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Effects of Methacrylate Exposure on Developing Zebrafish Embryos

Metakrilat Maruziyetinin Zebra balığı Embriyolarının Gelişimi Üzerine Etkileri

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Abstract

Aim: Methacrylate (MA), is widely used as a monomer in dentistry as well as medicine. MA derivatives have been used for a long time in biomedical devices, in restorative dental composites as well as contact lens materials also in bone cement. Zebrafish has become a popular used model organism in toxicity potential testing. Our aim in this study was to evaluate the effects of a MA derivative, polyethylmethacrylate (PEMA) exposure focusing on Nitric oxide (NO) and development of zebrafish embryo.

Materials and Method: Adult AB strain zebrafish were used in this study that were housed in aquarium rack system (Zebtec, Tecniplast, Italy) at 28 ± 1 °C maintained under a light/dark cycle between 14/10 h. Spherical embryos that were dividing normally were chosed and used for the analysis. Range-finding was applied and after that the zebrafish embryos were exposed to MA in well plates containing 20 embryos, having four replicates. Developmental effects and mortality rate were evaluated for 120h. NO levels of the embryos were evaluated using Griess Method.

Results: In present study no significant difference was observed in the NO levels of the embryos in the control group and in the MA exposed group. However, some developmental defects were observed in some of the MA exposed embryos. Lack of pigmentation was evident in one Dimethyl sulfoxide (DMSO) exposed embryo and pericardial edema was observed in some of the MA exposed embryos.

Conclusions: Our findings suggest that zebrafish embryos are useful models for the assessment of toxicity of dental MA and more research is necessary to determine the potential effects of MA exposure in embryos.

Keywords: Methacrylate, zebrafish, embryo, nitric oxide

Öz

Amaç: Metakrilat (MA), tıp ve diş hekimliğinde yaygın kullanım alanı bulan bir monomerdir. MA türevleri biyomedikal cihazlarda köklü bir role sahiptir ve restoratif dental rezinler, kontakt lens materyalleri ve kemik sementinde kullanılır. Zebra balığı, toksisite potansiyeli testinde en yaygın kullanılan balık türlerinden biridir. Bu çalışmanın amacı, bir MA türevi olan polietilmetakrilat (PEMA) maruziyetinin zebra balığı embriyosunun gelişimine ve Nitrik oksit (NO) düzeylerine etkisini değerlendirmektir.

Gereç ve Yöntem: Erişkin AB türü zebra balıkları, akvaryum rafı sisteminde (Zebtec, Tecniplast, İtalya) 27 ± 1 ° C'de 14/10 saat açık / karanlık bir döngüde tutuldu. Normal bölünen ve küresel embriyolar seçildi ve çalışmalar için kullanıldı. Doz aralığı bulma deneylerinden sonra, zebra balığı embriyoları 4 tekrar şeklinde 20 embriyo içeren kuyu plakalarında metakrilata maruz bırakıldı. Gelişimsel etkiler ve mortalite oranı 120 saatte değerlendirildi. Embriyoların nitrik oksit seviyeleri, Griess Metodu kullanılarak değerlendirildi.

Bulgular: MA maruziyeti ve kontrol gruplarında NO düzeyleri arasında anlamlı fark bulunmamıştır. Ancak MA maruziyeti bazı embriyolarda gelişimsel bozukluklara neden olmuştur. Dimetil sülfoksit (DMSO) kontrol grubunda pigmentasyon azalması, MA maruziyeti grubunda ise perikardiyal ödem gözlenmiştir.

Sonuçlar: Bulgularımız, zebra balığı embriyolarının, dental MA'ın toksisite değerlendirmesi için yararlı modeller olduğunu ve embriyolarda MA maruziyetinin potansiyel etkilerini belirlemek için daha fazla araştırmaya ihtiyaç olduğunu ortaya koymaktadır.

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INTRODUCTION

Methacrylates (MA) are important type of materials that show a broad range of properties. MA derivatives have been used for a long time in biomedical devices and are used in restorative dental materials as well as contact lens materials

also in bone cement (1). Biocompatibility or in other words, tissue compatibility is explained as the capability of a material to respond a suitable host response when applied. MA derived resins are used in dentistry, mostly in deceit of dentures as well as orthodontic appliances (2). On the other hand, these materials are considered to be cytotoxic, if they leach different potential toxic molecules, generally as residual monomer. Accordingly cytotoxic effects of some acrylic derived resins or their migrated components have been shown in many *in vitro* and *in vivo* experiments and also in cell based studies. These *in vivo* and *in vitro* studies are important to examine the long term clinical and molecular effects of these materials, moreover to aid in improvement of alternate resins (2).

Zebrafish (*Danio rerio*) is a small tropical freshwater fish naturally from Himalayan region are today has become a popular model organism that is used in human diseases. As a vertebrate zebrafish, is closer to humans when compared with the invertebrate model organisms *Caenorhabditis elegans* and *Drosophila melanogaster* (3). Zebrafish is genetically similar to humans and easier to house and care when compared with rodents. Zebrafish are externally fertilized and has nearly 200-300 offspring when compared with the 5-10 obtained from rodents. Zebrafish embryos grow and develop very quickly, and both embryo and larvae are transparent, which makes them available for observation and for manipulation during all stages of their development and therefore facilitates experimental techniques. Because of these reasons zebrafish has become a commonly used model organism for toxicity potential testing (4).

Nitric oxide (nitrogen monoxide, NO), is a gaseous molecule, that is derived from l-arginine, a basic amino acid. When it was discovered, it became a great surprise because although other hormones and regulators in our body are composed of proteins, lipid-derived compounds, or other molecules, none of which are gaseous. NO influences different enzyme systems as a co-factor in some important enzymes. In many ways, NO has been believed to act as a hormone and regulate many different processes. For example, it leads to vasodilation by inhibiting the contraction of vascular smooth muscle. It has a rapid action and it is found in the blood only for seconds. Other functions include inhibition of platelet aggregation and inhibition of leukocytes adhesion to endothelia. NO has distinct roles which are not completely understood yet regarding different diseases, including as diabetes, atherosclerosis, and also hypertension (5). Aim of this study was to investigate the possible effects of MA exposure on the NO levels and zebrafish embryo development.

MATERIAL AND METHODS

Maintenance of zebrafish

Wild type AB/AB Strain zebrafish were housed in an aquarium rack system (Zebtec, Tecniplast, Italy) at 28 ± 1 °C under a light/dark cycle of 14/10 h. Fish were fed twice a day using commercial flake food and live Artemia was also given as complement. Reverse osmosis water was used for the experiments and the water was supplemented with 0.018 mg L^{-1} Instant Ocean™ salt. After their natural spawnings the fertilized embryos were gathered then they were cultured, and they were staged according their developmental time and morphological criteria based on the methods that were described previously (4,6). Since embryos used in this study were younger than 5 days old, no licence is needed by Council of Europe (1986), Directive 86/609/EEC or Marmara University ethics committee.

Embryo exposure

For the exposure experiments newly fertilized eggs were selected, they were collected and rinsed two or three times in water before their use. The spherical embryos that were dividing normally were selected and they were used for all of the explained studies. Range-finding experiments were performed and then zebrafish embryos were exposed to the powder of one type of MA based product (ingredient: Polyethylmetacylate: PEMA) (Ufi Gel Hard; Voco GmbH, Cuxhaven, Germany), which is used as a chair-side relining material for acrylic based dentures. The exposure was made in well plates containing 20 embryos, having 4 replicates. Dimethyl sulfoxide (DMSO) was used as the solvent. Nitric oxide (NO) levels of the embryos were evaluated at the end of 72h using Griess Method (6). Each day the solutions for exposure were renewed with fresh solutions. When the exposure period ended, the embryos were washed two or three times with water and they were allowed for development until 120 hpf. Using a stereomicroscope (Zeiss Discovery V8, Germany) developmental parameters were monitored and documented. Every 24 hours mortality and hatching rate of the embryos were determined. Malformation images of were captured every 24 hours and calculated to find abnormal embryo percentage. Malformations and

abnormalities of the embryos including axial malformations, pericardial edema and yolc sac edema were evaluated. Furthermore interruption in development was determined by comparing the exposure group with the control embryos.

Biochemical Analyses

Zebrafish embryos that were at 72hpf were used for the biochemical analyses. The embryos were used as replicate pools consisting of 72 hpf zebrafish (n=5, 100 individuals per pool). Pools were prepared and for each pool hundred embryos were homogenized in 1ml PBS, this was followed by centrifuging step. For the biochemical parameters the supernatant was used.

NO Determination

NO method is performed based on the ability of nitrate reduction to nitrite using vanadium (III) chloride. Within an acidic environment, nitrite and sulfonylamide reacted with N-(1-Naphtyl) ethylenediamine dihydrochloride. This reaction led to the formation of complex diazonium compound. A colored complex was produced and it was measured at 540 nm using a spectrophotometer. Then the results were given as nmol NO/mg protein (6).

RESULTS

Mortality rates are given for the methacrylate and the control groups are given in Figure 1. Increased mortality was observed in the MA exposed embryos after 24h. Developmental stages as well as the malformations are given in Figure 2. Lack of pigmentation was observed in the DMSO group. Pericardial edema was observed in some embryos of the MA group (Figure 2). No significant differences were observed in NO levels between the groups (Figure 3).

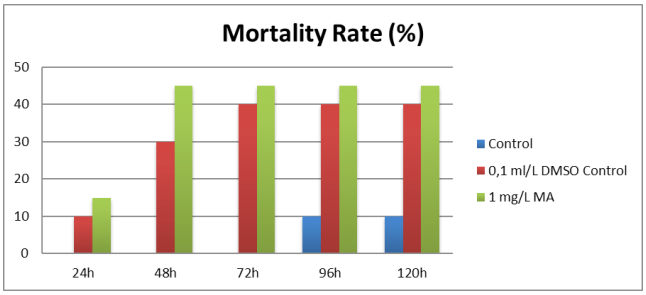

Figure 1. Mortality rates of the groups. MA: Methacrylate;h:hour

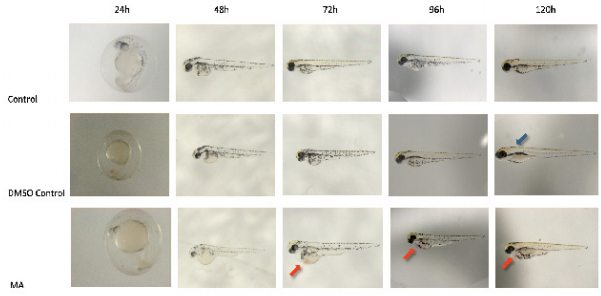

Figure 2. Development of the zebrafish embryos in the groups at 24 hpf, 48hpf, 72hpf, 96hpf and 120hpf. Blue arrow indicates decreased pigmentation and red arrow indicate pericardial edema. Hpf: hours post fertilization; MA: Methacrylate; DMSO: Dimethyl sulfoxide

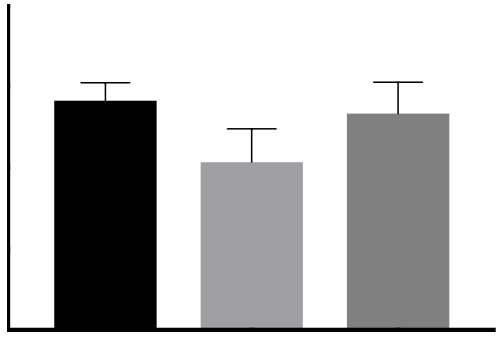

Figure 3. Nitric oxide levels of the embryos at 72 hpf (hours post fertilization); MA: Methacrylate; DMSO: Dimethyl sulfoxide. Replicate pools of 72 hpf zebrafish (n=5, 100 individuals per pool) were used. Values are given as mean±standart deviation.

DISCUSSION

A biocompatible material has a special structure of being non-destructive in the biological environments. Dental materials are known to release some substances into the oral environment in different degrees. Biopolymers are used in many areas in dentistry including cavity restorative materials, sealants, impression materials, cements, as well as orthodontic, habit breaking and oral and maxillofacial appliances. They are also used in dentin bonding agents and cleft palate plates as well as maxillary supports (7).

MA, is widely used in dentistry as well as medicine. Some adverse effects of MA has been reported related

with abnormal structures or lesions in different tissues. Both *in vitro* and *in vivo* clinical studies have shown that monomers may lead to irritation to mucous membranes and skin, eyes, and may lead to some conditions such as liver toxicity, allergic dermatitis, stomatitis, as well as disruption of central nervous system and even fertility disturbances (8-10). Since a very big part of the restorations are made using polymers and monomers, the dental staff using them may be suggested to be at a higher risk of deleterious effects of monomers than the patients (7).

Accordingly the present study aimed to evaluate the effects of MA exposure on developing zebrafish embryo. Zebrafish embryos have become a useful model organism to detect the toxicity of chemicals. NO metabolites are related with the genotoxic and carcinogenic effects of toxic substances and they play major roles in the regulation of DNA damage, lipid and protein modifications. NO can directly act as a radical and damage or protect the cell milieu or it can indirectly affect through its byproducts leading to oxidative and nitrosative stress. NO can also interact with mitochondria and inhibit respiration (11). In our study no significant change was observed in the NO levels of the embryos in the control group and in the MA exposed group. It may be suggested that the dose of MA used in this study did not cause an inflammatory or oxidant response leading to an increase in NO levels. However, some developmental defects were observed in some of the MA exposed embryos. Lack of pigmentation was evident in one DMSO exposed embryo and pericardial edema was observed in some of the MA exposed embryos. Various small molecules have been shown to cause extraordinary cardiac defects and disturb blood circulation in the zebrafish embryo. These defects can be the result of many different origins. For example, defects in cardiac progenitor cells or differentiation specification; heart tube morphogenesis, cardiac chambers, problems of atrioventricular canal and proper cardiac function (12). In our study, although MA exposure did not alter NO levels the mechanism underlying developmental defects need further investigations.

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