

Full Length Research Paper

Teratogenicity and brain aromatase-induction of monosodium glutamate in estrogen-responsive mosaic transgenic zebra fish *Danio rerio*

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Monosodium glutamate (MSG) has been used as a flavor enhancer for decades. It has various teratogenicity effects on tested animals but has not been examined in zebra fish model to date. This experiment was conducted to study the teratogenic effects of MSG on wild-type zebra fish embryos and also to study the estrogenic potential of MSG on the transient zebrafish embryos with a brain aromatase-based reporter gene. Different concentrations of MSG (0, 10, 50 and 100 µg/ml) were tested. Wild-type and transient embryos were exposed to the solutions at about 2 h post fertilization (hpf). Hatching and survival decreased in all treatments with significant difference ($p < 0.05$) at 50 and 100 µg/ml concentrations with control. Stunted skeletal structure was observed at 100 µg/ml treatment. At 96 hpf, MSG induced enhanced green fluorescence protein (EGFP) expression in the olfactory bulb at 100 µg/ml treatment. Various malformations were found in all treatments. The current results demonstrate that MSG or MSG-containing foods may harm the human offspring if they take it in a high dose. MSG in high concentration may disrupt the endocrine function. Zebra fish embryo with a brain aromatase-based reporter gene is a good model for the detection of estrogenic potential of any controversial chemical.

Key words: Monosodium glutamate (MSG), teratogenicity, aromatase, embryos, zebra fish.

INTRODUCTION

Zebra fish *Danio rerio* is a small fresh-water teleost. It has been used as a laboratory animal for several decades now (Feitsma and Cuppen, 2008). Zebra fish embryo *in vitro* model has many advantages such as easy breeding, large number can be maintained in a small space, and the generation time is relatively short (Kristensen, 1995; Luckenbach et al., 2001; Kristensen, 1995; Luckenbach et al., 2001). Moreover, because of rapid embryogenesis, transparency and *ex-utero*

development, it is considered an attractive organism for embryo toxicology research (Kristensen, 1995; Luckenbach et al., 2001; Chiranjib, 2011). Zebra fish was extensively used for studying the toxic effects of environmental pollutants (Baumann and Sander, 1984; Buchmann et al., 1993; Ensenbach and Nagel, 1995; Mizell and Romig, 1997; Chen et al., 2010; Hung et al., 2011; Shao et al., 2012; Liao et al., 2012). Also, zebra fish was used for the screening of drug candidates (Parg et al., 2002; Spitsbergen and Kent, 2003) including organ-specific toxicity and developmental toxicity. On this base, zebra fish embryo is a suitable model for studying and examining the effect of a wide range of compounds including also food additives, flavor enhancers and different controversial compounds.

In this experiment, we studied one of the most famous

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Abbreviations: MSG, Monosodium glutamate; hpf, hour post fertilization; EGFP, enhanced green fluorescence protein.

food additives, monosodium glutamate (MSG). MSG has been used as a flavor enhancer and food additive for a long time. Although, the food and drug administration (FDA) classified it as a food ingredient that is generally recognized as safe but the use of MSG still remains controversial (Williams and Woessner, 2009). MSG is the sodium salt of the amino acid glutamic acid and a form of glutamate. It is sold as a fine white crystal substance, similar in appearance to salt or sugar. It does not have a distinct taste of its own, but adds flavor to other foods with not fully understood mechanism (Food Science, 2007). Previous studies demonstrated that MSG has many biological effects in all tested animals especially in infants. These effects such as, brain damage potential, stunted skeletal development and hepatic toxicity (Olney, 1969; Pellett, 1977; Nemeroff, 1981; Farombi; Onyema, 2006; Diniz et al., 2004; Egbonu et al., 2009). It is also known to cause several systematic disorders, the so called 'Chinese restaurant syndrome' (Kenny, 1986). MSG was classified also as an exo-toxin, which play a role in the development of several disorders including certain endocrine disorders (Ikonomidou and Turski, 1995) and also may damage the brain especially, by oral intake without food (Walker and Lupien, 2000). Interestingly, the toxicity and teratogenicity of MSG have been studied using several kinds of laboratory animals but have not been examined in zebra fish model to date. This experiment was conducted to demonstrate the effect of different concentrations of MSG on wild-type zebra fish embryos and also to determine the estrogen-potential of MSG using estrogen-responsive mosaic transgenic zebra fish *D. rerio* model.

MATERIALS AND METHODS

Zebra fish maintaining

Zebra fish *D. rerio* were obtained locally (Green Fish, Korea) and were raised at our laboratory housing system (Daejong instrument industry, Korea). Adult zebra fish were maintained in glass aquariums with continuous re-circulating system, 14:10 h (light:dark photo-cycle) and 28.5°C. They were fed three times a day with a combination of dried blood worms (TetraWerke, Melle, Germany) and new hatched brine shrimp *Artemia* (San Francisco Bay Brand, Inc., Newark, CA, USA). Two pair of ripe fish males and females was separated with a barrier in a spawning box containing a mesh bottom to prevent the spawned eggs from being cannibalized. The boxes were incubated in 28.5°C incubator overnight. In the next day, the barrier was removed at the beginning of light period and the zebra fish started spawning. Fertilized eggs were obtained from matured zebra fish according to the manual by Westerfield (1995). We followed the care and treatment of the animals guidelines established by Institutional Animal Care and Use Committee, Seoul National University (Approval no. SNU-050418-2).

Plasmid construction and microinjection

Zebra fish brainaromatase-regulated reporter plasmid, pzfAroBEGFP was constructed according to Kim et al. (2009) by

linking the proximal promoter region of the zebra fish brain aromatase gene with enhanced green fluorescence protein (EGFP) reporter gene. The proximal promoter region of the zebra fish brain aromatase gene was amplified by PCR from zebra fish genomic DNA using a specific primer set as following: zfAroB-F(5'GCTCGAGGTTCAAAGCCCTCCCAAATA-3') and zfAroB-R(5'-CGGGATCCCGTCCTCAGGCTTCCATCAT-3') containing a restriction-digest adaptor (underlined) corresponding to XhoI and BamHI sites, respectively (Kazeto et al., 2004; Menuet et al., 2005) (Figure 1). PCR product was double digested by XhoI and BamHI and subcloned upstream of the EGFP gene in the T2KXIGvector (Kawakami et al., 2004). Fertilized eggs (one to two cell stage) were micro-injected with pzfAroB-EGFP vector and transposase mRNA. Transposase mRNA was synthesized *in vitro* using teratogenicity and brain aromatase-induction of monosodium glutamate in estrogen-responsive mosaic transgenic zebra fish *D. rerio* and the mMESSAGEmMACHINESP6Kit (AmbionInc.). The embryos were transferred to agarose ramps and were injected with DNA/RNA solution containing 25 ng/μl circular pzfAroB-EGFPDNA and 25 ng/μl transposase mRNA using a micro pipette secured in a micro manipulator (World Precision Instruments Inc., Sarasota, FL, USA).

Chemical solutions and experimental design

L-Glutamic acid, monosodium salt monohydrate (Powder, purity 98%) was purchased from Sigma-Aldrich Company (CAS. 6106-04-3). The powder was dissolved in the maintaining water to make the tested concentrations (10, 50 and 100 μg/ml) and the control was (0 μg/ml). The fertilized eggs were transferred into two groups; the eggs in the first group were micro-injected with the pzfAroB-EGFPDNA. The eggs in the other group were exposed directly to the MSG solutions. At about 2 hpf, wild-type and micro-injected embryos were transferred into 12-wells Petri dishes filled with the tested solutions and kept at a 14:10 h, light:dark cycles for four days. Each treatment and control group consisted of 30 embryos in three replicates. About 30% of the exposure solution was exchanged daily with fresh solution to renew the oxygen content and keep the concentration. Survival rate, hatching rate, morphological changes and EGFP expression were observed during the experiment until 96 hpf in all treatments.

EGFP detection in reporter zebrafish embryos

Zebra fish brain aromatase gene transcription is detectable at 5 hpf (Sawyer et al., 2006) and the micro-injected embryos were exposed to MSG immediately. After exposure, EGFP expression was monitored frequently using OlympusIX70 microscope equipped with NIBA2filter (λ_{ex} = 470 to 490 nm and λ_{em} = 510 to 550 nm).

Statistical analysis

Treatment-related effects on hatching rate, survival rate and body length were determined using one-way analysis of variance (ANOVA) followed by Tukey's. Significant difference occurred in a given parameter ($p < 0.05$). The entire statistical analysis was carried out using Graphpad Prism (version. 5).

RESULTS

Monitoring assay

The hatching rate, survival rate and total body length

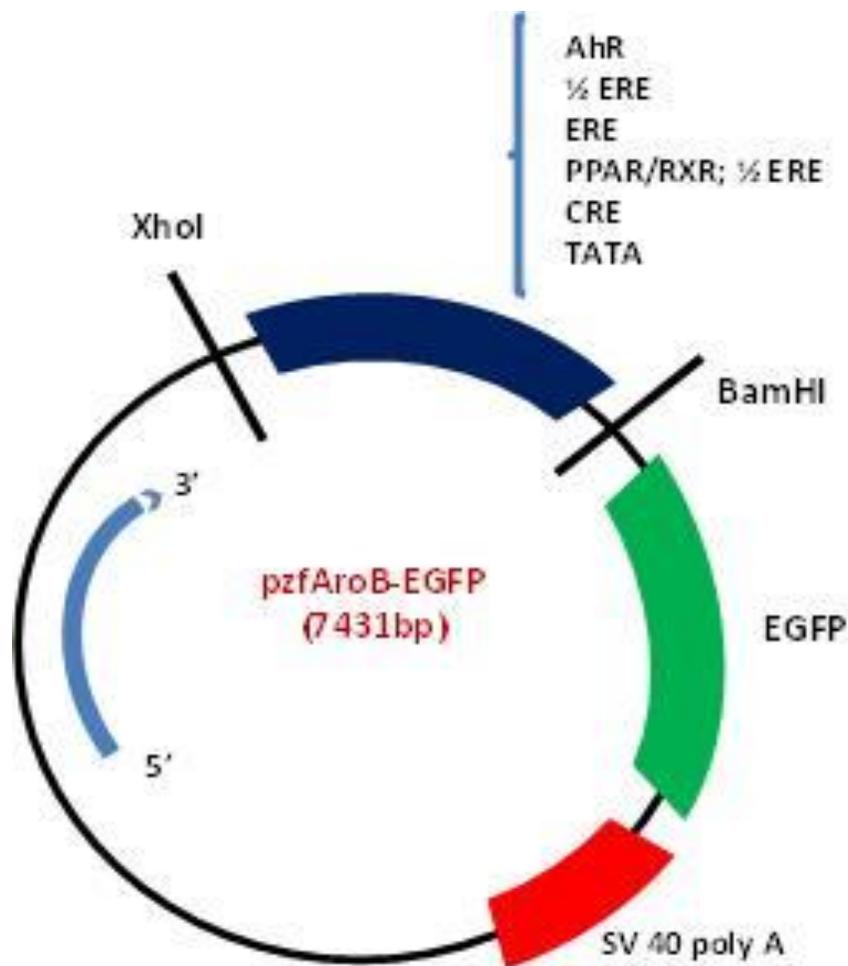


Figure 1. Schematic representation of the zebra fish brain aromatase-regulated reporter plasmid construct pzfAroB-EGFP. The proximal promoter region of the zebra fish brain aromatase gene was double-digested by XhoI and BamHI and then subcloned upstream of the EGFP gene in the XhoI/BamHI restriction sites of the T2KXIG vector. The gene amplification reaction conditions were as follow: denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s; annealing at 58°C for 30 s; and extension at 72°C for 45 s; and a final extension at 72°C for 7 min.

decreased in all treatments after MSG exposure. Hatching started at about 60 hpf and the over-all hatching rate was calculated at 96 hpf. Over-all, hatching rate was 83.33% in the control group but was 56.7, 46.7 and 36.7% in 10, 50 and 100 µg/ml treatment group, respectively. There was significant difference ($p < 0.05$) at 50 and 100 µg/ml treatments with control (Figure 2). The survival rate was 96.7% in the control group but decreased in MSG treatments, 66.7, 33.3 and 26.7% at 10, 50 and 100 µg/ml concentrations, respectively, with significant difference of ($p < 0.01$) at 50 and 100 µg/ml treatments with control (Figure 3). Total body length was measured at 96 hpf. It was about 4 mm in the control group and decreased in 10 and 50 µg/ml treatments, 3.77 and 3.67 mm, respectively but with no significant

difference ($p > 0.05$). The significant difference ($p < 0.01$) of total body length was found in 100 µg/ml MSG treatment (Figure 4).

Teratogenicity and morphological changes

Control group of wild-type embryos and transient transgenic embryos had no abnormal morphological changes during the experiment. Different malformations were observed in all MSG treatments in both wild-type embryos and transient transgenic embryos (Figure 5). There were different effects such as: elongated heart (B1), pericardial edema (B2 and C1), and yolk sac edema (C2), malformation in spinal cord (spinal kyphosis) (D).

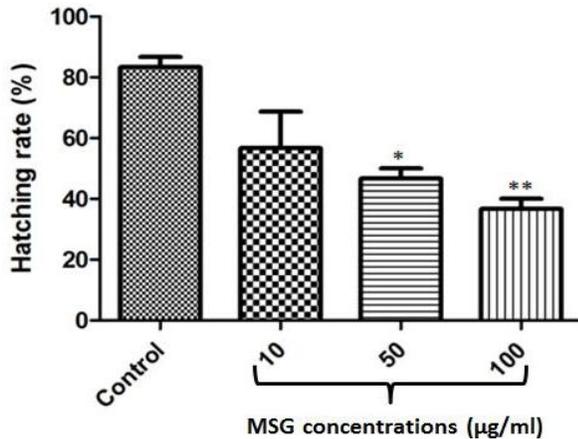


Figure 2. The effect of different concentrations of monosodium glutamate on hatching rate of zebrafish embryos. Embryos were exposed to MSG about 2 hpf. Over-all hatching rates were calculated at 96 hpf. The values were expressed as the mean \pm SE. *Means significant difference with control ($P < 0.05$).

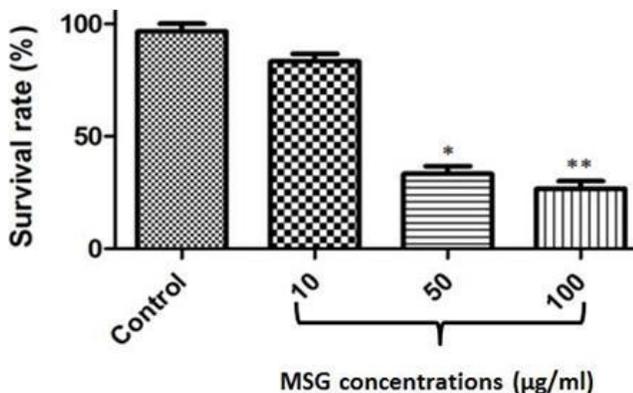


Figure 3. Effect of different concentrations of monosodium glutamate on the survival rate of zebrafish embryos. Embryos were exposed to MSG at 2 to 96 hpf. All values were expressed as mean \pm SE. *Means significant difference with control ($P < 0.05$). **Means high significant difference with control ($P < 0.01$).

In 100 µg/ml treatment, larvae did not grow normally and showed stunted skeletal structure (E). Total body length of these stunted larvae was about 3.17 mm with significant difference with control ($p < 0.05$). There was no significant difference between wild-type and micro-injected embryos in all measured parameters ($p > 0.05$).

MSG - induced aromatase expression in the mosaic reporter embryos

To determine the estrogenic potential of monosodium

glutamate, transient zebra fish embryos of aromatase promoter-driven EGFP reporter gene were exposed to same concentrations of MSG, immediately after micro-injection at about 2 hpf. We checked the EGFP signal frequently using the fluorescent microscope but did not observe any green fluorescence signal in the control groups of both wild-type and transient embryos. Also, we did not observe any signals in 10 and 50 µg/ml concentrations treatments until about 96 hpf. At about 96 hpf, we observed that some embryos in 100 µg/ml treatment induced green fluorescence signal in the head region. These signals were localized in the olfactory bulb of the larvae (Figure 6).

DISCUSSION

We have studied the toxicity of monosodium glutamate (MSG) in wild-type zebra fish embryos. We also, detected the estrogenic potential of MSG using our transient transgenic model. This is the first study in which MSG has teratogenicity on zebra fish embryos model. The wild-type and transient embryos were exposed to same concentrations of MSG (0, 10, 50 and 100 µg/ml). Hatching rate, survival rate and total body length decreased in all treated embryos with significant difference at 50 and 100 µg/ml treatments with control. The estrogenic potential was found in 100 µg/ml treatment at 96 hpf.

The toxicity and teratogenicity of MSG increased with the increase of concentration. All treated embryos had different effects such as elongated heart, cardiac sac edema, yolk-sac edema and spinal kyphosis. At 100 µg/ml concentration, the skeletal development was affected and significantly decreased the total body length. In our experiment, we observed that the larvae in 50 and 100 µg/ml treatments did not grow normally and showed a reduction in total body length. This finding is in line with the results of Olney (1969), Olney et al. (1980) and Elefteriou et al. (2003) in which infant mice had abnormal development and stunted skeletal structure after given doses of monosodium glutamate and were observed over a period of time. Therefore, great trials were conducted to illustrate the mechanisms of the teratogenicity of the animals' skeletal structure after they were exposed to MSG. We think that there is no direct effect to MSG on bone structure and stunting, but we think that MSG may cause a disruption of the endocrine function which is responsible for many systems in the body. This disruption may play a role in causing various malformations in the treated animals including stunted skeletal development and other malformations (Elefteriou et al., 2003). As mentioned previously, MSG is considered one of the excitotoxins compounds. Excitotoxicity is the pathological process by which nerve cells are damaged and killed by excessive stimulation by neurotransmitters such as glutamate and similar

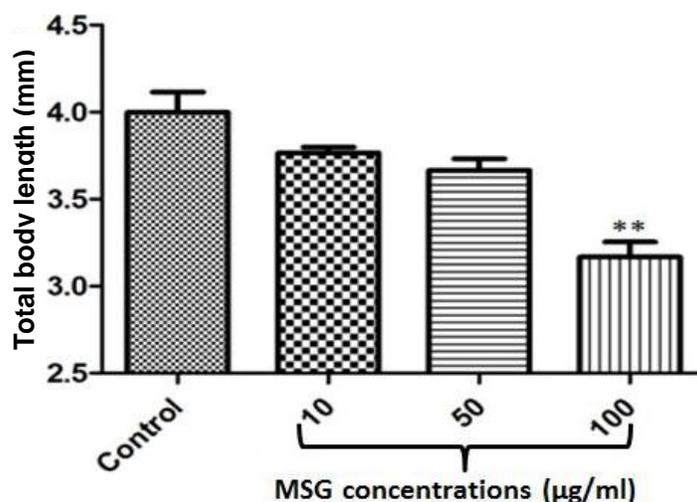


Figure 4. Effect of different concentrations of monosodium glutamate on the total body length of zebrafish embryos. Embryos were exposed to MSG at 2 hpf to 96 hpf. All values were expressed as mean \pm SE. *Means significant difference with control ($P < 0.05$). **Means high significant difference with control ($P < 0.01$).

substances. Excitotoxins play a critical role in the development of several neurological disorders, abnormal neural development, certain endocrine disorders and neuropsychiatric disorders (Ikonomidou and Turski, 1995). MSG-treated animals as neonates or in the first days of life were shown to suffer neuro-endocrine disturbances including; stunting, abnormalities of the reproductive system, and under-development of certain endocrine glands (Nemeroff, 1981). These results may lead us to consider MSG as a new member of the endocrine disrupting chemicals group. A considerable research effort has been focused on those endocrine disrupting chemicals (EDCs) that can modulate or/and disrupt the endocrine system invertebrates including teleost (Colborn et al., 1993; Sumpter, 1998).

The study's hypothesis regarding the teratogenicity of MSG, including the stunted structure, is all about the effects caused by the endocrine disruption after MSG exposure. To confirm this idea, we used our transient zebra fish model with brain aromatase (AroB)-based estrogen responsive reporter gene. Recently, our laboratory established this screening-system for detecting the endocrine disrupting chemicals (EDCs) using xenoestrogen-responsive transient transgenic zebra fish embryos with an AroB-based estrogen responsive reporter gene. The brain aromatase gene is a crucial enzyme that aromatizes androgens into estrogens, while its expression is up-regulated by estrogens (Tchoudakova et al., 2001; Kazeto et al., 2004). Also, aromatase expression has been used as a neural marker of estrogen effects in adults and embryos in previous studies with zebra fish (Kishida and Callard, 2001; Sawyer et al., 2006). In zebra fish, two aromatase genes, *cyp19a1* and *cyp19a2*, were predominantly

expressed in the ovaries and brain, respectively (Chiang et al., 2001; Kishida and Callard, 2001). Zebra fish embryonic P450aromB expression can be detected at 5 hpf (Sawyer et al., 2006) and as such, micro-injected zebra fish embryos were exposed immediately to MSG solutions. We found that MSG induced EGFP expression in the olfactory bulb (ob) of transient reporter embryos at 96 hpf in 100 µg/ml treatment. The location of EGFP expression showed acceptable agreement with that of estradiol induced AroB transcript expression (Menuet et al., 2005; Kim et al., 2009).

This result demonstrates that MSG can stimulate zebra fish brain aromatase gene expression. It was reported that the zebra fish brain aromatase gene can be a sensitive marker of xenoestrogens affecting the central nervous system in zebra fish juveniles and embryos (Kishida and Callard, 2001; Kazeto et al., 2004). These results may confirm our hypothesis regarding the effect of MSG on the endocrine function and may illustrate the reason for the teratogenicity findings such as; the elongated heart, cardiac sac edema that the disrupted endocrine system caused disruption to the vital processes in the body and development, which decreased the hatching rate and survival rate of all treated zebra fish embryos.

Conclusion

MSG induces toxic and teratogenic effects in zebra fish embryos. The effects of MSG increase with the increasing of concentration. High dose (100 µg/ml) of MSG may cause endocrine disruption and may also cause stunted skeletal development of the growing

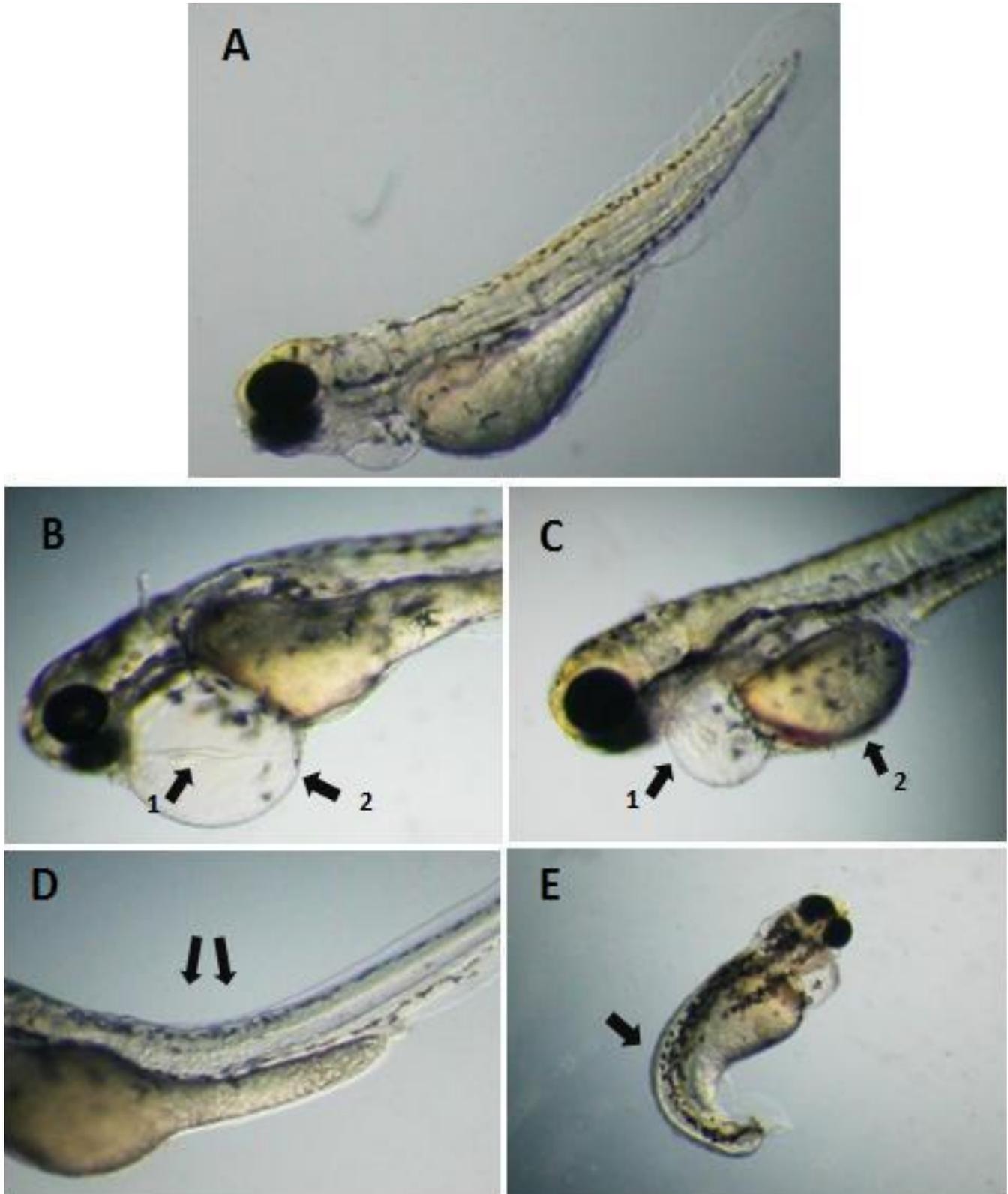


Figure 5. The toxicity of monosodium glutamate in zebra fish embryos. The embryos were exposed to MSG different concentrations (0, 10, 50 and 100 $\mu\text{g/ml}$) or Ringer's solution at 2 hpf to 96hpf. Malformations were detected using stereomicroscope. All embryos were treated with MSG showed malformation symptoms such as: elongated heart (B1), cardiac sac edema (B2 and C1), yolk sac edema (C2) and spinal kyphosis (D). Embryos were exposed to high concentrations (100 $\mu\text{g/ml}$) showed a stunted development (E). (A) Normal larvae at 96 hpf.

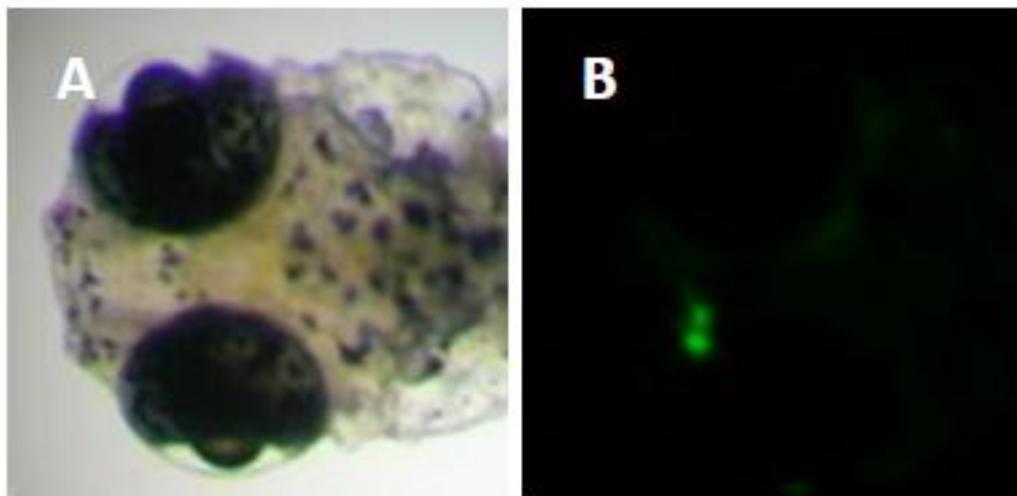


Figure 6. EGFP Expression in response to MSG exposure. PzfAroB-EGFP transient embryos that were exposed to high concentrations of MSG (100 µg/ml) at 96 hpf, revealed EGFP expression as a result of aromatase induction in the brain. (A) Bright field. (B) EGFP expression. The EGFP signal was localized in the olfactory bulb.

embryos. Based on the aforementioned results, MSG may harm the human offspring if they eat high dose of MSG-contained foods.

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