THE EFFECT OF MONOSODIUM GLUTAMATE ON BRAIN, LIVER, KIDNEY, TESTIS, OVARY, FERTILIZATION AND HATCHING RATE IN ZEBRAFISH (*DANIO RERIO*)

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ผลของโมโนโซเดียมกลูตาเมทที่มีต่อสมอง ตับ ไต อัณฑะ รังไข่ อัตราการปฏิสนธิ และอัตราการฟักในปลาม้าลาย (*DANIO RERIO*)

น.ส.ช่อทิพย์ สัจจาวิริยะ

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ช่อทิพย์ สัจจาวิริยะ : ผลของโมโนโซเดียมกลูตาเมทที่มีต่อสมอง ตับ ไต อัณฑะ รังไข่ อัตราการปฏิสนธิ และอัตราการฟักในปลาม้าลาย (*DANIO RERIO*). (
THE EFFECT OF MONOSODIUM GLUTAMATE ON BRAIN, LIVER, KIDNEY, TESTIS, OVARY, FERTILIZATION AND HATCHING RATE IN ZEBRAFISH (*DANIO RERIO*)) อ.ที่ปรึกษาหลัก : รศ. สพ.ญ. ดร.อรัญญา พลพรพิสิฐ, อ.ที่ปรึกษาร่วม : รศ. น.สพ. ดร.นพดล พิฬารัตน์,ผศ. สพ.ญ. ดร. เนาวรัตน์ สุธัมนาถพงษ์

โมโนโซเดียมกลูตาเมทเป็นสารประกอบเกลือโซเดียมของกรดกลูตามิกที่เป็นที่นิยมใช้เพื่อเพิ่มรสชาติอาหาร แม้ว่าจะมีความปลอดภัยในการบริโภคแต่พบว่ามีรายงานถึงผลเสียจากการได้รับผงชูรส การศึกษาทดลองนี้มีจุดประสงค์เพื่อศึกษาผลของโมโนโซเดียมกลูตาเมทที่มีต่อสมอง ตับ ไต อัณฑะ รังไข่ของปลาม้าลาย (*Danio rerio*) ที่ได้รับสัมผัสสารตั้งแต่ระยะตัวอ่อนไปจนถึงระยะโตเต็มวัย รวมทั้งผลต่ออัตราการปฏิสนธิและอัตราการฟักของไข่ปลาม้าลาย โดยการศึกษาลักษณะทางจุลพยาธิวิทยาที่เปลี่ยนแปลงไปของอวัยวะต่าง ๆ ของปลาม้าลายหลังจากสัมผัสกับผงชูรสที่มีความเข้มข้น 10, 100 และ 1000 พีพีเอ็มเป็นระยะเวลา 60 วัน เปรียบเทียบกับกลุ่มควบคุม ปลาม้าลายในแต่ละกลุ่มถูกนำมาผสมก่อนศึกษาความเปลี่ยนแปลงของเนื้อเยื่อในอวัยวะต่าง ๆ ผลการศึกษาพบว่า เนื้อเยื่อตับและไตในกลุ่มที่ได้รับสัมผัสกับผงชูรสเกิดรอยโรคที่มากขึ้นอย่างมีนัยสำคัญทางสถิติ (p-value < 0.05) เมื่อเทียบกับกลุ่มควบคุม โดยเซลล์ตับบวมขยายใหญ่และมีการคั่งเลือดเกิดขึ้น เช่นเดียวกันกับไตมีการคั่งเลือดและพบว่าท่อไตส่วนท้ายมีจำนวนลดลงในกลุ่มที่สัมผัสกับผงชูรส โดยความรุนแรงของรอยโรคในกลุ่มที่สัมผัสผงชูรสที่ความเข้มข้น 1000 พีพีเอ็ม มีความรุนแรงมากที่สุด รองลงมาคือกลุ่มที่สัมผัสกับผงชูรสที่ความเข้มข้น 100 และ 10 พีพีเอ็ม อย่างไรก็ดีในเนื้อเยื่อสมอง อัณฑะและรังไข่ไม่พบการเปลี่ยนแปลงที่แตกต่างจากกลุ่มควบคุม การศึกษาผลของผงชูรสต่ออัตราการปฏิสนธิและอัตราการฟักของไข่ปลาม้าลาย พบว่าเมื่อนำพ่อแม่ปลาม้าลายจากกลุ่มที่สัมผัสกับผงชูรสที่มีความเข้มข้น 1000 พีพีเอ็มมาทำการวางไข่ในตู้วางไข่ไม่พบว่ามีไข่จากพ่อแม่พันธุ์ในกลุ่มนี้ ส่วนในกลุ่มที่สัมผัสกับผงชูรสที่มีความเข้มข้น 100 พีพีเอ็ม มีจำนวนไข่น้อยกว่ากลุ่มที่สัมผัสกับผงชูรส 10 พีพีเอ็มและกลุ่มควบคุม เมื่อทำการสังเกตอัตราการรอดของไข่ปลาม้าลายที่ได้รับการผสมทุก 24 ชั่วโมงเป็นเวลา 120 ชั่วโมง พบว่าอัตรารอดในกลุ่มที่ได้รับสัมผัสกับผงชูรส 100 พีพีเอ็มที่อายุ 72 ชั่วโมงหลังการปฏิสนธิมีอัตรารอดประมาณ 25% ซึ่งน้อยกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ โดยไข่ของปลาม้าลายจากทุกกลุ่มที่รอดจนถึงชั่วโมงที่ 72 หลังการผสมสามารถฟักเป็นตัวได้ทั้งหมด ผลการศึกษาครั้งนี้สรุปได้ว่า ปลาม้าลายที่สัมผัสกับผงชูรสที่มีความเข้มข้น 10, 100 และ 1000 พีพีเอ็ม เป็นระยะเวลานาน 60 วัน มีรอยโรคที่ตับและไต และมีประสิทธิภาพของการสืบพันธุ์ลดลง

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Chortip Sajjaviriya :

THE EFFECT OF MONOSODIUM GLUTAMATE ON BRAIN, LIVER, KIDNEY, TESTIS, OVARY, FERTIL IZATION AND HATCHING RATE IN ZEBRAFISH (*DANIO RERIO*) . Advisor: Assoc. Prof. ARANYA PONPORNPISIT, D.V.M., Ph.D., D.T.B.V.M. Co-advisor: Assoc. Prof. NAPADON PIRARAT, D.V.M., Ph.D., D.T.B.V.P.,Asst. Prof. NAOWARAT SUTHAMNATPONG, D.V.M., Ph.D., D.T.B.V.M.

Monosodium glutamate (MSG) is composed of sodium salt and amino acids that is commonly used to enhance food taste. Although it is safe at consumption level, the adverse effect of MSG had been reported. The present research was intended to evaluate the effect of MSG on the brain, liver, kidney, testes, and ovaries of zebrafish (*Danio rerio*) which were exposed from embryo stage to adult stage, along with the analyzing of the fertilization rate and hatching rate of zebrafish embryos. From the observation of histopathological changing of different organs of zebrafish after exposed to 10, 100, and 1000 ppm MSG for 60 days compared with control group (zebrafish in each group were mating before sacrificed for study the histopathology. In the next day embryos of zebrafish were collected and analyzed for the numbers, survival rate and hatching rate). The results indicated that liver and kidney in MSG exposed group had significantly higher lesion score (*p-value* <0.05) compared to the control group. Generalized swelling of hepatocytes and blood congestion in the liver as well as the congestion and decreasing number of distal tubule in the kidney from all of the MSG exposed groups had been found. The severity of the lesions from 1000 ppm MSG exposure group was the highest following by the group of 100 and 10 ppm. However, no detectable lesions in the brain tissue, testes, and ovaries of every group were found. Zebrafish from the 1000 ppm MSG exposed group did not spawn after placing in the mating tank, while in 100 ppm MSG group was lower in the number of embryos than 10 ppm MSG and control group. The survival of fertilized embryos was observed every 24 hours for 120 hours. The result of survival rate in 100 ppm MSG group was significantly lower than the control group. All of zebrafish embryos that can be developed until 96 hours post fertilization were hatching completely. In summary, this study revealed that zebrafish exposed to MSG for 60 days showed liver and kidney lesions and reduced the reproductive performance.

Field of Study:	Veterinary Medicine	Student's Signature .....
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Chortip Sajjaviriya

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CHAPTER I

INTRODUCTION

Importance and Rationale

Monosodium glutamate (MSG) is a widely-used food additive and flavor enhancer in the food industry and household kitchen. The U.S. FDA has classified MSG as generally recognized as safe for consumption like other seasonings such as salt, pepper, and sugar (FDA, 2014). Even though is safe to be used, many studies show the adverse effects of MSG to the brain, liver, kidney and reproductive organs in rodent changes in histopathology and function were noted (Vinodini et al., 2008; Alao et al., 2010; Kumbhare et al., 2015; Sharma, 2015). Moreover, from the survey of Asians in rural Thailand shows high amount of MSG intake of 4.0 $\pm$ 2.2 g/day (Insawang et al., 2012). This is higher than in Vietnam (2.20 ± 1.80 g/day) (Thu Hien et al., 2013) and China (2.20 ± 1.60 g/day) (He et al., 2011). People who consume MSG were significantly risk of weight gain and metabolic syndrome consist of cardiovascular disease, abdominal obesity and insulin resistant (He et al., 2008; Chinna and Karupaiah, 2013).

Besides the experiment on rats, zebrafish is one of the popular animal models for toxicity study (Kinth et al., 2013) because of its high sensitivity to drug or compound, developmental change from embryonic stage to adulthood can be monitored easily and cost-effectively for husbandry (Hill et al., 2005). MSG over 100 mg/L exposed to zebrafish embryo showed teratogenicity in growth retardation, shrinkage of chorion, yolk sac and cardiac sac edema, lack of pigmentation, tail deformities and scoliosis (Abdelkader et al., 2012; Mahaliyana et al., 2016). The

median lethal concentration (LC50) of zebrafish embryo at 48 hpf is 1.525%w/v and at 96 hpf is 1.039%w/v (Ponpornpisit and Suthamnatpong, 2016). The neuronal effect of MSG was induced brain cell apoptosis at 1-day postfertilization of zebrafish embryo exposed to 10 μg/mL MSG for 24 - 72 hours which was associated with behavioral changes in decreased locomotor activities (Kurnianingsih et al., 2016). Furthermore, in recently study was reported that 15, 150 and 1500 ppm MSG was significantly high heartbeat of zebrafish embryo after 48 hpf (Suthamnatpong and Ponpornpisit, 2017).

All previous studies of MSG effect were done using early life stage of zebrafish but in larval to adult stages of zebrafish have not been evaluated and consequently. This study aims to investigate the effect of MSG on histopathological change of brain, liver, kidney and reproductive tissues in larval to adult stages as well as the related effect in reproduction of zebrafish.

Objectives of Study

1. To study the effect of monosodium glutamate on histopathological alteration of brain, liver, kidney, testis and ovary of zebrafish.
2. To study the related effect of monosodium glutamate on zebrafish life cycle.

Keywords (Thai): จุลพยาธิวิทยา โมโนโซเดียมกลูตาเมท ปลาม้าลาย

Keywords (English): histopathology, monosodium glutamate, zebrafish

Research question

1. What is the histopathological changes of brain, liver, kidney, testis and ovary of zebrafish after chronic exposure with monosodium glutamate?
2. Does monosodium glutamate have significant adverse effects on the reproduction of zebrafish?

Hypothesis

Chronic exposure to monosodium glutamate induces adverse effects on functional organs and reproduction of zebrafish.

Advantages of Study

The effect of the chronic exposure of monosodium glutamate on zebrafish functional test and life cycle test can be determined.

CHAPTER II

LITERATURE REVIEW

2.1 Zebrafish (*Danio rerio*)

2.1.1 Taxonomy

Phylum	Chordata
Class	Actinopterygii
Order	Cypriniformes
Family	Cyprinidae
Subfamily	Danioninae
Genus	*Danio*
Species	*D. rerio*

2.1.2 General feature

Zebrafish is a small freshwater fish. Its origin is in India and Myanmar (Engeszer et al., 2007; Arunachalam et al., 2013). The approximate weight of adult male and female fish is 0.5 $\pm$ 0.1 g and 0.65 $\pm$ 0.13 g, respectively. Female usually has the protruding belly for carrying eggs. A single mature female spawn at least 50 – 80 eggs per day. The size of zebrafish egg is around 0.8 – 1.5 mm. The outer side was enveloped by stable chorion, highly transparent and non-sticky. Male zebrafish is slenderer and has orange tint between blue longitudinal stripes which particularly evident at anal fin. Zebrafish embryo develop rapidly within one-hour post fertilization to cleavage stage then the organ and formation well develop within 24 hpf until hatching at 72 – 96 hpf which is becoming as a larvae stage then turn to be adult around 90 days post hatch (dph) (Kimmel et al., 1995).

Male Female

Figure 1 Male and female zebrafish

Table 1 Zebrafish stage

Age	Stage
0 – 72 hpf	Embryos
72 hpf – 13 dpf	Early larvae
14 – 29 dpf	Midstage larvae
30 dpf – 3 months	Juvenile
>3 months	Adult

(Wilson, 2012)

2.1.3 Food and feeding

Zebrafish is an omnivorous. The natural diet of zebrafish consists of zooplankton, phytoplankton, insects and algae. Zebrafish lead food through buccal cavity which has many taste bud then the food was led into the esophagus and pharyngeal pad and teeth for masticating. The first tooth bud of zebrafish starts around 48 hpf and completed of zebrafish pharyngeal dentition consists of three rows of teeth. Facial nerve (VII), glossopharyngeal nerve (IX) and vagus nerve (X) enters the branchial arch which receives sensory information from pharyngeal and gill area (Crucke et al., 2015).

Zebrafish larvae from the starting of fertilization until 72 hpf absorb nutrition from their yolk. Around 5 dpf, the fish digestive tract open and digestive enzymes start activity when they feed small size exogenous food such as paramecium or rotifer. From 14 dpf, the midlarval stage can consume artemia nauplii, bigger size zooplankton. In late larvae stage around 29 dpf to adult, combination food contains live feed, artemia nauplii, and dry flake food are usually provided for them.

For the brood fish, polyunsaturated fatty acid (PUFA) including linoleic (18:2 n-6), linolenic acid (18:2 n-3), eicosapentaenoic acid (20:5 n-3; EPA), docsahexaenoic acid (22:6 n-3; DHA), and arachidonic acid (20:4 n-6; AA) are source of energy and essential for pheromone, hormone and membrane component producing and the successful of spawning and hatching ability in zebrafish (Jaya-Ram et al., 2008). Around 30-53% Protein in zebrafish diet is required for growing up and carbohydrate is significantly needed for the zebrafish to gain in body weight (Ulloa et al., 2011).

2.1.4 Breeding and spawning

Mature male and female zebrafish are ready for mating around 3 months old, gonadal pheromone from the male fish can induce ovulation of the female. Courtship behavior appear by the male fish swim tight circle or flutter to the female then swim parallel and approach to wrap his body around female during spawning for stimulate oviposition and releasing sperm simultaneously. The technique for breeding the fish in the laboratory usually starts with select active male and female fish from the brood stock of sex ratio 1 male : 1 female or 3 male : 1 female which had no difference in the number of fertilization eggs per female (Ruhl et al., 2009). The mating crosses usually set up in the afternoon or early evening by choosing and transferring the zebrafish into a spawning tank with a crossing cage inside. In the next day morning, zebrafish spawn, and embryos drop through the mesh hole to the bottom tank which can be collected (Nasiadka, 2012).

2.1.5 Zebrafish organs development

Zebrafish embryos develop at first-hour post fertilization (hpf) and generate to blastula, gastrula, segmentation and pharyngula period around 2, 5, 10 and 24 hpf. The notable feature of pharyngula period (24 hpf) is somite, pigment in the retina and dorsolateral of skin together with tail extending from the yolk. Heartbeat and red blood cells can be observed from zebrafish embryos (Didier Y. R. Stainier et al., 1993). Normally, hatching period starts at 48 to 72 hpf.

Neurogenesis of zebrafish embryo starts at gastrulation stage around 6 to 10 hpf from the neuroectodermal epithelium. Zebrafish neurogenesis is similar to other vertebrates, but the developing time is expeditiously comparing to humans and rats. The zebrafish neurons directed by their genes to form the neuronal plate to

neuronal keel then developed to be the neural rod and neural tube. Finally, the neural tube is forming as the central nervous system including the brain, spinal cord, and nerves (Blader and Strahle, 2000; Schmidt et al., 2013).

The largest internal organ and important for metabolism is the liver. Within 22 to 24 hpf, endoderm starts to generate the liver from specific gene expression and differentiated into hepatoblast and maturing to hepatocyte until 50 hpf. After hepatocyte differentiation, the biliary system starts maturing at 3 to 5 dpf (Ober et al., 2003; Tao and Peng, 2009).

The kidney originates from mesoderm. The organogenesis is starting to form the pronephrons by pax2a and lhx1a gene expression since 12 hpf. Nephron cells and tubules continue to grow until 24 to 48 hpf. As pronephros mature, they are segmented as two nephrons with glomeruli at the midline of the embryo. The kidney is located on the dorsal wall of the body cavity and divided into head, trunk, and the tail portion (Drummond and Davidson, 2016).

The reproductive system of every individual of zebrafish begins with an ovary. At 2 weeks post fertilization (wpf) primordial germ cell develop and apparently see ovaries at 4 wpf (Maack and Segner, 2003). Testicular differentiation begins between 21 to 23 dph (Uchida et al., 2002) by the apoptosis of ovary. The zebrafish has no specific sex chromosomes like mammalian, however, sex determination is influenced by environmental factors such as temperature, pH and hormone sex reversal (Luzio et al., 2015; Ribas and Liew, 2017).

The rapid proliferative organ system which comparable to other higher vertebrates, sensitivity to the suspicious substance, easy and inexpensive to care are reasonable to use zebrafish as an animal model.

2.1.6 Histopathology of zebrafish organs

This study focuses to observe the development of zebrafish organs from larvae stage to adult which include brain, liver, kidney and reproductive organ of male and female zebrafish.

2.1.6.1 Brain

Neuron consists of cell body, axon and dendrite. The nucleus of neurons is large and round shape. The supporting cells in the brain is small size cell - glia cell, has difference type includes astrocytes, oligodendrocytes, Schwann cells and microglia. Although, glia cells have smaller size of nuclei, the number of this cells are larger than neurons (Barres, 2005; Lyons and Talbot, 2015). The brain of zebrafish is divided into five part as follow (Wullimann et al., 1996).

1. Telencephalon (Forebrain)

   The first anterior part of zebrafish brain associated with cranial nerve I or olfactory nerve. It plays an important role as chemoreception (Kasumyan, 2004). Olfactory bulbs located in the most rostral of the brain which is easily identified in this area.

2. Diencephalon

   The position of this part is between telencephalon and midbrain which can be divided into five subparts. They are the epithalamus, dorsal thalamus, ventral thalamus, posterior taberculum and the hypothalamus.

3. Mesencephalon (Midbrain)

   The mesencephalon or midbrain associated with cranial nerve II or optic nerves. The white matter of this part is the tectum opticum. The grey

zone in deeper layer is the periventricular gray zone of the optic tectum. The central of the midbrain is valvula cerebelli.

4. Metencephalon (Hind brain)

This part associated with cerebellum which controls the muscle and movement. The cerebellum locates in the median lobe of metencephalon and divides into three part includes vestibulolateralis, the corpus cerebelli and the valvular cerebelli.

5. Myelencephalon (Brain stem)

The rear part of zebrafish brain and it receives inputs from cranial nerve III to XII.

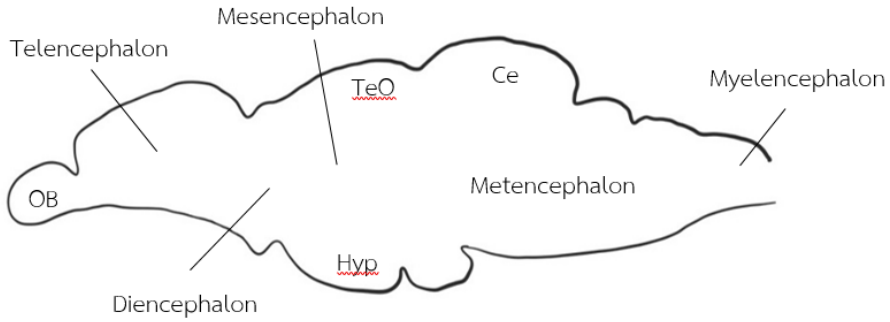


Figure 2 Normal structure of zebrafish brain

Telencephalon; Olfactory bulb (OB), Diencephalon; Hypothalamus (Hyp), Mesencephalon; Tectum opticum (TeO), Metencephalon; Cerebellum (Ce) and Myelencephalon

2.1.6.2 Liver

The liver plays an important role in metabolized nutrition, detoxification and synthesized of serum protein. It lies from esophagus to intestine and is composed of three lobes. The liver is surrounding the intestine and associated with pancreas and gallbladder. The portal vein and the hepatic artery are blood circulation of the liver. At the sinusoid, the collecting of blood is sending to central vein and flow from the hepatic vein to the heart. After that, blood flow to hepatic artery into the liver through capillary and the sinusoid. The shape of hepatocyte is polygonal-shaped cells which surround a sinusoid and form the hepatic cord. Zebrafish do not have Kupffer cells like mammalian liver, but macrophage can be seen in the liver and sometimes appear the area of macrophage accumulation with phagocytic cells containing pigment granule. Moreover, female hepatocytes are usually more basophilic than male (Wolf and Wheeler, 2018).

2.1.6.3 Kidney

Kidney plays roles to control water, electrolyte and acid-base balance in the body together with metabolic waste removal and the regulation of blood pressure. The nephron is a unit of kidney. It composed of three compartments as follow.

1. Blood filter calls ‘glomerulus’ and surrounding by Bowman’s capsule.
2. ‘The tubule’ which can secrete and/or absorb water and electrolyte and shows in proximal and distal part.
3. ‘The collecting duct’ is the final part of nephron where water reabsorption.

Higher vertebrate has three stage of kidney proceeds include pronephron, mesonephron and the last stage is the adult kidney or metanephron. This stage has a

greater number of the nephron and the arrangement is more complicated. In contrary, zebrafish kidney starts to form pronephron around 24 to 48 hpf and completely develop to be mesonephron in the adult stage (Drummond, 2000).

The kidney of zebrafish locates in the dorsal of body cavity which lies from anterior to posterior. The anterior (head) kidney contains lymphoid, hematopoietic, steroidogenic, and endocrine cells while the posterior (tail) kidney contains nephrons and lymphoid tissue. The proximal tubule difference from distal tubule by occurs eosinophilic tubules and prominent brush border. Under the microscope, the structure of the distal tubule and collecting duct are similar and difficult to determine (Menke et al., 2011).

2.1.6.4 Reproductive organ

Puberty period of adult zebrafish starts around 35 hpf in female and 45 hpf in male (Chen and Ge, 2013). Zebrafish reproductive organs in an adult stage can observe easily under the microscope.

Testes

Male reproductive organ appears as pair testis which located ventral of the gas bladder and dorsal of the liver. Spermatogenesis begins in the tubule of testes which connect with the spermatic duct. Testes of zebrafish can observe as deeply basophilic stains from lower magnification while in higher magnification can see more detail of lobule testes which separate by fibrous connective tissue. The inside of each tubule contains germinal epithelial cells as known as spermatogonia (Leal et al., 2009).

Spermatogonia are diploid cells of undifferentiated male germ cell. Their structure is pale and large cytoplasm with slightly granular nucleus. Next type of spermatogonia after mitotic division is the primary spermatocytes. These diploid cells recognize by deep basophilic nucleus and little cytoplasm. After first meiosis, secondary spermatocytes are occurring. They were divided from diploid to haploid cells which make them smaller than the primary spermatocytes with densely of nucleus but narrow cytoplasm. The second meiotic division changes secondary spermatocyte to spermatid and develops to be mature sperm which usually appears at the center of tubule. Sertoli cells are surrounding the cell cluster or spermatocyst in cytoplasm area. This cell has a similar size to spermatogonia and irregular nuclei. Leydig cells may present in the interlobular connective tissue of tubule which has round nuclei (Lacerda et al., 2014; Tzung et al., 2015).

Ovaries

The ovaries of female zebrafish are located from the ventral part of the gas bladder in the body cavity. They have two longitudinal lobes and contain varieties of oocyte stage. Oogenesis starts from primary oocyte divide and finally produce mature egg then pass into the oviduct and urogenital sinus through the urogenital pore. The difference stage of zebrafish oocyte process divided into four phases (Selman et al., 1993; Ozlem and Sema, 2007)

1. Primary growth phase

   In this first stage contains multiple nuclei and small oocytes between 0.08 to 0.16 mm diameter. The cytoplasm is strongly basophilic and can be seen cortical alveoli. the proportion between the oocyte growth phase in the

oogenesis and the volume of the ovary is increasing. The vitellogenic membrane begins to form and not thick.

2. Cortical alveolar phase

This stage oocyte appears cortical alveoli and thicker of vitelline envelope or zona radiata because of alveolin. The size of follicle and oocyte is increasing. The diameter of the oocyte in this stage is between 0.16 to 0.28 mm. The cortical alveoli contain polysaccharide and proteins and the content release when the zebrafish eggs are activated.

3. Vitellogenic phase

The oocytes are increasing in size from 0.28 to 0.74 mm in diameter. The center of oocyte present vitellus droplets or eosinophilic yolk granule which contain the yolk precursor protein and vitellogenin.

4. Mature oocyte

The diameter of oocytes in this final stage reach 0.74 to 0.76 mm. The yolk granules fill up entire cytoplasm of oocyte. the nuclear membrane breaks down which could not observe the nucleus. The vitelline envelope seems thicker because of the outer and inner zone have a gap which is easy to observe under the microscope.

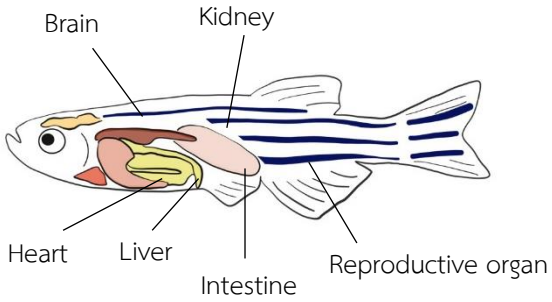


Figure 3 Zebrafish organs; Brain, Heart, Liver, Kidney, Intestine and Reproductive organs

2.2 Monosodium glutamate (MSG)

Out of the well-known taste include sweet, sour, salty and bitter, Ikeda discovered the characteristic taste called "*Umami*" implicated with monosodium glutamate (MSG) (1909). Monosodium glutamate is a non-essential amino acid consists of the sodium salt of glutamic acid. White, practically odorless crystals or crystalline powder of MSG is water soluble. Natural food such as tomato, seaweed, and parmesan cheese has a high amount of glutamate (Giacometti, 1979; Ninomiya, 1998). The process to produced MSG is fermentation by bacteria with natural source such as molasses from sugar cane or sugar beets, starch, seaweed, and corn sugar. To bring out pure monosodium glutamate crystal is precipitation and crystallization fermented broth (Li, 1965; Sano, 2009). Monosodium glutamate hydrolyzes to sodium and glutamic acid. The liver is the major organ metabolized amino acid although there is also evidence that glutamate is metabolized by the kidney (van de Poll et al., 2004). In addition, Burrin et al. (2008) reviewed that after MSG intake, glutamate acid is absorbed and metabolized at gastrointestinal tissues. Glutamate is the flavor enhancer that contact with glutamate receptors at the taste buds of the tongue and play roles as an essential neurotransmitter in human (Chaudhari et al., 2009).

2.3 Formulation of monosodium glutamate

Monosodium glutamate is a white crystalline powder that is easily soluble in water practically insoluble in ethanol or ether and odorless. Monosodium glutamate has molecular formula $C_5H_8NaNO_4 \cdot H_2O$ with the percentage of constituents contained in Monosodium glutamate including: glutamate 78.2%, Na 12.2%, H_2O 9.6%. In 1-gram Monosodium glutamate contains 0.122-gram Na. And the IUPAC name of monosodium glutamate is sodium;(4S)-4-amino-5-hydroxy-5-oxopentanoate; hydrate. The boiling point of monosodium glutamate is 225 °C and the melting point is 232 °C. The pH of monosodium glutamate is between 6.7 and 7.2 in 5 % solution (O'Neil, 2001)

Glutamate metabolism spreads widely to body tissues. Consumption of free glutamate will increase glutamate levels in blood plasma. Furthermore, glutamate in the mucosa of the small intestine will be converted to alanine and in the liver will be converted to glucose and lactate. MSG peak levels in plasma are influenced by the age of the experimental animal, the method of administration and the concentration of MSG in the solution. In newborns glutamic acid metabolism is lower than in adult animals. Giving parenteral MSG will give a different reaction with oral MSG administration because in parenteral administration, MSG does not go through the intestine and portal vein. Whereas in oral administration, MSG will go through the intestine to the portal and liver circulation. The liver has ability to metabolize glutamic acid to other metabolites. Therefore, if the administration of glutamate exceeds the capacity of the liver to metabolize, it can cause an increase in plasma glutamate (Yelamanchi et al., 2016).

Glutamate carries out several important functions in the metabolic processes in the body, including:

A. Substance for protein synthesis

It is estimated that 10-40% of glutamate is contained in protein. L-glutamic acid is an important ingredient for protein synthesis. Glutamic acid has physical and chemical characteristics that can become secondary structures of proteins called **α** chains (Yelamanchi et al., 2016).

B. Pair of transamination with **α**-ketoglutarate

L-glutamate is synthesized from ammonia and **α**-ketoglutarate in a reaction that is catalyzed by L-glutamate dehydrogenase (citric acid cycle). This reaction is important in biosynthesis of all amino acids. Absorbed glutamate is transamined with pyruvate in the form of alanine. Alanine from transamination from pyruvate, by the amino decarboxylic acid produces **α**-ketoglutarate or oxaloacetate. Glutamate which escapes from mucosal metabolism is carried through the portal vein to the liver. Some of the glutamate is converted by the intestine and liver in the form of glucose and lactate, then channeled to peripheral blood (Krebs, 1935; Choe and McGinty, 2001; Hu et al., 2010)

C. Precursor glutamine

Glutamine is formed from glutamate by glutamine synthase. This reaction is also important in the metabolism of amino acids. Ammonia will be converted to glutamine before entering the circulation. Glutamate and glutamine are links between carbon and nitrogen in the process of carbohydrate and protein metabolism (Yelamanchi et al., 2016).

D. Neurotransmitters

Glutamate is a major transmitter in the brain, functioning as a mediator to convey postnatal transmission. In addition, glutamate also functions as a precursor of the neurotransmitter Gamma Amino Butyric Acid (GABA) (Yelamanchi et al., 2016).

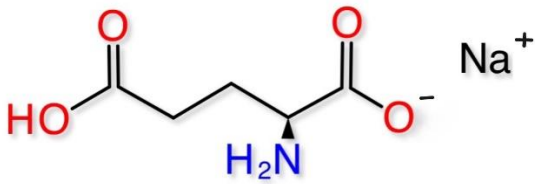


Figure 4 Monosodium glutamate structure

2.4 Effect of monosodium glutamate

U.S. Food and Drug Administration (FDA) classified MSG as substances that accept as safe like salt, pepper, vinegar and baking powder. However, the symptoms of burning, tightness, and numbness of the neck and face, occasionally accompanied by dizziness, headache, nausea, and vomiting occurring after consuming MSG were reported. These symptoms known as "Chinese restaurant syndrome" (Kwok, 1968) and many research show the adverse effect of MSG to several organ systems.

Glutamate is the major excitatory neurotransmitter in the central nervous system (Meldrum, 2000; Niciu et al., 2012). Neuronal effects of MSG on animal studies had been confirmed, for instance, male albino rats after fed with 3 g/kg/days MSG for 14 days had neurodegenerative change in the cerebellar cortex (Hashem et al., 2012). Postnatal 4 to 10 days of Sprague–Dawley rats had significantly fewer neurons in the brain after injected with 4 mg/g MSG (Foran et al., 2017).

MSG is associated with obesity and metabolic defects factor in human and animal model. Roman-Ramos et al. (2011) found levels of metabolic enzymes

include insulin, resistin and leptin were increased in mice serum which related to impaired glucose tolerance and lipid inflammatory. The report on neonate mice that received MSG for 75 days has shown histopathological change by infiltration of inflammatory cells around central vein in the liver parenchyma and the hepatocyte disruption associated with vacuolated cytoplasm and pyknotic nuclei (Bhattacharya et al., 2011).

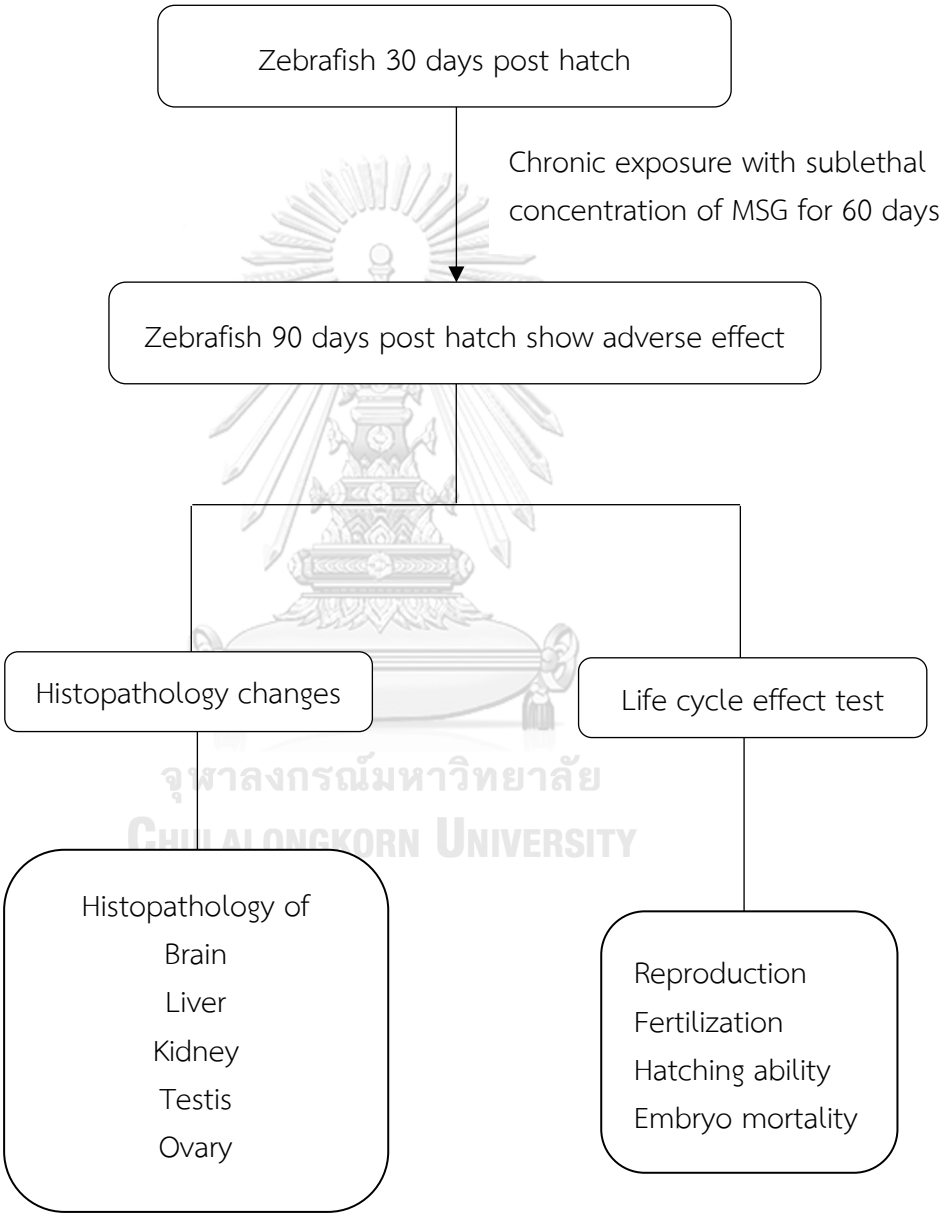
Chronic intake of MSG may induce kidney damage by oxidative stress which is caused by an accumulation of oxygen free radical in cells. From the experiment in rats focusing on the kidney, MSG was associated with urolithiasis and urinary tract obstruction from urine (Sharma et al., 2013). Both oxidative stress and alkaline urine can induce tubule-interstitial fibrosis and lead to kidney failure (Sharma et al., 2014).

The adverse effect of MSG on reproductive system was also found. Experiments in adult female Wistar rats treated with 0.08 mg/kg of MSG with the growers' mash had found cellular hypertrophy, degenerative and atrophic changes of the oocyte (Eweka and Om'iniabohs, 2011). In addition, the result after given higher dose of MSG in water found cellular hypertrophy, degenerative and atrophic changes in the ovary of Sprague-Dawley rats (Oladipo et al., 2015). Moreover, sperm count, and serum testosterone concentration was reduced, as well as abnormal testicular and sperm morphology was observed in five of six adult male rats samples (Dong and Robbins, 2015). According to the experiment of MSG feeding to Wistar-rats during pregnancy and breastfeeding had shown the lower length of the offspring at birth (von Diemen and Trindade, 2010) but have no effected to induced the body weight, serum glucose, triacylglycerides and total lipids which are the risk factors for obesity (Afifi and Abbas, 2011).

CHAPTER III

MATERIALS AND METHODS

3.1 Conceptual framework



3.2 Zebrafish husbandry and spawning procedure

Fifty males and females broodstock, wild type, long fin zebrafish were purchased from a local fish farm in Nakornpathom province. They were acclimatized in a fifty-liter PVC plastic tank at Veterinary Medical Aquatic Animal Research Center, Faculty of Veterinary Science, Chulalongkorn University, Bangkok for one month. The spawning process was done by placing five pairs of male and female zebrafish in a metal grating container overlay a five-liter plastic tank for one night. Their eggs were collected in the morning after the 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 optimum requirement with gently aeration. Half of the water tank were changed every day (OECD, 2013). The water temperature, pH, dissolved oxygen (DO), total dissolve solid (TDS), electrical conductivity (EC) and osmolarity were monitored. Zebrafish were fed twice daily with flake food (Tetramin, Germany) and *Artemia* nauplii.

3.3 Chronic exposure process

The 30 dph zebrafish larvae were randomly divided into 4 groups, in each group, 30 larvae were placed in the 1-liter plastic tank. Experimental groups consist of negative control and three different concentrations of MSG (L-glutamic acid monosodium salt hydrate, Sigma-Aldrich, USA) exposure groups. The exposure groups were added MSG in the water to be 10 ppm, 100ppm, 1000 ppm concentration, respectively. The negative control group was reared without MSG. The experiment was duplicated. All zebrafish were cared in static renewed system using carbon filter water. Fish in every tank was checked for abnormal behavior and mortality and fifty

percent of the rearing water was changed every day. Temperature, pH, dissolved oxygen (DO), total dissolved solid (TDS), electrical conductivity (EC) and osmolarity of water were monitoring during the experimental period. Live feed brine shrimp (*Artemia* nauplii) and flaked food were fed two times a day (Harper and Lawrence, 2010; Wang et al., 2011). The fish were reared in the testing solution for 60 days.

3.4 Histopathology

3.4.1 Histopathological examination

All 90 dph zebrafish of experimental groups were anesthetized in overdose solution of MS-222 (Monte and Varga, 2012; Leary et al., 2013) for ten minutes and confirmed dead by checked for fully lacking touching response then transferred the whole body to fix in 10% buffer formalin. Six zebrafish in each group were randomly selected to decalcify in 4% nitric acid and then subjected to general procedures for tissue dehydration processing using automatic tissue processor. After the process, tissue samples were embedded in paraffin wax in sagittal plane. The block of the sample was sliced into 6 µm thin section and mount onto the slide, place on a warm plate overnight, finally, stained the tissue sample by hematoxylin and eosin (H&E) (Mumford, 2004).

3.4.2 Histopathological scoring

The sections were carefully studied histopathology for the regressive changed, progressive changed and other appearing changes of brain, liver, kidney and reproductive organs (testis and ovaries) under the light microscope.

The tissue sections were studied for 5 areas per organ and scored each area by lesions. The lesion scores were categorized into 4 levels include score 0 if no

tissue changes have been detected, score 1 for mild tissue change (less than 25%), score 2 for moderate tissue change (25% - 50%) and score 3 for generalize tissue change (more than 50%) (Mumford et al., 2007; OECD, 2015).

Brain

For the brain, encephalitis, regressive changed (necrotic cells, karyolysis or karyorrhexis nuclei) and progressive changed (hyperplasia and hypertrophic cell) of neuron and microglial cell, tissue edema and hemorrhage including other specific findings were noted (El-Shobaki et al., 2016).

Liver

For liver, regressive changed and progressive changed of epithelial and supporting cells were noted. Regressive tissue change including fatty degeneration, the infiltration of white blood cell and melano-macrophage center, tissue edema and hemorrhage including other specific findings were checked (Bhattacharya et al., 2011).

Kidney

Kidney lesion of zebrafish was evaluated by the number of proximal and distal tubule of observed area and hydropic degeneration of tubular epithelium, cellular swelling along with the appearance of inflammation cells and red blood cells accumulation.

Testis

Testes of male zebrafish were observed and noted the intersex, regressive such as necrosis, atrophy and hypoplasia. Progressive change such as cellular

swelling and hypertrophic cells. The different stage of testis development was recorded.

Ovary

Zebrafish ovary was observed the difference of stage and noted the regressive and progressive change. The infiltration of inflammatory and red blood cells was recorded.

3.4.3 Sex ratio

After exposed with MSG for 60 days, all zebrafish from control, 10ppm, 100 ppm and 1000 ppm MSG exposed groups were confirmed male and female by wet mount and histopathology section under the light microscope. They were classified and compared the sex in each group between male and female then reported in the percent of sex ratio.

3.5 Effect on zebrafish embryo

Zebrafish were cultured and analyzed the survival. The day before collected embryo, 90 dph zebrafish from each group were placed in plastic tank for mating (Ruhl et al., 2009). Embryo were transferred into petridished contained carbon-filter water. Fertilized embryos were collected, and embryos survival were observed under stereo microscope at 24, 48, 72 and 96 hpf. The numbers of hatching larvae were recorded at 120 hpf.

3.6 Statistical analysis

Nonparametric statistic was used for analyzed the difference of lesion scores of zebrafish and survival analysis of zebrafish embryo. For determining the difference of histopathology lesions of brain, liver, kidney, testis, and ovary from

zebrafish of 10 ppm, 100 ppm and 1000 ppm MSG exposed groups and control group, Kruskal-Wallis test was used.

The descriptive statistics was used for analyzed sex ratio of zebrafish in every group and survival of zebrafish embryos. Then sex of each group were reported as percentages. The figures of the survival of zebrafish embryo in every 24 hours were reported as a graph in percentages of the survival of fertilized embryo until 120 hpf.

The survival and mortality rate of zebrafish embryos between the difference concentration of MSG groups and control group were compared by Kaplan-Meier survival curve and cox analysis.

The difference of mortality rate of zebrafish after exposed with MSG since 30 dph until 90 dph between the MSG exposed groups and control group were analyzed by Fisher's exact test.

The statistical analysis program was performed with SPSS ver. 22.0. Differences were considered significant when $p < 0.05$.

CHAPTER IV

RESULTS

4.1 Histopathological changes

After zebrafish exposed MSG for 60 days, histopathological examination of the brain, liver, kidney, testis, and ovary of each six fish from each group were performed. Whole body fish were fixed in neutral buffered formalin and subjected to general procedure of paraffin tissue section. The sections were stained with hematoxylin and eosin stain (H&E) then examined the histopathological lesions of the tissue under the light microscope. Histopathological lesions of each organ from each fish were scored to 4 scores include 0, 1, 2, 3 as described in materials and methods and the scores were compared between the control group and the MSG exposed groups (table 2).

4.1.1 Brain

For histopathological examination of zebrafish brain, neuron and microglial cell were observed for encephalitis (Infiltration of inflammatory cells), regressive changed (necrotic cells, karyolysis or karyorrhexis nuclei) and progressive change (hyperplasia and hypertrophic cell) including tissue edema and hemorrhage in the forebrain, midbrain, and cerebellum. From our observation, we could not find any notify lesions from the control group and all MSG exposed groups (Figure 5-6).

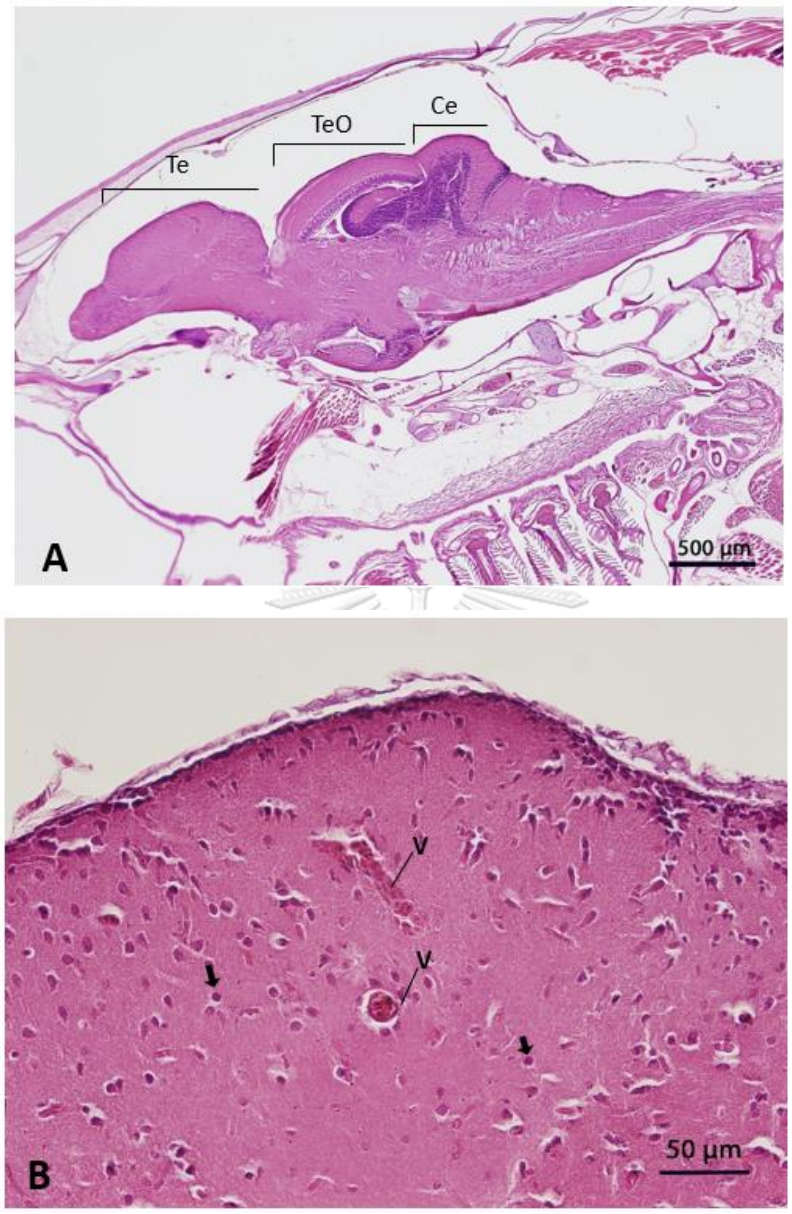


Figure 5 Histopathological finding of zebrafish brain

(A) Sagittal section of the brain of a 90 dph zebrafish from control group showed fully development of the telencephalon (Te) or forebrain, tectum-opticum (TeO) or midbrain, cerebellum (Ce) and spinal nerve (S). Normal gill tissue was noted (Gl) (H&E stain. 4X).

(B) Higher magnification of telencephalon presented normal neuron (arrow) and small blood vessels (V) fill with red blood cell (H&E stain. 40X).

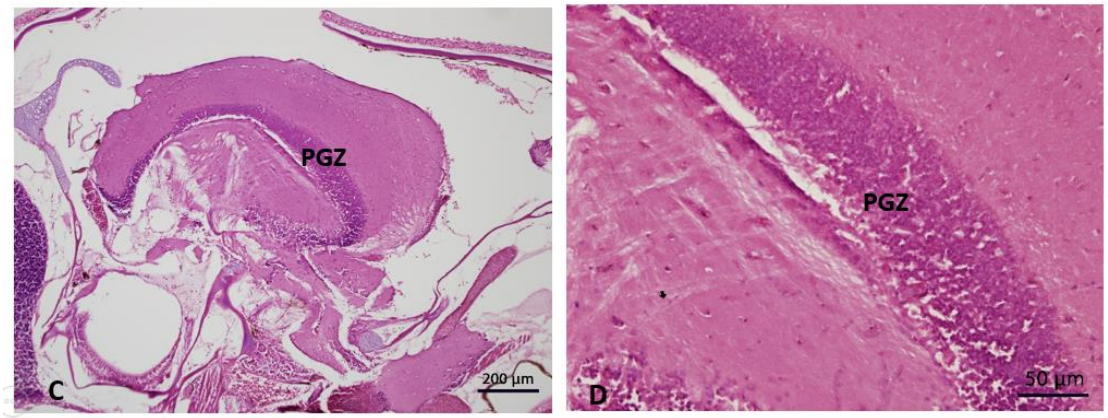


Figure 6 Histopathological finding of zebrafish brain in 1000 ppm MSG exposure group

(C) Tectum opticum of adult zebrafish from 1000 ppm MSG exposed group presented periventricular gray zone (PGZ) (H&E stain. 10X)

(D) A normal appearance of higher magnification of tectum opticum from figure C presented (PGZ) periventricular gray zone and neurons (arrow) (H&E stain. 40X).

4.1.2 Liver

For histopathological examination of zebrafish liver, fatty degeneration, the infiltration of white blood cells and melano-macrophage center, tissue edema and hemorrhage including other specific findings were checked but only degenerative change of the liver was detected in all experimental groups (Figure 7). Remarkable sinusoidal congestion and generalize ballooning degeneration of hepatocyte have been found in the highest MSG exposed group, the 1000 ppm group (Figure 7B-7D). The liver lesion score of the zebrafish exposed to 1000 ppm MSG was significantly different from the liver lesion score of the control group (p-value < 0.05) while the liver lesion score of the 10 ppm and the 100 ppm MSG exposed groups were not significantly different from the control group (Table 3).

4.1.3 Kidney

The zebrafish from all MSG exposed groups in the experiment presented remarkable degenerative change, renal congestion, and red blood cell infiltration into kidney tissue and accumulation between renal tubule (Figure 8). All of MSG exposed groups have less or absence of distal tubule compared with the control group. Kidney lesions scored of kidney congestion and the absence of distal tubule has been shown that all experimental groups (10 ppm, 100 ppm and 1000 ppm MSG exposed) were significantly different from the control group (p-value < 0.05) (Table 2).

4.1.4 Testes and ovaries

Histopathological lesions of male zebrafish have been observed for the degenerative change, necrosis and inflammatory process. Testes of zebrafish from the MSG exposed groups and the control group were looked up for the lesions and compared. All stages of spermatogenesis of the testis have been seen but the histopathological changes of all groups have not been found (Figure 9).

For the female zebrafish, sagittal sections of the ovaries have been done and observed for histopathological lesion and compared between the control group and MSG exposed groups. The result showed that the control group and all MSG exposed groups have no remarkable difference histopathological changed (Figure 10).

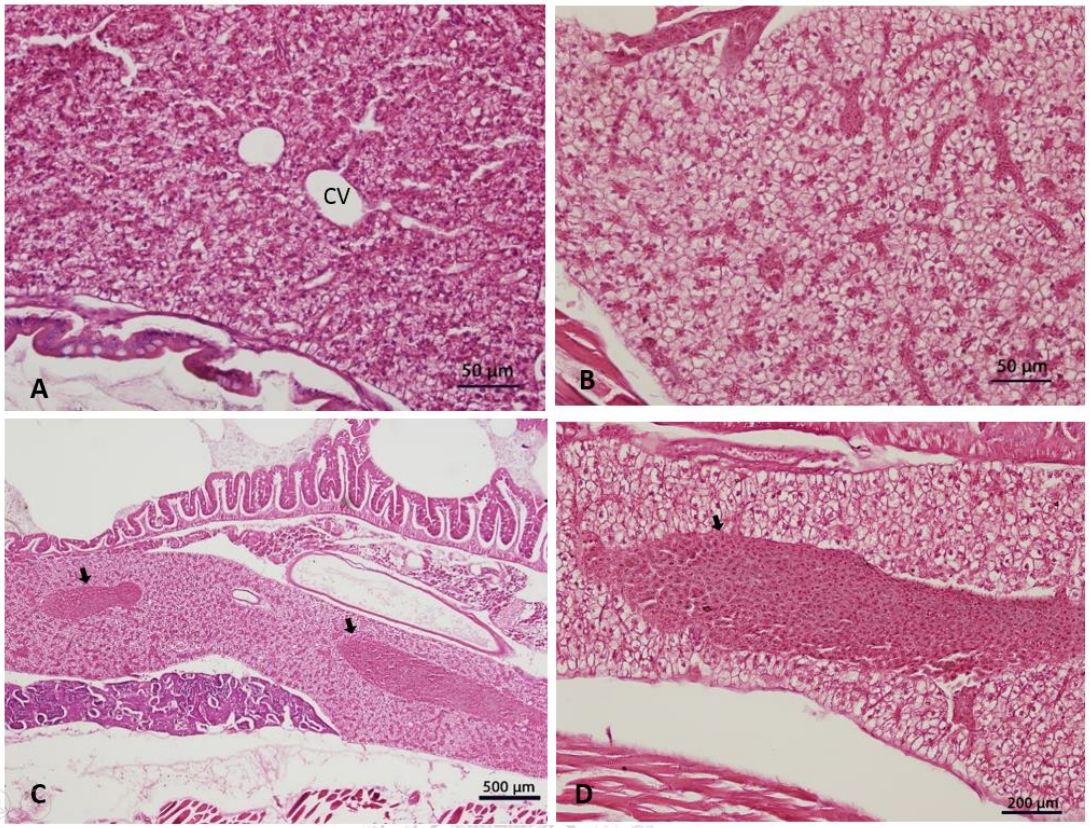


Figure 7 Histopathological finding of zebrafish liver

(A) Histopathological section of the liver of a 90 dph zebrafish from control group showed well organized hepatocyte around the central vein (CV) without red blood cell aggregation (H&E stain. 40X).

(B) Histopathological section of the liver of a 90 dph zebrafish from 1000 ppm MSG exposed groups showed generalized swelling of hepatocyte (H&E stain. 40X).

(C) Liver of the zebrafish from 1000 ppm MSG exposed groups showed hepatic vessels filled up with red blood cell. Normal gut epithelium, Intestinal pit, and white and red pulp of splenic tissue were noted (H&E stain. 4X).

(D) Higher magnification of the liver tissue section of the 1000 ppm MSG exposed groups presented hydropic degeneration of hepatocyte and blood congestion (arrow) (H&E stain. 10X).

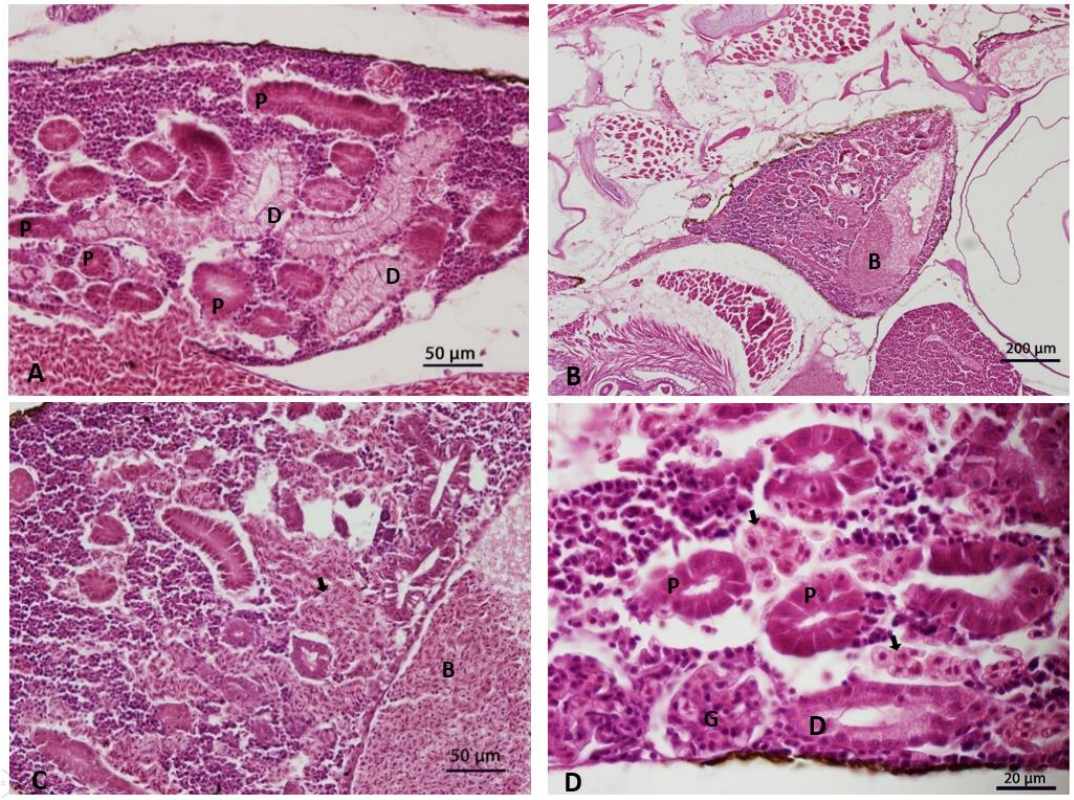


Figure 8 Histopathological finding of zebrafish kidney

(A) Histopathological section of anterior kidney of a 90 dph zebrafish from control group showed proximal (P) and distal tubule (D) without luminal debris and red blood cell infiltration (H&E stain. 40X).

(B) Histopathology overview of anterior kidney of a zebrafish exposed with 1000 ppm MSG for 60 days showed renal congestion (B) (H&E stain. 10X).

(C) Higher magnification of the figure B presented remarkable congestion (Bl) and red blood cell around the proximal tubule (arrow) (H&E stain. 40X).

(D) Sagittal section of kidney of a 90 dph zebrafish from 1000 ppm MSG exposed groups showed integrity of glomerulus (G) and proximal tubule (P) infiltrated with red blood cells around the tubule (H&E stain. 100x).

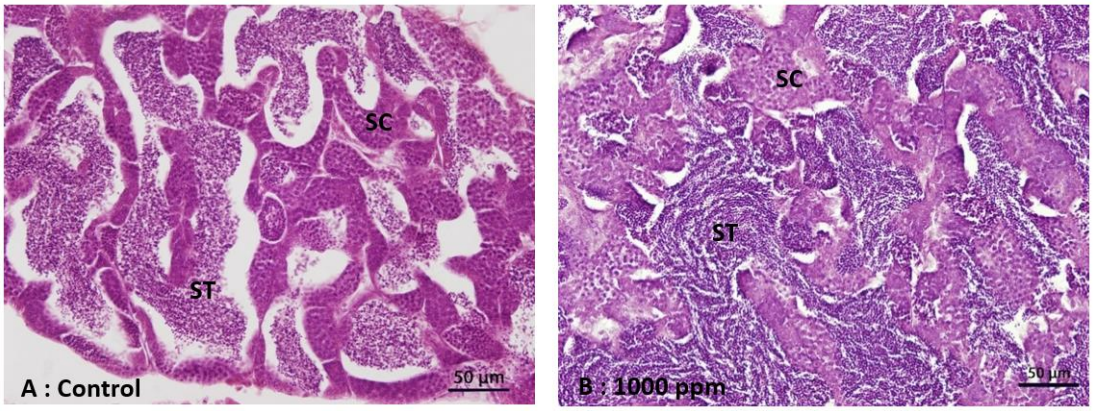


Figure 9 Histopathological finding of zebrafish testes

(A) Histopathology overview of the testis of zebrafish in the sagittal section of the control group showed normal structure of testis including spermatocyte (SC) and spermatid (ST) (H&E stain. 40X).

(B) Male zebrafish exposed with 1000 ppm MSG showed advanced development testis filled with spermatocyte (SC) and spermatid (ST) (H&E stain. 40X).

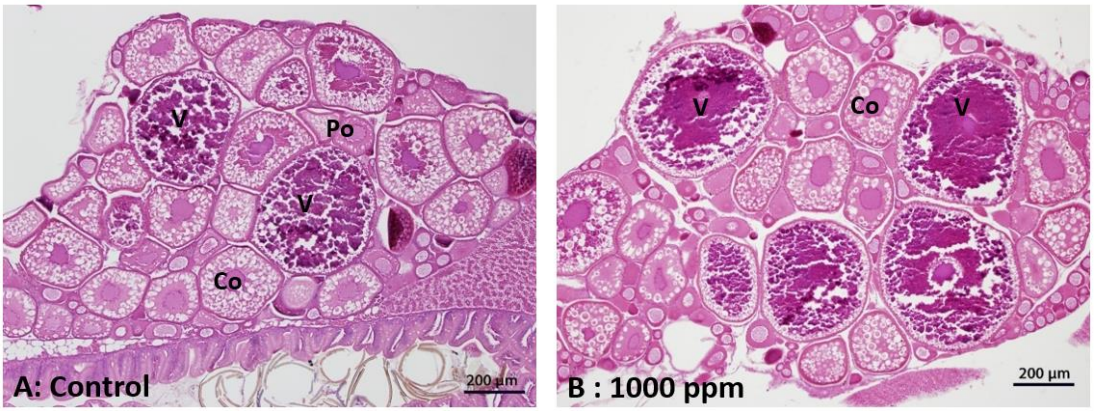


Figure 10 Histopathological finding of zebrafish ovaries

(A) Different stage of ovaries of zebrafish in sagittal section from control group showed primary oocyte (Po), cortical alveolar (Co) and vitellogenic stage (V) (H&E stain. 10X).

(B) Ovaries of zebrafish exposed with 1000 ppm MSG for 60 days showed normal structure of cortical alveolar (Co) and vitellogenic stage (V) (H&E stain. 10X).

Table 2 Histopathological lesion score of brain, liver, kidney, ovary and testis of zebrafish from control, 10ppm, 100ppm, and 1000ppm group.

	Control							
	Replication 1				Replication 2			
Sample	Brain	Liver	Kidney	Ovary and testis	Brain	Liver	Kidney	Ovary and testis
1	0	1	1	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	1	1	0	0	1	0	0
5	0	0	0	0	0	0	0	0
6	0	1	1	0	0	0	1	0

	10ppm MSG							
	Replication 1				Replication 2			
Sample	Brain	Liver	Kidney	Ovary and testis	Brain	Liver	Kidney	Ovary and testis
1	0	1	2	0	0	1	1	0
2	0	2	2	0	0	1	2	0
3	0	1	2	0	0	1	2	0
4	0	0	2	0	0	0	3	0
5	0	1	1	0	0	1	1	0
6	0	1	0	0	0	1	0	0

	100ppm MSG							
	Replication 1				Replication 2			
Sample	Brain	Liver	Kidney	Ovary and testis	Brain	Liver	Kidney	Ovary and testis
1	0	1	2	0	0	1	3	0
2	0	1	3	0	0	1	2	0
3	0	1	2	0	0	1	2	0
4	0	1	2	0	0	1	3	0
5	0	1	1	0	0	1	2	0
6	0	2	3	0	0	2	2	0

0 = Normal, 1 = Mild, 2 = Moderate, 3 = Severe

Table 2 Histopathological lesion score of brain, liver, kidney, ovary and testis of zebrafish from control, 10ppm, 100ppm, and 1000ppm group. (Cont.)

	1000ppm MSG							
	Replication 1				Replication 2			
Sample	Brain	Liver	Kidney	Ovary and testis	Brain	Liver	Kidney	Ovary and testis
1	0	2	3	0	0	2	1	0
2	0	2	3	0	0	1	2	0
3	0	1	3	0	0	2	3	0
4	0	1	2	0	0	2	2	0
5	0	2	2	0	0	2	3	0
6	0	2	1	0	0	2	2	0

0 = Normal, 1 = Mild, 2 = Moderate, 3 = Severe

Table 3 Kruskal-Wallis non-parametric test results from liver and kidney lesions score of adult zebrafish exposed with MSG.

	Liver			Kidney		
	Median	Mean±SD	P	Median	Mean±SD	P
Control (N=12)	0.5	0.50±0.522	-	0.00	0.33±0.492	-
10 ppm MSG	1	0.92±0.515	0.128	2	1.5±0.905	0.003*
100 ppm MSG	1	1.08±0.669	0.060	2	2.25±0.622	<0.001*
1000 ppm MSG	2	1.75±0.452	<0.001*	2	2.25±0.754	<0.001*

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

4.2 Sex ratio

All 30-mortal fish of each group were classified sex from wet mount and histopathology section. Zebrafish in the control group was 48% female while in 10 ppm 100 ppm and 1000 ppm MSG was 41%, 48%, and 38%, respectively. Male was 52% of the control group, 59% of 10 ppm MSG, 52% of 100 ppm MSG, and 62% of 1000 ppm MSG. In each group male and female ratio was approximately 1:1.

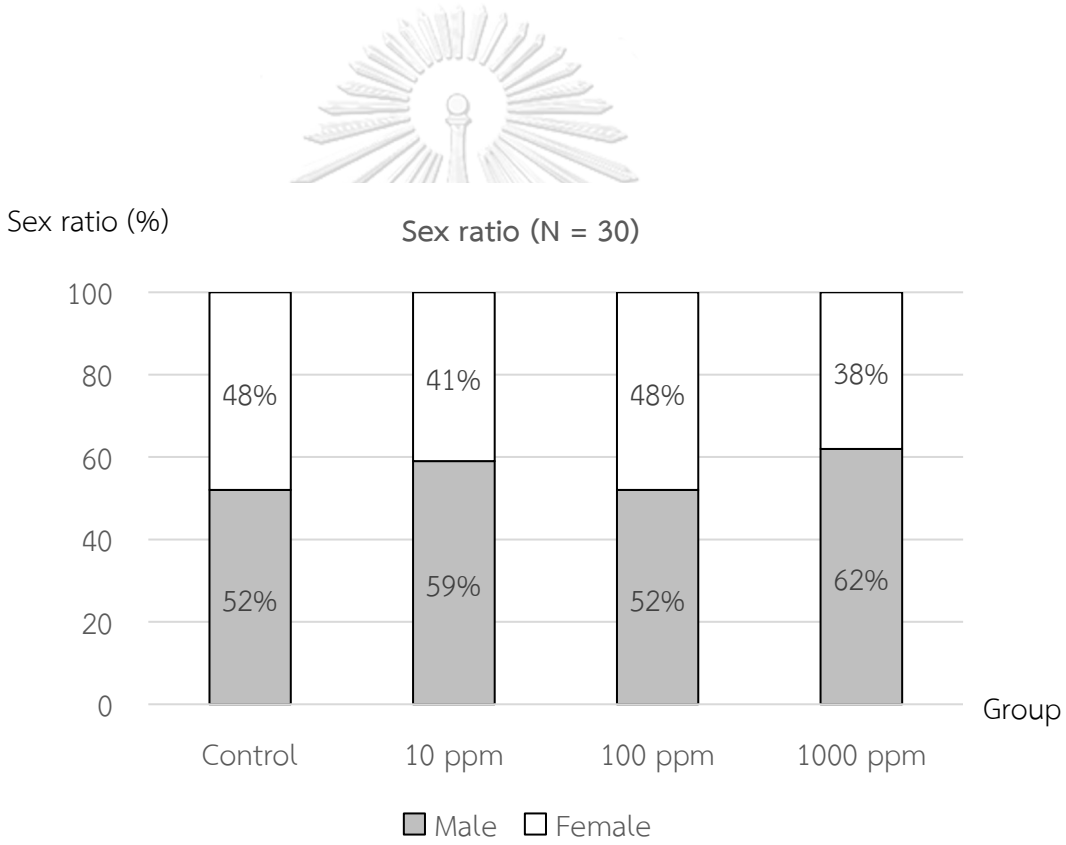


Figure 11 Sex ratio of zebrafish exposed with MSG from 30 dph until 90 dph with MSG concentrations of 0, 10, 100, and 1000 ppm.

4.3 The effect on reproduction

Male and female zebrafish of each group were mated before sacrificed and collected embryo in the next day. For the fertilized embryos result It has been found that the number of fertilized embryos were decreased in concentration dependent response as following, control group (408 embryos), 10 ppm MSG group (291 embryos), 100 ppm MSG group (36 embryos) and 1000 ppm MSG group had no embryo spawning (Table 4). For the embryo survival observation every 24 hours until 120 hpf, the decrease in the survival number according to the concentration dependent response also has been found (Table 4 and figure 12).

From Cox univariate analysis, the risk of mortality from 100 ppm MSG exposure was 24.56 times of control (Hazard ratio: 24.56, 95% CI: 15.00 - 40.22; $p < 0.05$). Figure 13 showed the decrease of survival rate of zebrafish embryo from control, 10 ppm and 100 ppm MSG group during 0 hpf to 120 hpf which zebrafish embryo survival rate between 100 ppm MSG group and the control group was significant difference *p*-value < 0.05.

Table 4 Number of survival embryos

	0 hpf	**24 hpf**	**48 hpf**	**72 hpf**	**96 hpf**	**120 hpf**
Control	408 (100%)	382 (93.63%)	379 (92.89%)	379 (92.89%)	379 (92.89%)	379 (92.89%)
10 ppm MSG	291 (100%)	250 (85.91%)	249 (85.57%)	249 (85.57%)	249 (85.57%)	249 (85.57%)
100 ppm MSG	36 (100%)	30 (83.33%)	13 (36.11%)	9 (25.00%)	9 (25.00%)	9 (25.00%)
1000 ppm MSG	0	NA	NA	NA	NA	NA

NA = Not Available

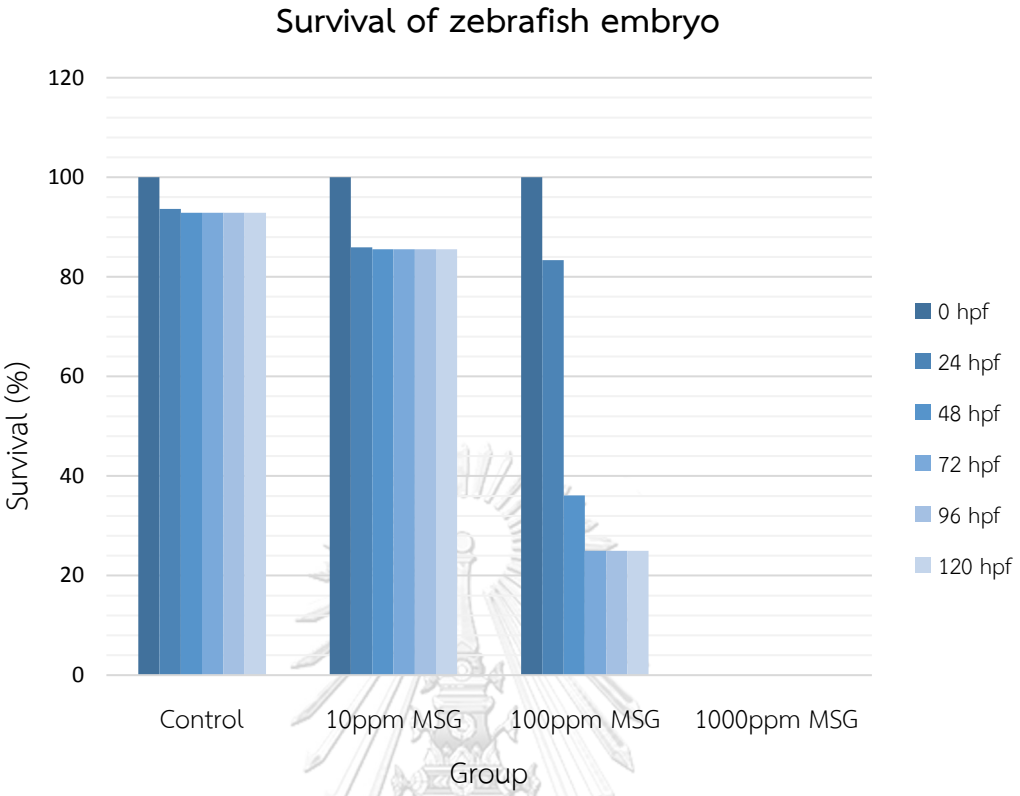
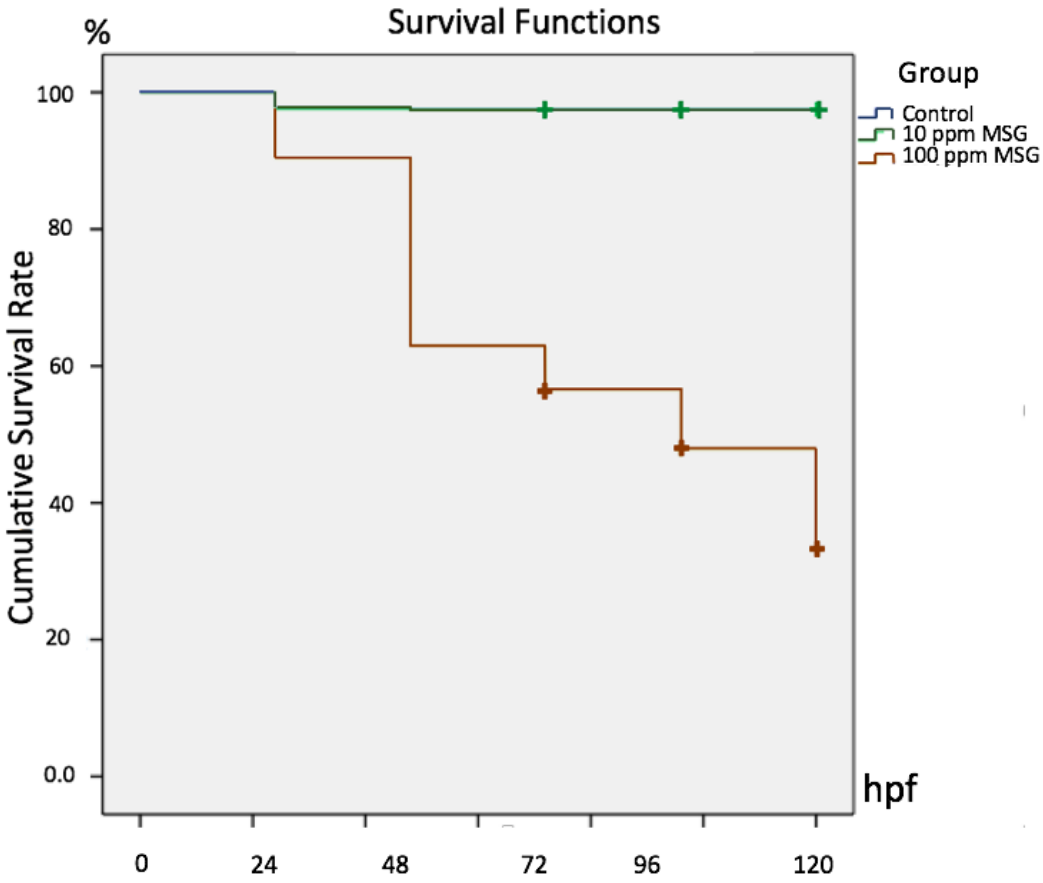


Figure 12 Percentage of zebrafish embryo survival until hatching from 0 to 120 hpf in control, 10ppm, 100 ppm, and 1000 ppm MSG exposure.



Cox regression	
95% CI	15.00 – 40.22
Hazard ratio	24.565
p-value	<0.000
Log rank	
Chi-square	467.443

Figure 13 Survival rate and time of zebrafish embryo

Survival rate of zebrafish embryo from control, 10 ppm and 100 ppm MSG group every 24 hours until 120 hpf by Kaplan-Meier survival analysis with significant of p-value < 0.05. Stair-steps show time of mortality. Cox regression analysis showed the risk of mortality between difference concentration of MSG group.

4.4 Other results

Beside the histopathology and reproductive effects, mortality rate of the zebrafish at 90 dpf and water quality was monitored regularly during the test period. Fisher's exact analysis test of mortality rate in every experimental group was not significantly difference from control group (table 5).

Table 5 Mortality rate of zebrafish at 90 dph by fisher's exact test

MSG concentration (ppm)	Mortality rate (%)	
	Replication 1 (n = 30)	Replication 2 (n = 30)
Control	33%	46%
10	43%	40%
100	30%	36%
1000	40%	46%

An average of water temperature in the control group was 27.4+0.7 °C, pH was about 7.51+0.06, dissolve oxygen (DO) was 3.42+0.75 mg/L, total dissolved solids (TDS) was 187.75ppm and electrical conductivity (EC) was 375.25 µS. Whereas after added difference concentration of MSG in the water, other three experimental group were monitored water parameter as the control group. An average water temperature in 10ppm, 100 ppm, and 1000 ppm MSG group were 27.6+0.5 °C, 27.43+0.67 °C, and 27.45+0.65 °C respectively. pH was about 7.41+0.11, 7.48+0.11, and 7.51+0.03. For dissolve oxygen (DO) was 3.77+0.16 mg/L, 3.44+0.75 mg/L and 3.73+0.23 mg/L. Total dissolved solids (TDS) was 193ppm, 215.75ppm, and 351.5 ppm. For an electrical conductivity (EC) was 387µS, 434.5µS, and 706.75µS. (Table 6).

Table 6 Water quality of the experimental groups during the test period

	Control	10 ppm MSG	100 ppm MSG	1000 ppm MSG
Water temperature (°C)	27.4±0.7	27.6±0.5	27.43±0.67	27.45±0.65
pH	7.51±0.061	7.41±0.11	7.48±0.11	7.51±0.03
DO (mg/L)	4.95±0.09	3.88±0.06	4.63±0.18	3.89±0.25
TDS (ppm)	187.75	191.75	215.75	351.5
EC (µS)	375.25	385.5	434.5	706.75
Osmolarity (mOsm/kg H_2O)	3.33	3.33	4.33	16

CHAPTER V

DISCUSSION

5.1 Histopathological changes

MSG, at different concentration were tested in zebrafish from larvae stage to adult stage. Then the brain, liver, kidney, testis and ovary were observed by histopathological study. The histopathological results of the liver in all MSG exposed groups showed the swelling of hepatocyte and blood congestion. The swelling and congestion lesions were similar to the lesion of the liver and the kidney of rats after intraperitoneal injection with MSG (Ortiz et al., 2006). Same histopathology change was also found in Wistar rat treated with MSG concentration 1.6 mg/g body weight by appearance of the congestion in the central vein of the liver and also occurred hepatocyte degeneration (Shrestha et al., 2018). Because of the glutamate receptor in the portal blood thus it may contribute to the swelling of liver cells which caused by changing in ion concentrations and water influx into the hepatocyte (Gill and Pulido, 2001; Wallig and Janovitz, 2013).

Blood congestion was found in kidney of zebrafish from all MSG exposed groups. Similar to Anil et al. (2015) reported the congestion in kidney vascular of adult Wistar rats after given MSG 6 mg/g body weight for 45 days. Intriguing of the proximal and distal tubule ratio in 1000 ppm MSG group appeared the number of proximal tubules were higher than distal tubule.

Proximal tubule function is absorbing water and ions and the absorption of ions is higher than distal tubule (Zhuo and Li, 2013).The ion regulation ability of the kidney in freshwater fish is more important than saltwater fish in ion regulation (M.

Mehedi Hasan et al., 2017) which some species of saltwater fishes lack of distal tubule (Hickman and Trump, 1969; Reimschuessel, 2001).

Contini et al. (2017) founded that MSG induced rat kidney showed histopathological alterations and decreases the excretion of Na, K and water from glomerular hyperfiltration. However, zebrafish kidney has ability to regenerate neonephron and restorage of the proximal tubule after its injury and damage (Diep et al., 2011) thus, proximal tubules were shown up after suspected that MSG damaged to nephrons and chronic MSG exposure may lead to adaptation in kidney structures.

Blood congestion in liver and kidney was indicated to the alteration of vessel, according to Suthamnatpong and Ponpornpisit (2017) found that MSG induced tachycardia, pericardial edema and coagulation in zebrafish larvae, thus, might ascribe that MSG effect to cardiovascular system which induce blood congestion in liver and kidney of adult zebrafish after chronic exposure.

Moreover, MSG contain sodium ion, thus it may associate with the congestion of zebrafish (Flores-Lopes and Thomaz, 2011). Kidney, liver and gill are the major organs that contact with environmental substances especially toxicity substances (Maetz and Garciaromeu, 1964). Same as the histology changes in gills, liver and kidney from Tilapia fish which in all of these organs showed the blood congestion after exposed with aluminum (Hadi and Alwan, 2012).

Histopathological study of the brain of zebrafish from 10 ppm, 100 ppm, and 1000 ppm MSG group have showed no lesions different from the control group. In contrast, from the previous study found that MSG can cause apoptosis of brain cells of zebrafish larvae (Kurnianingsih et al., 2016) and increased cell hypertrophy and

tissue edema in the region of the nucleus lateralis tuberis near pituitary gland area after 24-hours post injected MSG into the adult goldfish brain (Peter et al., 1980). However, in this study the zebrafish had long term exposed to MSG from larvae stage to adult stage, therefore regeneration of the cell may occurred as similar as several studies that have report about the regenerative responses of the telencephalon of adult zebrafish happen after injured by stabbing or given the excitotoxic by injection through the brain (Kroehne et al., 2011; Schmidt et al., 2014; Skaggs et al., 2014)

Zebrafish testis and ovaries tissue section of different MSG expose group were compared with control group and there was no histopathological lesion. Found in all groups. Although there had no study of MSG effect in term of histopathology lesion of zebrafish, the effect of MSG on testis and ovary of rodents has been reported. The atrophy of seminiferous tubules and loss of spermatocyte, spermatid along with exfoliation of germ cells of male rats were found after received MSG through intraperitoneal injection for 14 days (Nosseir et al., 2012). Another experiment on rats has been done by given MSG 30 and 60 g/kg body weight to the rat for two months, the result showed that there were testicular alteration, seminiferous tubule atrophy and sloughing of spermatocyte, spermatids and immature germ cells in the tubule (Alalwani, 2014), whereas ovaries of the female rats degenerated with many lesions included the damage of basement membrane thus the theca folliculi and the zona granulosa were separated coupled with degenerative and atrophic changes of oocyte (Eweka and Om'iniabohs, 2011) after treated with MSG in concentration of 0.08 mg/kg body weight for 14 days.

Besides the purposed organs in this study, gills of zebrafish were also observed. The gill tissue sample of 1000 ppm MSG groups showed blood congestion

in the primary lamellae (Figure 14). The alteration of the gill tissue may affect the gas exchange and other functions regarding osmoregulation, gas-exchange, acid-base regulation and nitrogenous waste excretion (Evans et al., 2014). The gill vascular damage usually found when exposed with high dose of irritant substances and the prolong exposure to the elements (Mallatt, 1985; Puntoriero et al., 2018). Therefore, the gill tissue of zebrafish that exposed to high concentration of MSG for a period of time may exhibit the lesion.

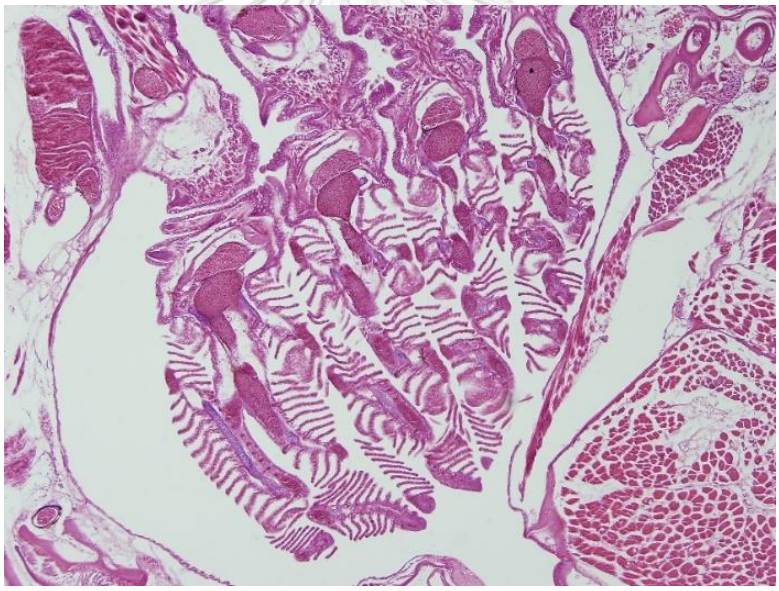


Figure 14 Histopathological finding of zebrafish gill

Blood congestion in the primary gill lamellae from 1000ppm MSG exposed group (H&E stain. 10X).

5.2 Sex ratio

About 30 dph, zebrafish were differentiated the sexual from female to male or develop into mature female. As the result after classified the sex of zebrafish from MSG experimental group and control group showed the ratio of male and female in each group about 1 male: 1 female. Thus, the sex ratio in each group was not the factor in the difference of spawning embryo numbers.

5.3 The effect on reproduction

Fertilized embryos of zebrafish from the control group and all difference concentration of MSG exposed groups were collected and observed morbidity and mortality. In the morning after transferring male and female zebrafish into the same tank, 1000 ppm MSG group did not have any spawning embryos, while in 100 ppm MSG the group was lower in the number of embryos than 10 ppm MSG and control group. From the results, the survival rate of embryos has tendency to decrease on higher MSG concentrations exposure, the survival analysis showed significant lower survival of the 100 ppm MSG group compared with the control group. Even though the number of spawning embryos in each MSG experimental group was different, there were no difference lesions of histopathological study of testis and ovary between all MSG exposed groups and the control group.

The previous study demonstrated that MSG related to brain aromatase gene of zebrafish which affect estrogen releasing (Abdelkader et al., 2012). In addition, Iamsaard et al. (2014) reported the decreased of plasma testosterone level in male rats after received 6 g/kg body weight MSG for 30 days similarly to Okoye et al. (2016) discovered the lower of testosterone levels in serum of male rabbit after received 1 g/kg body weight for 56 days. The effect of MSG on female reproductive

organ was studied in rats, Mondal et al. (2018) found the duration of proestrus, estrus and metestrus phases decreased and diestrus phase increased .They also found the level of serum LH, FSH and estradiol which secreted from anterior pituitary were increased after given MSG to virgin female rats for 30 and 40 days. Until now, there is no literature described about the histopathology of male and female zebrafish reproductive organ tested with MSG. This could imply that there might be another impairment caused by MSG beyond histopathological abnormality, but the decreasing of reproduction might cause of MSG impaired the released of gonadal hormone from the brain. Therefore, further study is needed to explore this issue in the zebrafish.

5.4 Other results

During the MSG exposed for 90 days, zebrafish was healthy, and all group did not showed any abnormalities which the survival rate of adult zebrafish in the control and every concentration group were not different. Higher dose of MSG affects the changing of water quality parameters by increasing of TDS, EC and osmolarity of water which hypothesized that these parameters were higher from MSG added (Moore et al., 2008; Lawrence and Mason, 2012). However, there were not affected to zebrafish survival rate.

Osmoregulation of freshwater fish was associated with gills and kidney and in zebrafish. Ion homeostasis was regulated by ionocytes which found in gills and skin (Hwang and Chou, 2013; Zhu et al., 2018). Kammerer et al. (2010) exposed sea water which had high water osmolarity to tilapia fish then plasma osmolarity results of this fish was increased rapidly. According to the lesion at gills and kidney, higher water osmolarity in 1000 ppm MSG group might affected to the osmoregulation control at

gills and kidney even the survival of zebrafish was not different between the control group and MSG exposed group.

From the observation of histopathological lesion in zebrafish organ development after chronic exposure of zebrafish, we concluded that MSG induced histopathological changes of kidney and liver of zebrafish. The changing of brain, testis and ovary were not presented but the reproductive function and the survival of the zebrafish offspring were decreased depending on the concentration of MSG.

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