Original Article

Chronic toxicity of monosodium glutamate on the reproductive system and some internal organs of zebrafish (*Danio rerio*)

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Abstract

Monosodium glutamate (MSG) is commonly used to enhance the tastes food. It is composed of sodium salt and amino acids. Although MSG is considered safe at the consumption level, its adverse effects have been reported. The objective of this study is to evaluate the effects of MSG on the brain, liver, kidney, testes, and ovaries of zebrafish (*Danio rerio*) which were chronically exposed to MSG from the embryo stage to the adult stage. Additionally, the fertilization rate and hatching rate of zebrafish embryos were also analyzed. Zebrafish were randomly divided into four groups and treated with MSG at 0, 10, 100 and 1,000 mg/l, respectively for 60 days. Before sacrifice for histopathological study, the fishes in each group were mated and the embryos were collected and analyzed for the numbers, survival rate and hatching rate. The results showed that the liver and kidneys of fishes in the MSG-exposed groups had a significantly higher lesion score ($P<0.05$) compared to the control group. All fish from the MSG-exposed groups had diffuse hepatocyte swelling and hepatic congestion. Renal congestion and a decrease in the number of distal convoluted tubules were observed. Zebrafish from the 1,000 mg/l MSG-exposed group did not spawn after placing them in the mating tank. The number of embryos from the 100 mg/l MSG-exposed group was lower than the 10 mg/l MSG-exposed group and the control group. The survival rate of fertilized embryos (after 120 hours) from the 100 mg/l MSG-treated group was significantly lower than the control group. In summary, the results from this study showed that exposure to MSG for 60 days induced lesions to the liver and the kidneys and decreased the reproductive performance of zebrafish.

Keywords: chronic toxicity, histopathology, monosodium glutamate, zebrafish

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Introduction

Monosodium glutamate (MSG) is widely used as a food additive and flavor enhancer in both the food industry and the household kitchen. The increase in MSG consumption has resulted in a massive disposal of high organic pollutant which has increased the chemical oxygen demand (COD) and ammonia in wastewater which is harmful to the environment (Liu *et al.*, 2007, Jiang *et al.*, 2015). Water pollution is one of the major issues that causes serious concern since the chemical waste that gets into the water can cause illness in animals and human and poison the marine life.

Even though the U.S. FDA has classified MSG as generally recognizably safe for consumption like other seasonings such as salt, pepper and sugar (FDA, 2014), studies in rodents have revealed the adverse effects of MSG on the brain, liver, kidney and reproductive organs (Vinodini *et al.*, 2008; Alao *et al.*, 2010; Kumbhare *et al.*, 2015; Sharma, 2015). In addition, previous studies have indicated that people who regularly consume MSG have a higher risk of becoming obese and having metabolic syndromes (He *et al.*, 2008; Chinna and Karupaiah, 2013).

At present, one of the most popular animal models in research is the zebrafish (Kinth *et al.*, 2013). It is widely used especially in toxicity studies due to its high sensitivity to chemical compounds and easy to maintain and inexpensive husbandry costs. This model is also a vertebrate, which gives it superiority over other models. Another advantage is that zebrafish eggs develop externally, allowing researchers to study their development easily (Hill *et al.*, 2005). The zebrafish is therefore considered as a very valuable replacement species for higher vertebrates in toxicity study.

Previously there have been some studies using zebrafish as a model to study the effects of MSG. Abdelkader *et al.* (2012) and Mahaliyana *et al.* (2016) reported the effects of MSG (100 - 500 mg/l) on zebrafish embryos such as the teratogenic effect, growth retardation, shrinkage of chorion, yolk sac and cardiac sac edema, lack of pigmentation, tail deformities and scoliosis. Kurnianingsih *et al.* (2016) reported the neuronal effect of MSG on neuron apoptosis accompanied with behavioral changes and decreased locomotor activity at day 1 postfertilization of zebrafish embryo after exposure to 10 µg/ml MSG for 24 - 72 hours. The adverse effects of MSG on the cardiovascular system such as tachycardia in zebrafish embryos at the age of over 48 hours post-fertilization (hpf) have also been reported (Suthamnatpong and Ponpornpisit, 2017).

As described above, earlier studies on the effect of MSG on zebrafish were done using fishes at an early life stage but the chronic toxicity of MSG (from larval to adult stages) on zebrafish has never been evaluated before. The toxicity test of MSG on the whole life of the zebrafish in this study is very important in veterinary science as it represents the vertebrate species. The aim of this study was to investigate the effect of long-term exposure (from larval to adult stages) of sublethal dose of MSG on the histological structures of the brain, liver, kidneys and the reproductive tissues as well as the related effect on the reproduction and the offspring of zebrafish.

Materials and Methods

The present experiment was performed under the guidelines documented in the ethical principles and guidelines for the use of animals for scientific purposes, edited by the National Research Council of Thailand and approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Science, Chulalongkorn University, in accordance with university regulations and policies governing the care and use of experimental animals, approval no. 1731073.

Zebrafish husbandry and spawning: Fifty male and female broodstock, wild type, long fin zebrafish were purchased from a local fish farm in Nakhonpathom province. They were acclimatized in a 50-liter PVC plastic tank for one month at the Veterinary Medical Aquatic Animal Research Center, Faculty of Veterinary Science, Chulalongkorn University, Bangkok. The spawning process was done by placing five pairs of male and female zebrafish in a metal grating container overlaying a five-liter plastic tank for one night. Their eggs were collected in the morning after broodfish spawning and kept in a new plastic tank for three days until the larvae hatched. The hatching larvae were reared for thirty days under natural photoperiod, pH and water temperature at optimum requirement with gentle aeration. Half of the water tank was changed and the quality checked every day (OECD, 2013). The zebrafish were fed twice daily with flake food (Tetramin, Germany) and brine shrimp (*Artemia* nauplii).

Chronic exposure process: The 120 zebrafish larvae at 30 days post-hatching (dph) were randomly divided into 4 groups (30 larvae per group3) and placed in a 1-liter plastic tank. Experimental groups consisted of the control groups (without MSG) and three MSG-treated groups at different concentrations (10, 100, and 1,000 mg/l). MSG (L-glutamic acid monosodium salt hydrate, Sigma-Aldrich, USA) was added into the water to make a final concentration of 10, 100, and 1,000 mg/l, respectively before placing the fish into the tank. In order to evaluate the chronic effect of MSG and to prevent the fish from dying during the experiment, this study used MSG at a concentration lower than the median lethal concentration (LC_{50}) of MSG in zebrafish which was reported as 15,200 mg/l at 48 hpf and 10,300 mg/l at 96 hpf (Suthamnatpong and Ponpornpisit, 2017).

The concentrations of MSG used in this study were selected based on data from our previous study which demonstrated that the LC50 of zebrafish embryo at 48 hpf is 15,200 mg/l and at 96 hpf is 10,300 mg/L (Suthamnatpong and Ponpornpisit, 2017). In order to study the effect of chronic exposure of MSG, the 10-time lower dilution of the LC50 on zebrafish embryo was chosen on the grounds that our present study evaluated the chronic effect of MSG, the selected

concentrations of MSG was deemed appropriate and would not kill the fish during the long-term experiment. The control group was reared without MSG. The experiment was done in duplicated manner.

All zebrafish were cared for in a static renewed system using carbon filter water with fifty percent of the rearing water being changed every day. Fish in every tank were checked for abnormal behavior and mortality together with the monitoring temperature, pH, dissolved oxygen (DO), total dissolved solids (TDS), electrical conductivity (EC) and osmolarity of water during the experimental period. Newly hatched brine shrimp and flaked food were fed two times a day. The fish were reared in the testing solution for 60 days.

Histopathological examination: Zebrafish of all groups were anesthetized at 90 dph in an overdose solution of MS-222 (Monte and Varga, 2012; Leary *et al.*, 2013) for ten minutes. Six zebrafish from each group were randomly selected and the whole body of the fish was preserved in 10% formalin solution and then decalcified in 4% nitric acid. All specimens were subjected to tissue dehydration processing using an automatic tissue processor and embedded in paraffin wax in a sagittal plane. Six-micron thick; serial sections were cut and stained with hematoxylin and eosin (H&E).

Histopathological scoring: Microscopic examinations were carried out in all selected organs; brain, liver, kidney and reproductive organs (testis and ovaries) under a light microscope by a certified veterinary pathologist. The tissue sections were studied for 5 areas per organ and scored for each area by lesions. The lesion scores were categorized into 4 levels include score 0 for no tissue changes, score 1 for mild tissue change (less than 25%), score 2 for moderate tissue change (25% - 50%) and score 3 for generalized tissue change of (more than 50%) (Mumford *et al.*, 2007; OECD, 2015).

Effect of MSG on the zebrafish embryo and sex ratio: Zebrafish were cultured and analyzed for survival. The day before the collection of embryos, 90 dph zebrafish from each group were placed in a plastic tank for mating. Embryos were transferred into petridishes containing carbon-filtered water. Fertilized embryos were collected and embryo survival was observed under stereomicroscope at 24, 48, 72 and 96 hpf. The numbers of hatching larvae were recorded at 120 hpf. In addition, the sex of all zebrafish from the control, 10, 100 and 1,000 mg/l MSG-exposed groups were confirmed by wet mount and histopathology section under a light microscope.

Statistical analysis: Nonparametric statistics were used for analyzing the difference of lesion scores of zebrafish and survival analysis of zebrafish embryo. The Kruskal-Wallis test was used for determining the difference of histopathology lesions of the brain, liver, kidneys, testis and ovaries from zebrafish of 10, 100 and 1,000 mg/l MSG-exposed groups and the control group. The sex ratio of all groups of zebrafish was analyzed using descriptive statistics and the survival of zebrafish embryos. Data were recorded every 24 hours as percentages of survival of fertilized embryos until they reached 120 hpf. The survival and mortality rate of all groups of zebrafish embryos were compared by using Kaplan-Meier survival curve and cox analysis. The difference in the mortality rate of all groups of zebrafish after exposure to MSG from 30 dph to 90 dph was analyzed by Fisher's exact test. The statistical analysis program was performed with SPSS version 22.0. Differences were considered significant when $P < 0.05$.

Results

Histopathology of internal organs: Histopathological analysis revealed the presence of degenerative changes in the liver from all groups of zebrafishes (Figure 1). Zebrafish exposed to MSG at the highest concentration (1,000 mg/l) had the most remarkable sinusoidal congestion and generalize hydropic degeneration of hepatocytes (Figure 1B-1D). The liver lesion score of 1,000 mg/l MSG-exposed group was statistically different from the control group ($P < 0.05$).

Kidneys from all MSG-exposed groups had remarkable degenerative changes and renal congestion (Figure 2). All of the MSG-exposed groups showed significant increased atrophy of distal convoluted tubule compared to the control group. The kidney lesion score of kidney congestion and the absence of distal convoluted tubule of all MSG-exposed groups were statistically different from those of the control group ($P < 0.05$). The present histological examination of the specimens from brain, ovaries and testes of all groups indicated that there were no remarkable lesions.

Effects on the reproductive system: Decrease in fertilization success in males and females after prolonged treatment with MSG during their development was found. The MSG-treated groups had fewer fertilized embryos than those of the control group. The number of embryos decreased in a concentration-dependent manner respectively as follows: 408 (control group), 291 (10 mg/l MSG group), 36 (100 mg/l MSG group) and 0 (1,000 mg/l MSG group).

The survival of embryos was examined every 24 hours until 120 hpf. The MSG-treated groups had a lower survival number of embryos compared with the control group and the survival number decreased in a concentration-dependent manner (Figure 3).

From Cox univariate analysis, the risk of dying from 100 mg/l MSG exposure was 24.56 times of the control (Hazard ratio: 24.56, 95% CI: 15.00 - 40.22; $P < 0.05$). Figure 4 showed the survival rate of embryo from the control group and the MSG-exposed groups (10 mg/l and 100 mg/l) from 0 hpf until 120 hpf. The survival rate of zebrafish embryos from the 100 mg/l MSG-exposed group and the control group were significantly different ($P < 0.05$).

The sex of fish from all groups was classified from wet mount and histopathology section. The rates of female fish of the control group, 10, 100 and 1,000 mg/l MSG-treated groups were 48%, 41%, 48%, and 38%, respectively. The rate of male fish was 52%, 59%, 52%, and 62%, respectively. The male and female ratios of all groups were approximately 1:1.

The water quality was monitored regularly during the test period (Table 2). Fisher's exact analysis of the mortality rate of the zebrafish at 90 dpf in every experimental group was not significantly different from the control group.

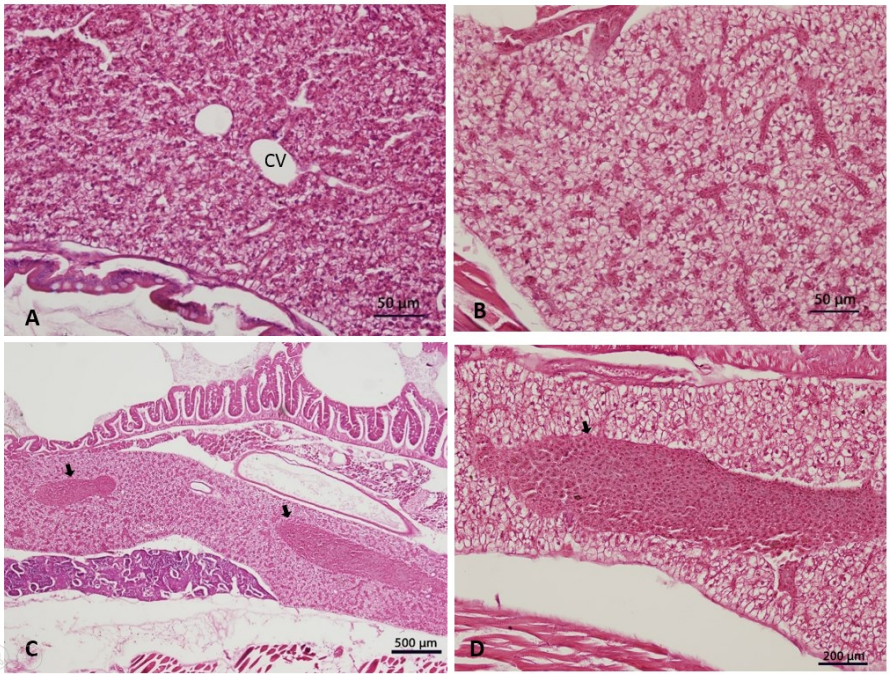

Figure 1 Histopathology of the liver of zebrafish (90 dph). (A) control group showed well organized hepatocytes around the central vein (CV) (B) 1,000 mg/l the MSG-exposed group had generalized swelling of hepatocytes (C) the 1,000 mg/l MSG-exposed group showed hepatic vessels filled up with red blood cell. Normal gut epithelium, intestinal pit, and white and red pulp of splenic tissue were noted. (D) the 1,000 mg/l MSG-exposed groups showed hydropic degeneration of hepatocyte and blood congestion (arrow). Hematoxylin and eosin (H&E) staining.

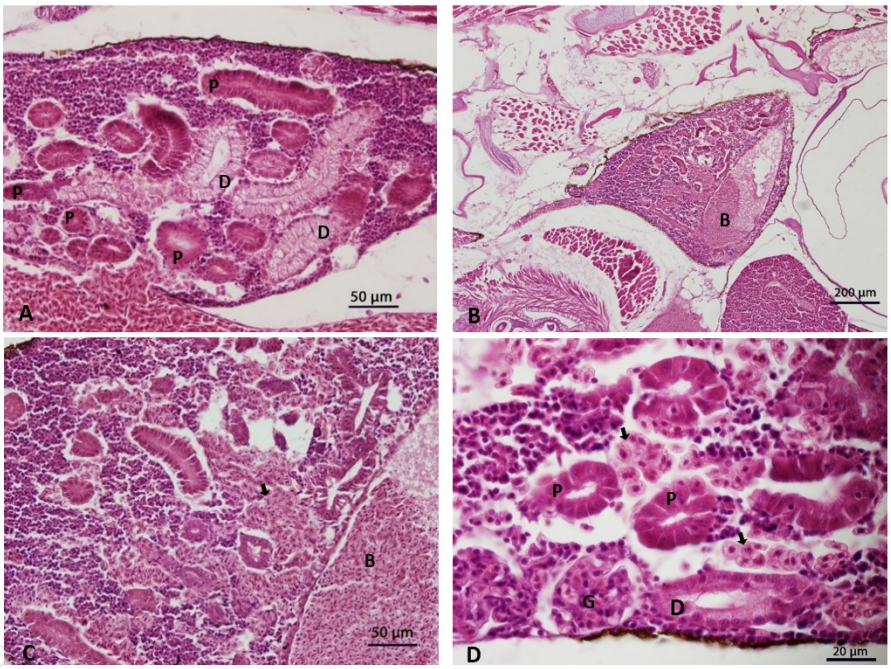

Figure 2 Anterior kidney of zebrafish (90 dph). (A) the control group had normal proximal (P) and distal convoluted tubules (D) (B) 1,000 mg/l the MSG-exposed group showed renal congestion (B) (C) the higher magnification of figure B showed remarkable congestion (B) (D) sagittal section of kidney from 1,000 mg/l MSG-exposed groups showed integrity of glomerulus (G) and proximal convoluted tubule (P). Hematoxylin and eosin (H&E) staining.

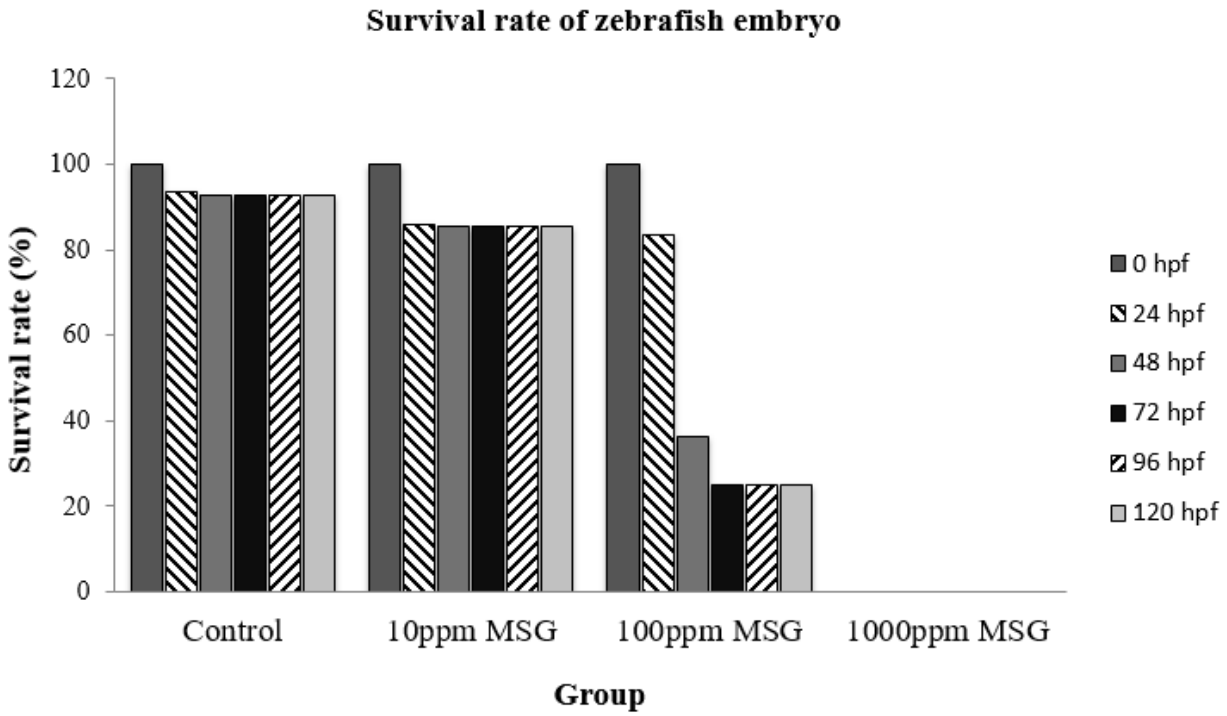

Figure 3 Percentage of zebrafish embryo survival until hatching (from 0 to 120 hpf). From the left: control group, 10 mg/l, 100 mg/l, and 1,000 mg/l MSG-exposed groups.

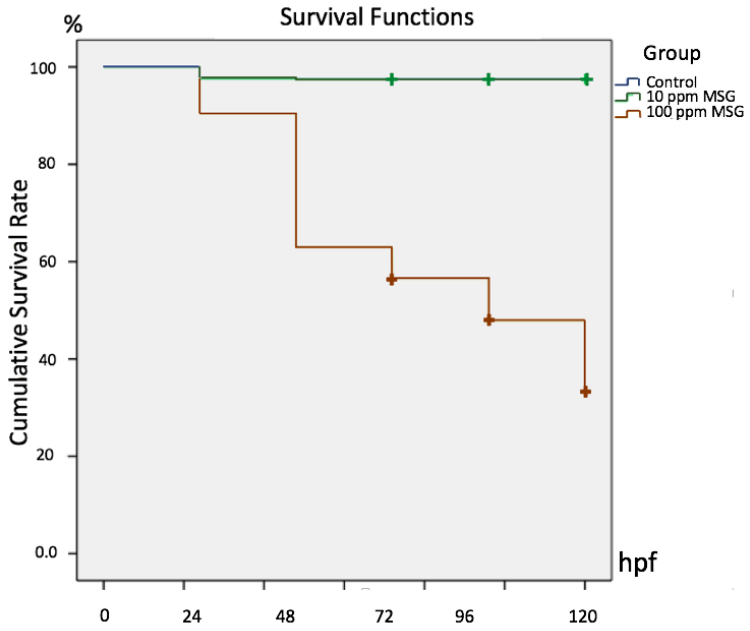

Figure 4 Survival rate of zebrafish embryos from control, 10 mg/l and 100 mg/l MSG-exposed groups observed every 24 hours until 120 hpf by Kaplan-Meier survival analysis with a significance of $P < 0.05$.

Table 1 Kruskal-Wallis non-parametric test results from liver and kidney lesions scores of adult zebrafish exposed to MSG.

	Liver		Kidney	
	Median	**Mean±SD**	**Median**	**Mean±SD**
Control (N=12)	0.5	0.50±0.52	0.00	0.33±0.49
10 mg/l MSG	1	0.92±0.52	2	1.5±0.91*
100 mg/l MSG	1	1.08±0.67	2	2.25±0.62*
1,000 mg/l MSG	2	1.75±0.45*	2	2.25±0.75*

*Kruskal-Wallis test, significant difference between each group and control group ($P < 0.05$)

Table 2 Average of water quality parameter

	Water temperature (°C)	TDS (ppm)	EC (µS)	DO (mg/L)	pH
Control	27.40±0.70	187.75	375.25	3.42±0.75	7.51±0.06
10 mg/l	27.6±0.5	193	387	3.77±0.16	7.41±0.11
100 mg/l	27.43±0.67	215.75	434.50	3.44±0.75	7.48±0.11
1000 mg/l	27.45±0.65	351.50	706.75	3.73±0.23	7.51±0.03

Discussion

Our results showed that exposure to MSG induced swelling and congestion in the liver of zebrafish similar to those found in the liver and kidneys of rats after intraperitoneal injection with MSG (Ortiz *et al*., 2006). In accordance with this study, Wistar rats treated with MSG (1.6 mg/g body weight) also had similar histological changes in the liver such as hepatic congestion and degeneration of hepatocytes (Shrestha *et al*., 2018). Glutamate receptors were identified in the portal blood vessel of the liver. Thus, manipulation of glutamate receptors by MSG may have contributed to the hydropic change of hepatocytes as a result of electrolyte imbalance and water influx into the hepatocytes (Gill and Pulido, 2001; Wallig and Janovitz, 2013). In spite of the fact that exposure to MSG caused a gradual *degeneration* of the liver and tended to have a dose-dependent effect, only the liver lesion score of 1,000 mg/l MSG-exposed group was statistically different from the control group ($P < 0.05$). Still the chance of a causal relationship cannot be disregarded. It is known that dose-response relationships can be affected significantly by time. Therefore, the relationship of the MSG exposure to the outcome may have been influenced by a latent period such that partial effect from MSG exposure at concentration lower than 1,000 mg/l were evident.

The histological observations of the kidneys from all groups of MSG zebrafish showed congestion. Similar to our results, Anil *et al.* (2015) reported renal congestion of adult Wistar rats after being given MSG (6 mg/g body weight) for 45 days. Additionally, our present study found congestion in the primary lamellae of gill tissue of zebrafish from the MSG-exposed group (1,000 mg/l). The abnormality of gill tissue may disrupt the gas exchange and other functions regarding osmoregulation, acid-base regulation and nitrogenous waste excretion. Vascular damage for gills usually occurs after exposure to an irritant substance at a high dose or prolong exposure (Puntoriero *et al*., 2018).

High concentrations of MSG may disturb the quality of water and result in the rise of several parameters including TDS, EC and osmolarity. It was hypothesized that the level of these parameters could be raised due to the presence of MSG in water (Moore *et al*., 2008; Lawrence and Mason, 2012). Although the changes in these parameters of water quality did not affect the survival rate of zebrafish in this study, the exposure to high concentrations of MSG for a long period caused damage which resulted in the lesions of the gill tissues. Gills and kidneys of freshwater fish play an important role in osmoregulation. In zebrafish, the ionocytes found in the gills and skin actively regulate the ion homeostasis (Hwang and Chou, 2013; Zhu *et al*., 2018). Kammerer *et a*l. (2010) reported that plasma osmolarity of tilapia increased rapidly following exposure to high water osmolarity (25% seawater). Thus, in the 1,000 mg/l MSG-treated group the exposure to high concentration of MSG may have interfered with the osmoregulation control of gills and kidney. However, there was no statistical difference in survival rate between the MSG-treated groups and the control groups of zebrafish.

In the 1,000 mg/l MSG-treated group, the numbers of proximal convoluted tubules were higher than the distal convoluted tubule (higher proximal to distal renal tubule ratio). The absorption of water and ions occurs mostly at the proximal convoluted tubule than in the distal convoluted tubule (Zhuo and Li, 2013). Furthermore, in freshwater fish, higher demand is placed on the kidneys to maintain stable concentrations of blood salts. The ability of the kidney to regulate ion balance is more important in freshwater fish than saltwater fish (Hasan *et al*., 2017).There are studies reported that some species of saltwater fishes lack distal convoluted tubules (Reimschuessel, 2001). A study of the effect of MSG in rat kidneys (Contini *et al*. 2017) indicated that the addition of MSG to the diet decreases the excretion of Na^+, K^+ and water with glomerular hyperfiltration which the NaCl retention was accompanied by histopathological alterations of the kidneys. However, the kidneys of vertebrate animals including those of zebrafish possess a remarkable potential to regenerate after an injury or nephrotoxic insult (Diep *et al*., 2011). Thus, this repair mechanisms may lead to the formation of new proximal convoluted tubules after MSG-induced injury to the nephrons.

Similar to our results, the effect of MSG on the testis and ovary of rodents was reported. Atrophy of seminiferous tubules and loss of spermatocyte and spermatid along with exfoliation of germ cells of male rats were found after exposure to MSG through intraperitoneal injection for 14 days (Nosseir *et al*., 2012 and Mondal *et al*,. 2018) as well as experiments on rats receiving MSG at 30 and 60 g/kg body weight for two months (Alalwani, 2014). Also Eweka and Om'iniabohs (2011) reported degenerated ovaries together with damage to the basement membrane, the theca folliculi and the zona granulosa were separated and atrophic changes of the oocytes in female rats after treated with MSG at 0.08 mg/kg body weight for 14 days.

Our study demonstrated that the survival rate of embryos tended to decrease after exposure to high concentrations of MSG. The survival analysis showed that the 100 mg/l MSG-exposed group had significantly lower survival rate of embryos when compared to the control group. Even though the

number of spawning embryos of each MSG-exposed group was statistically different, the lesions of testis and ovary of the MSG-exposed groups and the control group were not different.

MSG-induced expression of the brain aromatase gene which affects estrogen release was reported in zebrafish (Abdelkader *et* al., 2012). In addition, Iamsaard *et al.* (2014) reported a decrease in plasma testosterone levels in male rats after receive MSG at 6 g/kg body weight for 30 days. Similarly, Okoye *et al.* (2016) demonstrated that male rabbit had lower level of testosterone in serum after administration of MSG at 1 g/kg body weight for 56 days. At present, there is no detailed study on the mechanism of action of MSG-induced histopathological lesions in the reproductive organs of male and female zebrafish. The mechanism of action of MSG may involve other pathways beyond histopathological abnormality. The negative impact of MSG on reproduction may involve inhibitory actions on the release of the gonadal hormones from the brain. Further study in zebrafish is needed to explain this issue.

In zebrafish, the sexual differentiation from female to male or development of immature into mature female occurs at around 30 dph. The sex ratio of male and female zebrafish was 1:1 after exposure to MSG for 60 days in MSG-treated groups. Thus, the decrease in spawning numbers of embryos after exposure to MSG compared to the control group was not due to a change in sex ratio.

In conclusion, the results from this study clearly demonstrated that chronic exposure to MSG during the developmental stages from 30 dph to 90 dph induced histopathological changes in the kidneys and liver of zebrafish. Furthermore, the reproductive function and the survival of zebrafish offspring were decreased in a concentration-dependent manner, although there were no observable lesion in the brain, testis and ovary.

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