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*Original Article*

# Effects of monosodium glutamate on heart beat and the embryonic development of zebrafish

**Naowarat Suthamnatpong[1] Aranya Ponpornpisit[2*]**


## *Abstract*

The effect of monosodium glutamate (MSG) on zebrafish, *Danio rerio*, embryo was investigated at different concentrations of ten-fold dilution of ten times lesser than the median lethal concentration ($LC_{50}$). Zebrafish embryos in the MSG treated groups (40,000 ppm and 80,000 ppm) reached highest mortality rate at 100 % within 48 hpf and 96 hpf which is significantly different ($p<0.05$) from negative control group. According to the Probit analysis, the LC50 of MSG on zebrafish embryo at 48 hpf and 96 hpf were 15,200 ppm and 10,300 ppm, respectively. Under the observation, effects based on the toxicity endpoint composed of lethal, sublethal and malformation effects appeared within 144 hpf. Exposure to MSG at 1,500 ppm and 150 ppm significantly produced sublethal effects and malformation development to zebrafish embryo. MSG induced cardiotoxic effects in zebrafish embryo was observed at 15, 150 and 1,500 ppm. Our results demonstrate that zebra fish embryo is a good model for the detection of toxic potential of chemical substances. Also, our data suggest the need to reconsider the safety of MSG and elucidate its mechanism of adverse reaction in further studies.


**Keywords:** MSG, monosodium glutamate, zebrafish, toxicity test

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## Introduction

Nowadays home cooking has declined, mostly people consume ready-to-eat or frozen food products. Almost every type of prepackaged food is likely to contain food additives to enhance flavor, color, texture and smell, or as preservatives (Kawakita, 2000). Even in the drinks such as milk and soft drinks are loaded with food additives in various quantities (Kritsunankula and Jakmuneeb, 2011). Therefore, most people cannot easily avoid eating food additives altogether since they are already added in the daily diets. Some food additives can have a negative health impact on children compared to adults and have been linked to things like obesity and attention deficit hyperactivity disorder (ADHD) (McCann et al. 2007).

MSG is one of the best-known flavor enhancer (Whitney and Rolfes 2015). It produces a unique flavor that referred to as a "sixth flavor" or sometimes described in Japanese as umami, which translates to "savory" (Kurihara, 2015). The chemical name of MSG is monosodium L-glutamate monohydrate (Yamaguchi and Kimizuka 1979). Chemical structure of MSG ($C_5H_8NNaO_4$) is similar to an important neurotransmitter of the nervous system, glutamate (Filer, 1979; Kirk, 2007) in vertebrate.

MSG is considered safe at levels normally consumed by the general population (Beyreuther et al., 2007), but some people reported unusual symptoms after consuming food with MSG such as feeling numbness around mouth and face, hot flashes, headaches, nausea, difficult breathing, sweating and other hypersensitivity reactions. These symptoms became known under the names "Chinese food syndrome" and "monosodium glutamate symptom complex" (Yang et al., 1997; Williams and Woessner, 2009; Husarova and Ostatnikova 2013). Additionally, MSG toxic effect on central nervous system, digestive system and reproductive system has been reported in chick embryo, rat, mice and numerous animal studies (Veronika and Daniela, 2013)

There are several ways to test the toxicity of chemicals such as $LC_{50}$ test, acute toxicity test, chronic toxicity test, specific life stage test, life cycle test etc. The studies using different life-stage of fish was widely use and it has been proved that the result could be predicted by the results of the early life stage test (Tamer et al., 2012) Organization for Economic Co-operation and Development (OECD) recommended using one of 3 types of fish for fish embryonic toxicity tests (FET) including: zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*) (Braunbeck and Lammer, 2006). The test is done by exposing the chemical product to the fish at embryonic stage and observe for lethal, sublethal and malformation effects (OECD, 1992; Braunbeck and Lammer, 2006; OECD, 2007; ISO, 1999)

Recently, zebrafish, *D. rerio* has become a very popular vertebrate model in toxicological studies and has contributed to the understanding of the potential toxicological and ecotoxicological impact of chemical compounds on the human and aquatic environment (Braunbeck and Lammer, 2006; Xiaoshan et al., 2007; Brown, 2016; Orn, Holbech and Norrgren, 2016) Zebrafish have also been shown to be a good model for studying drug toxicities because several known toxicants in mammalian models or humans have similar effects in zebrafish (Carlsson and Norrgren, 2004; Hill et al., 2005; MacRae and Peterson, 2015).

Zebrafish is a freshwater tropical fish of the cyprinidae family and a type of oviparous with transparent, non-adherent eggs so it is very convenient to visualize of embryonic development and this is a major aid in examination of developmental disorders (Carlsson and Norrgren, 2004). At 24 hpf the embryo has developed eyes and a tail. At 48 hpf, pigmentation can be seen on the eyes and body, the development of the heart and the body circulation can also be observed. It is possible to count the heartbeat and thereby determine the heart frequency. The embryo undergoes a fast development and the larvae hatches in 96 hpf at 26°C (Carlsson and Norrgren, 2004; Braunbeck and Lammer, 2006; Xiaoshan et al., 2007; OECD, 2007)

In this study, we examined the $LC_{50}$ of MSG on zebrafish embryo and eleuthero embryo included the effect of MSG solution emphasized on heart beat and the zebrafish embryonic development as well as the other lethal, sublethal and malformation effects to increase concerning the MSG risk effect in vertebrate.

## Materials and Methods

***Maintenance of brood fish and embryo collection:*** Brood stock were maintained in fiberglass tank, about sixty mature male and female, long fin zebrafish was kept together supplied with twenty liters carbon filtered water. They were fed with frozen tubifex worms in the morning and dried feed (TetraMin, Tetra GmbH, Germany) in the afternoon. They were cared for in the veterinary medical aquatic animal research center laboratory that allowed exposure to natural light and dark hours with the highest and lowest temperature fluctuating between 25-28°C.

One day before the embryonic toxicity test was performed, ten males and five females zebrafish were selected and transferred to a stainless steel spawning cage in a plastic aquarium contained four liters of carbon filtered water. In the next morning, all brood fish were move back to the rearing tank and the embryos were collected by pouring the water through filter sieve and rinsed with standard water. The embryo then transfer in a petri dish with the standard water to a research room for experiments (Braunbeck and Lammer, 2006, Ponpornpisit et al. 2013).

***Preparation of test solutions:*** Standard water (ISO, 1999) contained $CaCl_2 2H_2O$, $MgSO_4 7H_2O$, $NaHCO_3$ and KCl and deionized water, was prepared and used as negative control solution. For positive control, 4 ppm of 3, 4-dichloroaniline (Merck, 98% purity) diluted with standard water was used. MSG stock solution at 320,000 ppm was freshly prepared on the same day before starting the test to minimize the half-life degradation effect by simply mixing distilled water with the powder of L-glutamic acid monosodium salt hydrate (Sigma, 99% purity). The stock solution then diluted with standard water to reach the twofold serial dilutions between 5,000 ppm – 160,000 ppm for zebrafish embryo and eleuthero embryo $LC_{50}$ test. For

the embryonic toxicity test, the stock solution was prepared at 10,000 ppm and diluted with standard water to be 1,500 ppm, 150 ppm and 15 ppm.

***The $LC_{50}$ test:*** Zebrafish embryo and eleuthero embryo LC50 test were performed at six MSG two-fold serial dilutions between 5,000 ppm – 160,000 ppm. Two replicates of twelve embryos per concentration were prepared by placing one embryo per well in a 96 wells plate including the negative and the positive controls group. The embryos were observed under stereomicroscope at 48 hpf and 96 hpf for five mortality signs including coagulation embryo, lack of somite formation, non-detachment of the tail, lack of heart beat and hatching failure. Recording data was analyzed by Probit method to obtain the LC50.

***Fish embryonic toxicity test:*** For fish embryonic toxicity test, total 120 fertilized embryos were divided into 5 groups by transferred into 100 ml beakers containing 50 ml of each MSG testing solutions at 1,500 ppm 150 ppm and 15 ppm including one negative and one positive control group. Thereafter each embryo was transfer into each well of 96 wells plate together with 250 microliter testing solution using wide tip micropipette. The plates then covered with parafilm and placed in a temperature controlled room at 26°C without changing the testing solution throughout the testing period.

The plates were accessed at 24, 48, 96 and 144 hpf to observe developmental abnormalities. The lethal, sublethal (reversible side effect) and malformation (irreversible side effect) endpoints were performed under a stereomicroscope (Table1). Fisher's Exact Test (Langsrud, 2016) was used to calculate data for Fisher's Exact significant different between the MSG group and the negative control group.

**Table 1** Mortality rate of zebrafish embryo at 48 hpf and 96 hpf

| MSG concentration (ppm) | Mortality rate 48 hpf (%) | | Mortality rate 96 hpf (%) | |
|---|---|---|---|---|
| | Replication1 n = 12 | Replication2 n = 12 | Replication1 n = 12 | Replication2 n = 12 |
| Negative control | 8 | 0 | 8 | 0 |
| 5,000 | 0 | 0 | 17 | 33 |
| 10,000 | 8 | 8 | 25 | 25 |
| 20,000 | 92 | 92 | 92 | 100 |
| 40,000 | 100 | 92 | 100 | 100 |
| 80,000 | 100 | 100 | 100 | 100 |
| 160,000 | 100 | 100 | 100 | 100 |
| Positive control | 100 | 100 | 100 | 100 |

***Observation endpoints at 24, 48, 96 and 144 hpf:***

***24 hours post fertilization:***

Lethal effect
- coagulation embryo
- lack of tail formation
- lack of eye formation
- lack of somite formation
- lack of detachment of the tail-bud from the yolk sac

Sublethal effect and malformation
- less or over movement of embryo

***48 hours post fertilization:***

Lethal effect
- coagulation embryo
- lack of tail formation
- lack of eye formation
- lack of somite formation
- appearance of yolk edema
- appearance of heart edema
- lack of detachment of the tail-bud from the yolk sac

Sublethal effect and malformation
- lack of blood circulation
- lack of pigment formation
- heart beat (observation two times at 30 hpf and 48 hpf; at 30 hpf "tachycardia" if heart beat > maximum heartbeat of control group ± 2 sd (170 beat/min) "bradycardia" if heart beat < minimum heartbeat of control group ± 2 sd (134 beat/min); at 48 hpf "tachycardia" if heart beat > maximum heartbeat of control group ± 2 sd (193 beat/min) "bradycardia" if heart beat < minimum heartbeat of control group ± 2 sd (151 beat/min).

***96 hours post fertilization:***

Lethal effect
- coagulation of embryo
- appearance of yolk edema
- appearance of heart edema
- other abnormality appearance (other than indicated above)

Sublethal effect and malformation
- no hatching
- lateral recumbency
- appearance of abnormal body curvature

***144 hours post fertilization:***

Lethal effect
- no hatching
- hatching dead (die after hatching)
- other abnormality appearance (other than indicated above)

Sublethal effect and malformation
- head down
- upside down
- lack of movement
- lateral recumbency

- appearance of abnormal body curvature

***Compliance with Ethical Standards:*** All animal husbandry and experimental conditions were approved by Chulalongkorn University Animal Care and Use Committee. Protocol number 1531073. Documentation is available upon request.

## *Results*

Zebrafish embryos in the group that exposed to MSG reach highest mortality rate at 100 % within 48 hpf and 96 hpf after exposed to MSG at concentration of 40,000 ppm and 80,000 ppm which a significantly different ($p<0.05$) from negative control group (Table 1).

Subsequently calculated with the Probit analysis, the LC50 of MSG on zebrafish embryo at 48 hpf and eleuthero embryo at 96 hpf were 15,200 ppm and 10,300 ppm, respectively.

The toxicity test were applied to individual embryo at the concentration of 15 ppm, 150 ppm and 1,500 ppm. The results showed that there was no observable effect (NOEC) in the group of the embryo tested at 15 ppm at 24 hpf whereas the lowest observed effect concentrations (LOEC) was 150 ppm at 24 hpf (Figure 1).

However significant sublethal and malformation effect did occur in all MSG exposed group after 48 hpf (Figure 1). The effects included high frequency of heart beat, pericardial edema and lack of embryo blood circulation. The adverse effects continued to happen in 96 hpf and 144 hpf increase with other abnormality such as scoliosis and lateral recumbency especially in the highest test concentration group (Figure 2 and 3).

Regarding to the heart effect, average heart beat at 30 and 48 hpf of the embryo in negative control group were 152 and 172 beat/min, respectively but the heartbeat of the embryo exposed to MSG at 1,500 ppm was higher than that the control group significantly. The effect also significantly visible in every MSG tested group when observed at 144 hpf.

In the positive control group, which embryo was exposed to 3, 4-dichloroaniline, 100% lethal effect within 48 hpf was noted.

At 26°C, average embryo hatching number is not significant different in all MSG exposed and negative control group. All tested solution has the same range of pH at 7.4 the conductivity and total dissolved solid in MSG concentration was elevate in range.

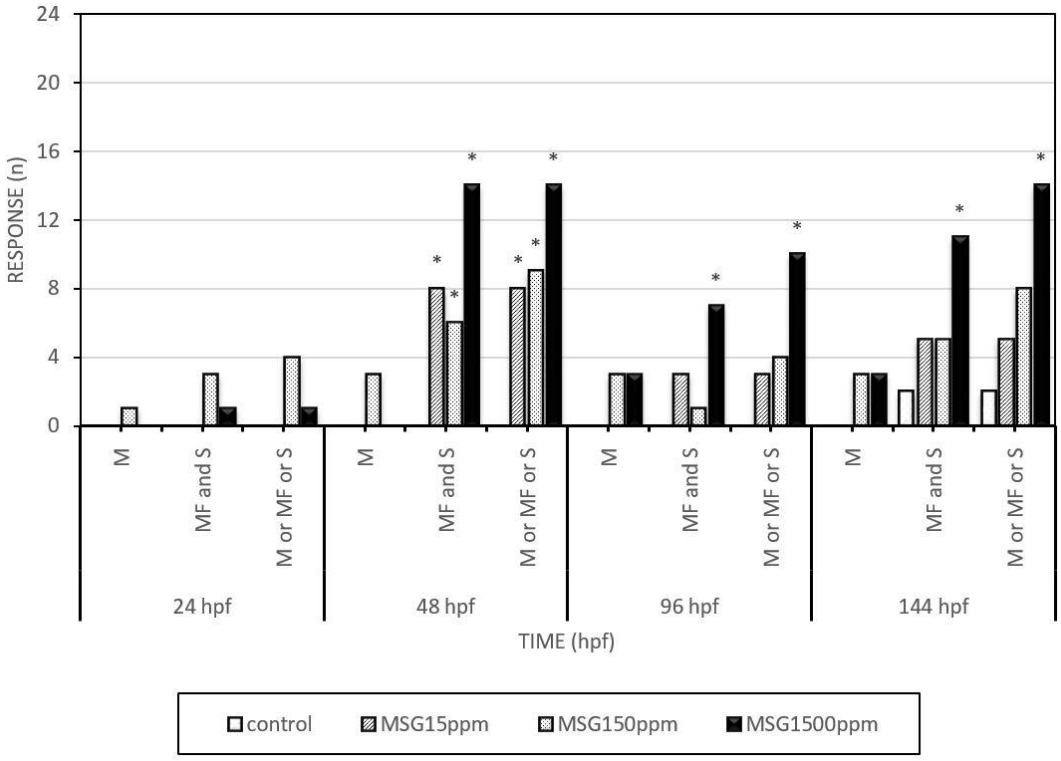


**Figure 1** Mortality (M), malformation (MF) and sublethal response (S) of zebrafish embryo exposed to difference concentration of MSG at 24, 48, 96 and 144 hpf (* mean significant difference from control)

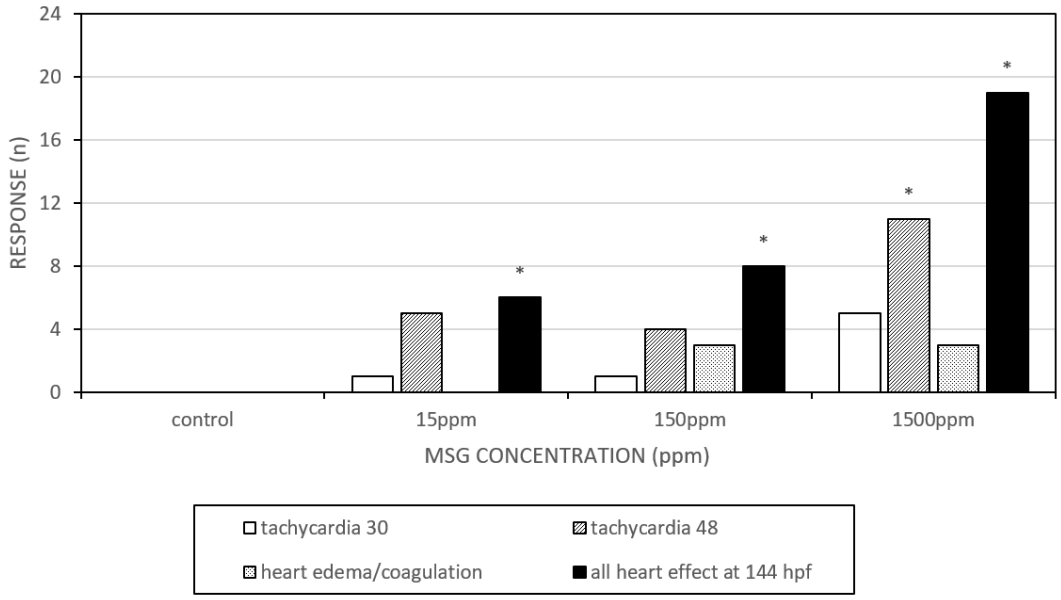


**Figure 2** Number of zebrafish embryo showed signs of heart defect including tachycardia, pericardial edema and coagulation, after exposed to difference concentration of MSG at 30, 48 and 144 hpf (* mean significant difference from control)

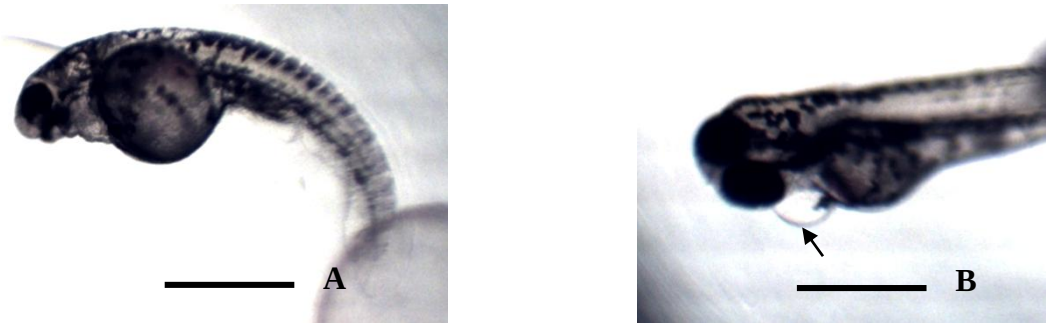


**Figure 3** Hatching zebrafish embryo show significant abnormality of scoliosis, lateral recumbency (A) and pericardial edema (B arrow) Bar = 2 mm.

## *Discussion*

Exposures to MSG are unavoidable since it is used increasingly in large amount worldwide as food additives (He, 2011). Unfortunately, the potential hazards of MSG are not completely identified. Hence, in this study we have investigated the toxicity of MSG using zebrafish as a model to assess multiple toxicological endpoints including lethality, cardiotoxicity and external phenotypic abnormalities. We conducted the test with six MSG two-fold serial dilutions between 5,000 ppm – 160,000 ppm. Our study demonstrated that the LC50 of MSG were 15,200 ppm and 10,300 ppm for 48 and 96 hpf, respectively.

These results are in agreement with the per oral LD50 of MSG in rats and mice (15,000–18,000 mg/kg body weight), suggesting a very low acute toxicity of MSG to the tested animals. In human, FDA have affirmed that MSG at levels normally consumed by the general population is safe and there is no evidence linking MSG used in food to any serious, long term medical problems in the general population (Walker and Lupien, 2000).

Zebrafish embryonic developmental study is a very sensitive tool to demonstrate the morphological and developmental abnormalities at a very low toxicity of chemical concentration. In this study, we did not find adverse effect of MSG within the test level upon early development stage of the zebrafish (24 hpf) by the observation technique. The observation endpoint used in this study could not measure the early response of the embryo to MSG.

Although we did not see any different response between MSG exposed and unexposed group within 24 hpf, we have found that MSG has certain impact on the embryo cardiac function causing tachycardia at the time the heart develops which is later than 24 hpf. These results are similar to cardiotoxic effects that has been described in zebrafish exposed to triclosan (Alisha, 2016) and hexabromocyclododecane (Wu, 2013).

Another reversible response included lateral recumbency, head tilt and upside down of the hatching larva was also noted. A progress example of malformation effect was lacking blood circulation, the appearance of abnormal body curvature, pericardial edema, yolk edema or abdominal edema.

Similar to the results of our study, Mahaliyana, et al. (2016) found that their zebrafish

embryos had normal embryonic development after exposure to the low concentrations of MSG such as 10, 30, 50 ppm and after exposure to MSG at high concentrations between 100 ppm - 500 ppm, the observable deformities occurred, such as growth retardation, shrinkage of chorion, yolk sac edema, lack of pigmentation, tail deformities and scoliosis.

Mahaliyana described that their acute toxicity experiment was performed for a 4-day period but did not specify the exactly the time point (in hpf unit) in which the abnormalities of the embryonic development occurred, while we reported the observed abnormalities at 24, 48, 96 and 144 hpf. Therefore, it is difficult to precisely compare our results and their results, since the degree of toxicity of chemicals varies according to embryonic phase.

Our results on abnormal embryonic development are also similar to the effects of ethanol on zebrafish embryo reported by Merkel et al. (2014). They found that after zebrafish embryos exposed to alcohol, some fishes had abnormal longer tails, and some fishes had developed kinks near the tips of their tails. These abnormalities that occurred to the zebrafish embryos after exposure to MSG and ethanol proved that these substances which seem to be safe, actually have negative impact to the physical development of fish embryos.

MSG at high concentration has been shown to produce the endocrine dysfunction and malformation in zebrafish embryo including elongate heart, pericardial edema and spinal kyphosis (Tamer, 2012). Kurnianingsih et al. (2016) also demonstrated that prolonged exposure of MSG at 10 µg/mL increased apoptosis of brain cells, stereotypic behavior and decreased locomotor activity of zebrafish larvae at early developmental stages.

In the present study, MSG induced cardiotoxic effects in zebrafish embryo was observed. We found that after 48 hpf, MSG exposure at the concentration of 15, 150 and 1,500 ppm, induced cardiotoxic effects including abnormal heart rate, pericardial edema and lack of embryo blood circulation.

Researchers acknowledge that the zebrafish is an excellent model for testing drug-induced cardiotoxicity (Brown, 2016). The first organ to develop in zebrafish embryo is the heart. By 22 hpf the heart starts beating and is fully functional by 48 hpf. The development of the cardiovascular system requires a coordination of tightly regulated expressions of ion channels and complex metabolic processes. At present the precise mechanisms of cardiotoxicity of MSG is not known. It is possible that MSG as an excitotoxin may interfere with the neurotransmission, the conduction system, and the cardiac function. The first proof that amino acids could work as excitatory neurotransmitters in animals emerged from the experiments in 1950s (Hayashi, 1954) in which topically applied MSG to motor cortex resulted in tonic convulsions. Nowadays the excitatory amino acid receptors are generally accepted as an important receptor that mediate synaptic excitation in the vertebrate central nervous system.

The cardiovascular system is very important for the survival and health of all living creatures, further study on the impacts of MSG exposure to zebrafish's cardiovascular system and the mechanisms of cardiac toxicity is warranted. If the result like what we have found also occur in mammal, parent should be concern of limit amount MSG consumption to avoidance the malformation of their offspring. The results from previously mentioned studies and our present study strongly indicated that MSG exposure had negative impacts on zebrafish embryo model, especially on the early developmental stages. It is also possible that in human, children may be more sensitive to MSG compared to adults therefore MSG should be used with caution in this population.

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# บทคัดย่อ

## ผลของโมโนโซเดียมกลูตาเมตต่ออัตราการเต้นของหัวใจและพัฒนาการของตัวอ่อนปลาม้าลาย

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การบริโภคอาหารสำเร็จรูปส่งผลให้ผู้บริโภคได้รับสารปรุงรสที่ผสมอยู่ในอาหารดังกล่าว และอาจส่งผลต่อสุขภาพได้โดยเฉพาะในเด็กและผู้สูงอายุ งานวิจัยในสัตว์ทดลองพบว่าปลาม้าลายมีความเหมาะสมที่จะนำมาใช้เป็นโมเดลการศึกษาทางด้านพิษวิทยา โดยเฉพาะอย่างยิ่งการศึกษาที่เกี่ยวข้องกับผลกระทบด้านสุขภาพร่างกายของมนุษย์ และการตอบสนองต่อการสัมผัสสารพิษที่ได้รับในปริมาณน้อย การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความเป็นพิษและผลของสารละลายโมโนโซเดียมกลูตาเมต ต่อการเต้นของหัวใจและพัฒนาการของตัวอ่อนปลาม้าลาย ตัวอ่อนปลาม้าลายกลุ่มที่ได้รับโมโนโซเดียมกลูตาเมตที่ 40,000 และ 80,000 พีพีเอ็ม มีอัตราการตาย 100 % ภายใน 48 hpf และ 96 hpf ซึ่งมีความแตกต่างอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุมลบ ($p < 0.05$) พบว่าระดับความเข้มข้นที่ทำให้ตัวอ่อนตายที่ร้อยละห้าสิบ (LD 50) ที่เวลา 48 ชั่วโมงเท่ากับ 15,200 พีพีเอ็ม และที่เวลา 96 ชั่วโมงเท่ากับ 10,300 พีพีเอ็ม โดยการวิเคราะห์ด้วย Probit analysis จากนั้นทำการทดสอบผลของโมโนโซเดียมกลูตาเมตที่ความเข้มข้น 15 พีพีเอ็ม 150 พีพีเอ็ม และ 1,500 พีพีเอ็ม ต่ออัตราการเต้นของหัวใจและพัฒนาการของตัวอ่อนปลาม้าลายทดสอบที่ระดับความเข้มข้นที่ต่ำกว่า LD 50 (ที่ 48 ชั่วโมง) 10 เท่า โดยสังเกตความผิดปกติระดับต่าง ๆ รวมถึงความผิดปกติของพัฒนาการเป็นระยะ ๆ ภายในเวลา 144 ชั่วโมง พบว่าโมโนโซเดียมกลูตาเมตที่ความเข้มข้น 150 พีพีเอ็ม และ 1,500 พีพีเอ็ม ทำให้หัวใจของตัวอ่อนปลาม้าลายเต้นเร็วขึ้น และมีพัฒนาการของร่างกายผิดปกติ ผลของงานวิจัยนี้สร้างความตระหนักเกี่ยวกับผลกระทบจากการได้รับโมโนโซเดียมกลูตาเมตต่อพัฒนาการของสิ่งมีชีวิตวัยอ่อน นอกจากนี้ควรทำการศึกษาเพื่อทบทวนความปลอดภัยและเพื่อให้ทราบรายละเอียดของกลไกที่ทำให้เกิดผลไม่พึงประสงค์ของโมโนโซเดียมกลูตาเมตต่อไป

**คำสำคัญ:** ผงชูรส โมโนโซเดียมกลูตาเมต ปลาม้าลาย ความเป็นพิษ

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