) were randomly divided into two groups (n=10) which were: Control Group (Cr) and Experimental Group (Exp). Rats in the Exp group had oral adminstration of MMA diluted with water (120mg/kg/day) for four weeks, five days a week.

Tissue Preparation and Immunostaining

At the end of the treatment, animals were sacrificed, and their hepatic tissues were dissected and subsequently fixed in 10% neutral buffered formalin. Then, the tissue samples were processed and subsequently embedded in paraffin. Next, 5µm thick paraffin-embedded sections were prepared for immunostaining with anti-HSP70 and iNOS antibodies. So the sections were deparaffinized and rehydrated. After that, antigen retrieval was performed by processing the sections in the reveal solution for 2 minutes in the Decloaking chamber. After cooling the sections down to room temperature, they were incubated with 3% hydrogen peroxide in methanol, for 5 minutes, in order to block the endogenous peroxidase activity. Then, after washing the sections in Phosphate Buffered Saline (PBS), some of them were incubated with anti-HSP70 antibody (sc-32239, Santa Cruz Biotechnology, CA, USA), whereas others were incubated with anti-iNOS antibody, according to the dilutions recommended by the manufacturers. Then, the sections were rinsed off with PBS before and after being incubated with biotinylated secondary antibody.

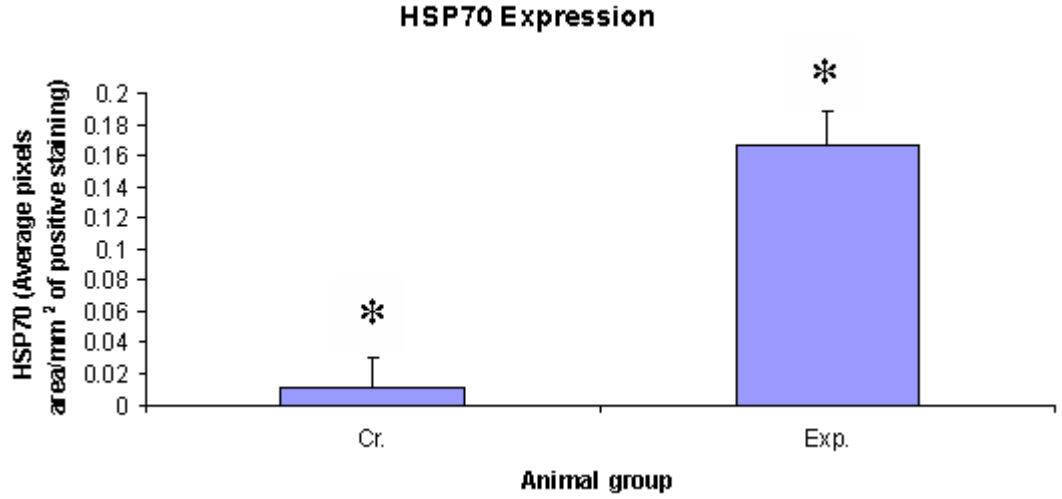

Figure 3: Expression of HSP70 in the liver. The level of HSP70 expression increased significantly in the experimental group following the administration of MMA compared to control group (P<0.01,*).

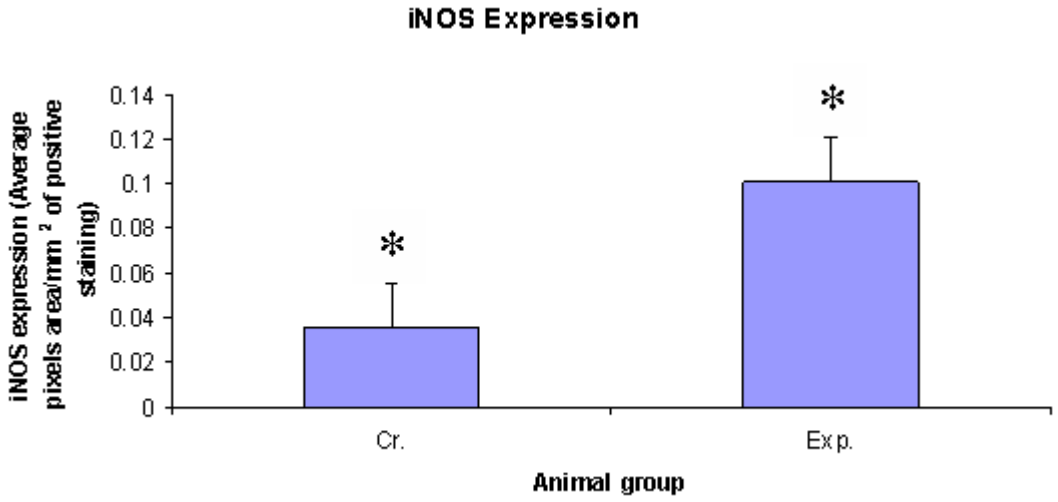

Figure 4: Expression of iNOS in the liver. The level of iNOS expression increased significantly in the experimental group following the administration of MMA compared to control group (P<0.01, *). Cr.: control group, Exp.: experimental group.

Next, samples were incubated with streptavidin horse radish peroxidase (sc2019, Santa Cruz Biotechnology, CA, USA) for 15 minutes at room temperature and washed with PBS. 3′-Diaminobenzidine[35] was applied until the desired staining intensity was reached. Finally, the slides were washed with tap water to stop the reaction. Negative control sections were processed without the primary antibody (phosphate buffered saline). All sections were then counterstained with hematoxylin, and viewed under the light microscope (BIO2T, BEL, Engineering, Italy).

Data Collection and Statistical Analysis

Digital camera (Video Head, BEL, Engineering, Italy) was used to photograph three randomly selected regions per liver section. Ten sections from each animal of all 10 animals in each group were analyzed by counting the total pixels area occupied by positive staining in each of the selected regions in each liver section and computing it as a proportion of the total pixels in each field in the sections, using Adobe Photoshop software[36,37]. Independent samples t-test was used to statistically compare HSP70 and iNOS expression in liver sections between the 2 different groups. Differences in HSP70 and iNOS expression were considered statistically significant at p value < 0.05.

RESULTS

Immunohistochemical staining revealed low level of HSP70 expression (Fig. 1A) and of iNOS (Fig. 2A) in livers from the control groups. However, HSP70 and iNOS expression was obviously increased in the liver following the administration of MMA (Fig.1B and 2B, respectively) To investigate the effect of MMA on liver, we compared the expression of iNOS and HSP70 between experimental and control groups. Hepatocytes from experimental rats showed significant increase in the expression of iNOS

compared hepatocytes in comparison to control rats ($P < 0.01$) (Fig. 3). Likewise, HSP70 expression was significantly increased in hepatocytes from the experimental rats compared to that in hepatocytes from the control rats ($P < 0.01$) (Fig. 4).

DISCUSSION

We investigated the mechanism of hepatic toxicty of MMA on a sampole of rats. Our study illustrates two important findings: Firstly, oral administration of MMA in rats significantly increased the hepatic expression of HSP70. Secondly, iNOS expression was significantly elevated in the liver following the oral administration of MMA in rats. Suggesting that MMA may exert toxic effects on liver via increasing the expression of INOS and HSP70.

Heat Shock Protein70 (Hsp70) is one of the most universally and strongly induced chaperons[38,39] and its induction is considered as a useful indicator of toxicity [40]. Consequently, we decided to investigate alterations in the hepatic expression of HSP70 following MMA administration in an attempt to evaluate the toxicity of MMA to the liver.

Previous studies reported toxic effects of MMA on the liver through decreasing the level of glutathione (GSH)[34,41]. Other studies reported rapidly increased level of HSP70 in cells that are affected by toxic compounds, providing immediate repair of intracellular damaged proteins and preventing intracellular structures from further injury[42,43]. Consistent with previous findings, the results revealed significant increases in the expression of HSP70 in the liver of rats following oral exposure to MMA. The findings of our study are in line with the previous studies, which illustrated an increased level of HSP70 upon exposure to biological stress such as heat, high pressure, and toxic compounds[44]. It was proposed that biological stress act to damage proteins, causing unfolding or aggregation of them[45]. Thus, it can be inferred that the expression of HSP70 increased probably to counteract the cellular stress that might have been induced subsequent to the exposure to MMA.

Inducible nitric oxide synthase produces nitric oxide (NO). Large amounts of NO have been shown to be toxic to cells[46]. Elevation in cellular NO can inhibit a variety of metabolic processes, such as mitochondrial respiration, and can cause direct damage to DNA[47]. It is also suggested that iNOS to interact with superoxide (O_2^-) forming the free radical peroxynitrite (ONOO-)[47,48] leading to oxidative damage, nitration, and S-nitrosation of biomolecules, including proteins, lipids, and DNA[32,33]. The hepatotoxicity of MMA is obvious and proved through the reduction of glutathione (GSH), which is an antioxidant that plays an important role in the defensive systen agaist oxidative stress[34,41,49-51]. Consistently, our results revealed significantly increased expression of iNOS in the liver following exposure to MMA. Thus, it can be concluded that exposure to MMA causes hepatotoxicity probably due by increasing iNOS expression that leads to the formation of free radicals and subsequent oxidative stress and damage.

CONCLUSION

It can be concluded that exposure to MMA causes hepatotoxicity probably due to inducing biological stress, as indicated by increased hepatic HSP70 expression, and causing oxidative damage, as indicated by increased hepatic iNOS expression, following exposure to MMA.

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CONFLICT OF INTEREST STATEMENT

None of the authors declared any conflict of interest.

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