Investigating the effects of monosodium glutamate using zebrafish as an animal model

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Under the guidance of

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DECLARATION

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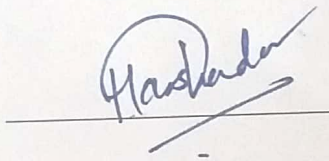

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APPROVAL SHEET

This thesis, entitled **"Investigating the effects of monosodium glutamate using zebrafish as an animal model"** by **Harshada S Jadhav** is approved for the degree of Master of Technology.

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ABBREVIATIONS

ATP	-	Adenosine triphosphate
ALS	-	Amyotrophic Lateral Sclerosis
ANOVA	-	Analysis of Variance
a.u.	-	Arbitrary unit
ASD	-	Autism Spectrum Disorder
BCG	-	Bacillus Calmette–Guérin
BOD	-	Biological oxygen demand
CAT	-	Catalases
CRS	-	Chinese restaurant syndrome
DCFDA	-	2',7' –dichlorofluorescin diacetate
dpf	-	Day post fertilization
EPM	-	Elevated plus maze
FET	-	Fish embryos acute toxicity test
FDA	-	Food and drug association
FEV1	-	Forced expiratory volume
GRAS	-	Generally Recognized as Safe
GSH	-	Glutathione
GPX	-	Glutathione peroxidase
GR	-	Glutathione reductase
GST	-	Glutathione-s-transferase
GMP	-	Good Manufacturing Practices
g/kg	-	Gram/killogram
hrs	-	Hours
hpf	-	Hours post fertilization
LD 50	-	Lethal dose 50
MDA	-	Malondialdehyde
µg/ml	-	Microgram per milliliter
µl	-	microliter
µM	-	micromolar
mg/g	-	Milligram/gram
mg/kg	-	Milligram/killogram
mg/L	-	Milligram/liter
ml	-	milliliter
M.W.	-	Molecular weight
MSG	-	Monosodium glutamate
OFT	-	Open field test
OECD	-	Organisation for Economic Co-operation and Development
ppm	-	Parts per million

ROS	-	Reactive oxygen species
RO	-	Reverse osmosis
SD	-	Standard deviation
SEM	-	Standard error of the mean
SOD	-	Superoxide dismutase
TRH	-	Thyrotropin-releasing hormone
TDS	-	Total dissolved solids

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The main goal of the food industry is to provide consumers with fresh, well-preserved, and palatable food. In the early 20th century, the food industry was blooming. Yet, many were battling to conserve the taste of packed and processed food. MSG came as a perfect solution. Today, it stands as the most commonly used flavor enhancer in the food industry. Yet, concerns were raised after several incidences of Chinese restaurant syndrome (CRS) emerged. To investigate this issue, many short-term human trials and animal studies were conducted. The data obtained were ambiguous, inconclusive, and sometimes irrelevant to the human level of consumption. In this study, we used zebrafish as an animal model to investigate the effect of MSG on development, behavior, and oxidative stress. Embryos were treated with food-grade MSG for 4 days with concentrations varying from 50 to 50,000mg/L at two different developmental periods.MSG induced growth retardation, mortality, and delayed hatching when exposed in the cleavage period at a higher concentration. No change in thigmotaxis and oxidative stress were observed in the 3dpf larvae treated with 500mg/L of MSG. Our study strongly supports the harmful effect of MSG during early period of development when consumed in higher concentration. Thus, we suggest exercising caution in the consumption of MSG, especially in children.

CONTENT

1.INTRODUCTION

1.1 Monosodium Glutamate (MSG)

Monosodium glutamate is the sodium salt of glutamic acid. It is a crystalline white/off-white powder with M.W of 169.112 g/mol. This odorless compound is highly soluble in water and insoluble in alcohol and ether. It is the most commonly used flavor enhancer in the food industry (1). The Food and Drug Association (FDA) has categorized MSG under "Generally Recognized as Safe (GRAS) "list. According to the FDA, its use should be by Good Manufacturing Practices (GMP)(2).

In developed countries, the estimated daily dietary intake of MSG is 0.3-1.0 gram/day. In recent years, its excessive and undefined usage in the food industry has raised concerns; mainly due to the emerging cases of Chinese Restaurant Syndrome (CRS). The occurrence of CRS is most prevalent in countries with the highest level of MSG consumption (3).

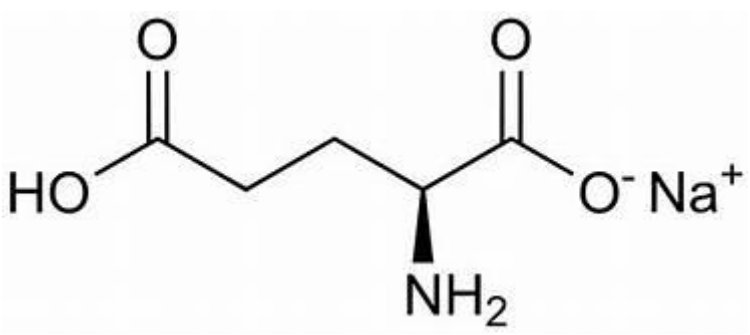

Figure 1. Structure of Monosodium Glutamate

Figure 2.Food products containing MSG: Knorr soup, Pringles, Cheetos and Maggi (clockwise)

1.2 Oxidative stress

Oxygen is an essential gas in the living world. The generation of adenosine triphosphate (ATP) takes place through oxidative phosphorylation. Although this process is critical for the production of energy, it can cause severe damage to the structural integrity of the cell by forming reactive oxygen species (ROS). Hence, the body is under a constant attack by this malicious oxygen. In order to prevent damage, the cell has evolved with a system of antioxidant mechanisms that nullifies the harmful effects of reactive species. Any perturbation to this balance can lead to a state called oxidative stress (4). Neurological pathologies and disorders such as schizophrenia, Autism Spectrum Disorder (ASD), Amyotrophic Lateral Sclerosis (ALS), depression, and anxiety are associated with oxidative stress (5–9).

1.3 Anxiety

Anxiety is a state of mind wherein the subject presumes danger/fear without its actual existence. Occasional anxiety is reasonable and sometimes necessary for flight /fight response. It becomes a disorder when without any real occurrence of danger; a persistent feeling of

disturbance, turmoil, worry, and confusion exists. There are different types of anxiety, such as existential, social, trait-based, test/performance, choice/decision making, and generalized. The most common symptoms include rapid heartbeat, profuse sweating, confusion, irritability, restlessness, and sleeplessness. Most of the patients facing anxiety are victims of a troubled childhood, sexual/physical/substance abuse, and trauma. It can also have a genetic base (10).

1.4 Zebrafish as an animal model

Zebrafish are widely used animal model due to their small size, low cost, easy maintenance, external fertilization, high fecundity, and transparent embryos. Researchers use this model extensively for toxicological profiling of commonly used preservatives, drugs, flavor enhancer, and pesticides. With the advancement in the imaging and labeling techniques, these transparent larvae can provide critical insight at the systemic, organ, cellular, and sub-cellular levels. Due to external fertilization, early developmental defects and changes can be easily captured with precision. Behavioral assay to study condition like anxiety is well-developed in this model. Hence, we used zebrafish larvae to study developmental, behavioral, and oxidative changes due to MSG.

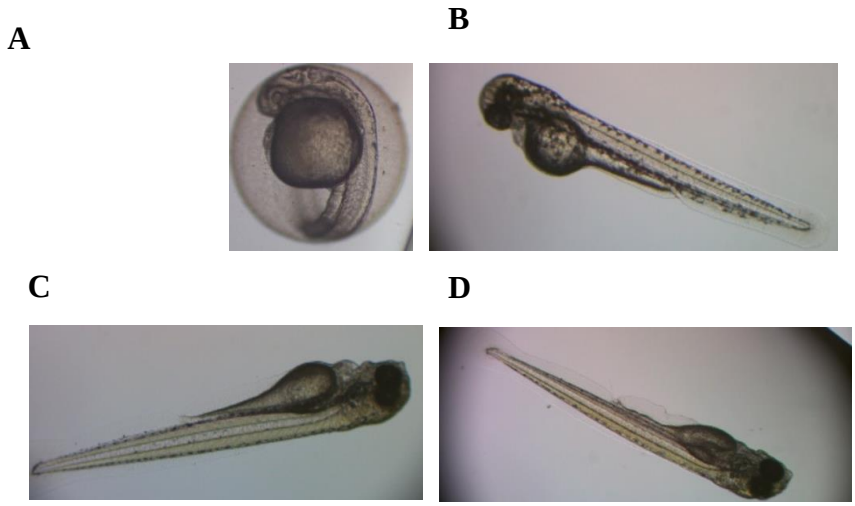

Figure 3. Zebrafish larvae as a model organism: Panel A) Representative image of a 24 hpf embryo; Panel B) Representative image of a 48 hpf larva; Panel C) Representative image of a 72 hpf larva Panel D) Representative image of a 96 hpf larva

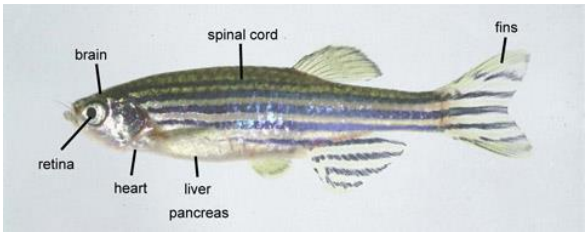

Figure 4: Mature adult zebrafish (90 days – 2years)

2.REVIEW OF LITERATURE

2.1 Effect on MSG

2.1.1 Human studies

In the late 20th century, studies on the usage of MSG accelerated due to increasing cases of palpitation, flushing, headaches, restlessness, muscle tightness, and tingling/numbness after consumption of Chinese food. This condition of hypersensitivity to Chinese food is called "Chinese restaurant syndrome." A table of different case studies and human trials on MSG and its safety enlisted as below:

2.1.1.1 Case studies

Table 1.Case studies are showing the adverse effects of MSG.

Case study	Conclusion	References
1.Four female with fibromyalgia (for 2 to 17 years) (11)	Fibromyalgia symptoms reduced with the cessation of MSG in the diet. Consumption of MSG caused the reoccurrence of symptoms (11).	Smith et al. *Ann Pharmacother*. 2001 Jun;35(6):702-6.
2.A 7 months old infant developed granuloma after BCG injection (12).	Monosodium glutamate (used as a carrier/stabilizer) in the BCG vaccine caused foreign body granuloma (12).	Chiu et al. *J Am Acad Dermatol*. 2006 Aug;55(2 Suppl):S1-5.
3.A 15-year-old white girl is showing orofacial granulomatosis on the consumption of MSG (13).	Allergic reaction to MSG.MSG-restricted diet lead to resolution of the facial swelling (13).	Oliver et al. *Oral Surg Oral Med Oral Pathol*. 1991 May;71(5):560-4

2.1.1.2 Human trials:

Table 2.Human trials and their conclusion on the effects of MSG

Aim of the study	Study design	Conclusion	References
1.To evaluate the reaction to MSG using a multiphase, multicenter, double-blinded placebo-controlled crossover study design(14).	130 MSG-sensitive volunteers participated. Challenge A: 5g MSG without food; Challenge B (to evaluate reproducibility and consistency): Individuals who responded in challenge A were rechallenged again with 5g of MSG and placebo; Challenge C: Rechallenged the individuals who responded to both the previous challenges (only MSG responders); Challenge D: MSG responders in challenge C were rechallenged thrice with food (14).	Large doses of MSG consumed without food can elicit CRS symptoms. Responses were neither severe nor consistent (14).	Geha RS et al. *J Allergy Clin Immunol*. 2000 Nov;106(5):973-80.

2. To determine whether MSG can induce bronchoconstriction in asthma patients who claim to be MSG-intolerant (15).	In this study, twelve subjects with asthma and claims of being MSG-intolerant participated. Challenged with 1g of MSG (day 1), 5g of MSG (day 2) and 5g of lactose (placebo-day3) in the morning after overnight fasting. At the end of three days, nonspecific bronchial hyperresponsiveness was measured. FEV1 and peak expiratory flow rate evaluated for all control and challenge days. Soluble inflammatory marker activity was determined (15).	This study concluded that MSG does not induce asthma (15).	Woods RK et al. *J Allergy Clin Immunol*. 1998 Jun;101(6 Pt 1):762-71.
3. To study the short-term neuroendocrine effect of a hefty dose of MSG on fasting males (16).	Fasting males challenged for four days with 12.7g MSG on day 1, an MSG vehicle on day 2, an IV injection of TRH on day 3 and a high protein diet on day 4 (16).	High plasma level of glutamate causes a minimal effect (if any) on hypothalamic and pituitary function (16).	Fernstrom JD et al. *J Clin Endocrinol Metab.* 1996 Jan;81(1):184-91.

4. To study the CRS symptoms in self-identified MSG sensitive subjects (17).	No. of participants= 61. Type of study= Double-blinded and placebo-controlled. Challenge 1: 5g of MSG and placebo given to the subjects in a double-blind way. Challenge 2: Positive responders to the test chemical rechallenged with 1.25, 2.5, 5 g of MSG, and placebo (17).	In this study, MSG-sensitive subjects showed CRS symptoms in a statistically higher rate compared to the placebo group (17).	Yang WH et al. J Allergy Clin Immunol. 1997 Jun;99(6 Pt 1):757-62.
5. To study whether MSG causes flushing (18).	Flushing in six subjects was studied using laser Doppler velocimeter to monitor changes in facial cutaneous blood flow after challenge with MSG and pyroglutamate (18).	MSG can rarely (if any) provoke flushing (18).	Wilkins JK. *J Am Acad Dermatol.* 1986 Aug;15(2 Pt 1):225-30

These pioneering studies focused on studying the physiological effect of MSG rather than behavioral effects. No studies are available that relates MSG and anxiety in human subjects.

2.1.2 Animal models

2.1.2.1 In Mice

Among the pioneering studies on the effect of MSG in mice, Olney JW observed severe brain lesions (intracellular edema and neuronal necrosis) in newborn mice when given a subcutaneous injection of 0.5 to 4 mg/g (19). In one study, Kunming filial mice treated with MSG (2.5mg/g or 4mg/g) at 17-21 day of pregnancy, gave birth to offsprings that showed impaired Y-maze discrimination learning (at 60 days) without visible damage to the hypothalamus area (20). Recently, a group of scientists observed an increase in the depression-like condition in young mice (4 to 5 weeks old) treated with 2.5g/kg of MSG. Also, such a depressive condition was observed even in treated adults (9 to 10 weeks) at a higher rate than the healthy controls (21).

2.1.2.2 In Rats

Palaez et al. observed neural necrosis in hypothalamic arcuate nuclei of neonatal rats (22). In another study, a 4mg/g subcutaneous injection of MSG given at postnatal day 2,4,6, 8 and 10, reported a reduction in the pituitary weight by 30% and 40% at the age of 6 and 12 months respectively(23). Using the same dose of MSG at postnatal day 1,3,5, and 7, González-Burgos et al. observed severe damage to the prefrontal cerebral cortex (24). One study linked physiological changes in the brain to changes in the behavior of neonatal rats. This group of researchers found that with an increase in the level of glutamate and catecholamines in the brain tissue, there was a decrease in spatial memory and learning (25). A recent study on male albino rats has reported a significant reduction in cognitive functions even at a low dose of 1/20 of LD50 given through gavage(26). Apart from its effect on the CNS, many studies have shown that MSG causes obesity and other metabolic disorders (27–29).At a dose of 4mg/g of MSG, the levels of testosterone and size of the testes in sexually matured rats were found to be lower than the controls (30).

2.1.2.3 In Zebrafish

In the past few years, MSG toxicity studies on zebrafish have gained impetus. One study published in the year 2016, observed severe abnormalities like yolk and pericardial edema, lack

of pigmentation, tail bending and scoliosis along with developmental defects such as growth retardation at a concentration of 100 to 500mg/L (31). A year later, another group found the LC50 of MSG to be 15,200 ppm and 10,300 ppm in 48hpf and 96hpf embryos, respectively. The same study observed cardiotoxicity at a low concentration of 15ppm, developmental malformation at 150ppm, and sublethal effects at 1,500ppm (32). Kurnianingsih et al. treated embryos with 10µg/ml of MSG and observed increased apoptosis in brain tissue and a decrease in locomotor activity. They concluded that at the early developmental stage, MSG could increase the risk of brain damage and chances of stereotypic behavior (33).

2.2 MSG and oxidative stress

Table 3.Studies showing the relationship between MSG and oxidative stress

Aim of the study	Study design	Conclusion	References
1.To study the effect of MSG on hepatic microsomal lipid peroxidation, calcium, ascorbic acid, glutathione, and its dependent enzyme in adults male mice (34).	Subcutaneously injected 4mg/g of MSG in adult male rats for six days (34).	Increase in lipid peroxidation in the hepatic microsomes. The level of glutathione (GSH) significantly reduced. An increase in the activity of glutathione-related enzymes such as GST, GR, GPX was found (34).	Choudhary et al. *Toxicology Letters*. Volume 89, Issue 1, December 1996, Pages 71-76
2. To study the effect of Vitamin E on MSG-induced hepatoxicity and oxidative stress in rats (35)	A dose of 0.6mg/g was given through gavage for 10 days to rats (35).	Significant increase in the level of lipid peroxidation, decrease in glutathione (GSH) with the increased enzymatic activity of glutathione-s-transferase (GST), catalase and superoxide dismutase (SOD) was observed in the liver (35).	Onyema et al. *Indian Journal of Biochemistry and Biophysics.* Volume 43, Issue 1, February 2006, Pages 20-24.

3. To study the effect of Vitamin C, Vitamin E and quercetin on oxidative damage and genotoxicity caused due to MSG in the rat model (36)	4mg/g of MSG given intraperitoneally for 10 days. The treatment group was given additional 200mg/kg of Vitamin C in saline solution, 200mg/kg of Vitamin E in corn oil and 10mg/kg of quercetin in corn oil along with MSG (36).	MDA (a biomarker for lipid peroxidation) remarkedly increased in the liver, kidney and brain tissue. The decrease in GSH, increased activity of GST, SOD, and CAT were observed. Vit E, Vit C, and quercetin successfully recovered different tissues (brain, kidney, and liver) from the oxidative stress (36).	.*Human and Experimental Toxicology*. Volume 25, Issue 5, May 2006, Pages 251-259
4. The effect of dietary administration of MSG on lipid peroxidation and antioxidant status in the brain was studied (37).	For 14 weeks, mice were fed (30% w/w) MSG containing diet (37).	The dietary consumption of MSG in large quantities increases the weight of cerebrum with a corresponding increase in lipid peroxidation and reduction in CAT activity (37).	Adebayo et al. *Asian Journal of Clinical Nutrition.* Volume 3, Issue 2, 2011, Pages 71-77

2.3 MSG and anxiety

Table 4.Studies showing the role of MSG in causing anxiety

Aim of the study	Study design	Conclusion	References
1.To investigate whether MSG causes depression-like condition and anxiety in young rats (38).	Male and female Wistar rats received a subcutaneous injection of 4g/kg/day during 1st to the 5th postnatal period. The behavioral test was performed on 60th to 64th postnatal day (38).	Due to a dysfunction in the serotonergic system, MSG-treated rats are more susceptible to develop anxiogenic and depression-like behavior (38).	Quines et al. *Life Sciences.* Volume 107, Issues 1–2, 27 June 2014, Pages 27-31.
2. To study the effect of monosodium glutamate and aspartame on behavioral and biochemical parameters of male albino mice (39).	The dose of MSG: 8mg/g. Mode of administration: Oral (in drinking water). Duration of treatment: One month. Anxiety test: The elevated plus-maze with two open and two closed arms (39)	Both MSG and aspartame (individually and combined) showed an increase in anxiety and fear (39).	Abu - Taweel, Gasem. (2016). *African Journal of Biotechnology*. 15. 601-612. 10.5897/AJB2015.15199.
3.To study the effect of commonly used flavor enhancer on the behavior of mice when given orally (40).	Mice received an oral dose of 10mg/kg of MSG for 21 days. One group of control and pretreated mice (n=20) were allotted for the open-field test (OFT) and another	At the dose used in the study, it was concluded that MSG was associated with anxiety-related behavior (40).	Onaolapo, O.J., Aremu, O.S. & Onaolapo, A.Y. *Naunyn-Schmiedeberg's Arch Pharmacol* (2017) 390: 677.

	group for elevated plus maze (EPM) test. OF and EPM tests were performed on day 1 and 21(40).		https://doi.org/10.1007/s00210-017-1371-6

3.SCOPE OF THE STUDY

3.1 Aim

Due to different study designs and animal models, the data on the effects of MSG is still inconclusive and biased. In this project, we did a dose-dependent toxicity study to observe the effect of MSG on zebrafish larvae when the larvae were exposed to MSG at two different developmental periods. In additional, we investigated whether MSG elicits anxiety-like behavior and oxidative stress in this model organism.

3.2 Objectives

- To analyze the differences in the %abnormalities, mortality rate, and hatching rate in zebrafish embryos, when the exposure to the larvae was initiated at two different developmental periods namely cleavage and blastula.
- To find whether MSG induces anxiety-like behavior.
- To study the effect of MSG on oxidative stress.

4.MATERIALS AND METHODS

4.1 Zebrafish housing

Adult female and male fish were purchased from a local supplier and maintained in 10L and 6 L tanks filled with RO water. All the fish were acclimatized to laboratory condition for two weeks before using them for experiments. The temperature (26ºC ± 2) and oxygen levels were maintained using a heater and aerator, respectively. E3 media (0.0595M NaCl, 0.021M KCl, 0.039M $CaCl_2.2H_2O$ and 0.048M $MgCl_2.6H_2O$: pH 7.2, sterile) was added to each tank to maintain the total dissolved solids (TDS) concentration**.** The fish were fed with pelleted diet twice daily. Cleaning of the tanks took place on every alternate day.

4.2 Zebrafish mating and collection of embryos

Our laboratory uses a breeding chamber to facilitate spawning and mating of female and male fish. This breeding chamber is made up of two containers: one with mesh-like openings at the bottom that fits onto the second container. The mesh-like design prevents adult fish from eating/damaging the embryos by collecting them efficiently in the second container. Healthy adult fish in the ratio of 2 females to 1 male is kept in the dark condition for 12 hrs ; followed by 1 hr of light condition on the next day. The embryos are produced during the light condition and are collected in a 90mm petri dish using Pasteur pipette (1 ml). They are washed thrice with RO water and then stored in E3 media. The petri dish is kept in a BOD incubator with appropriate labeling until use.

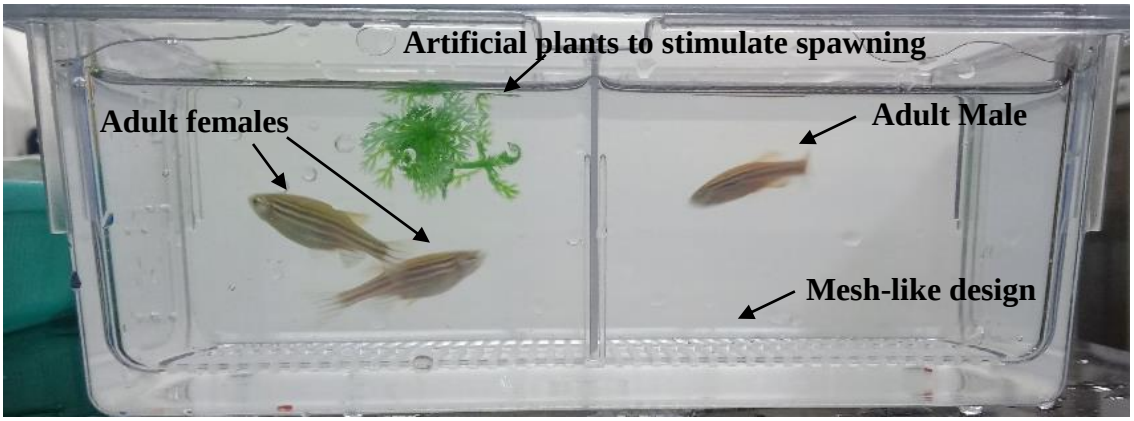

Figure 5.Setup of a breeding tank.

4.3 Preparation of MSG solution

MSG was purchased from a local spice shop. The product is a registered trademark owned and licensed by AJINOMOTO CO., INC.TOKYO, JAPAN. This company is the first one to produce AJI-NO-MOTO commercially.

Figure 6. Food-grade MSG used for the study

4.3.1 Preparation of the stock solution

Total volume prepared=100ml

1. Stock concentration =100,000 mg /L

Dissolved 10 grams of food grade MSG in 100 ml of RO water.

2. Stock concentration=1000mg/L

Dissolved 0.1 grams of food grade MSG in 100 ml of RO water.

4.3.2 Preparation of the working solution

Total volume= 10ml

Table 5. Preparation of working concentration

Dose	Volume added from a stock solution (in ml)	The volume of E3 media added (in ml)	
50	0.5	9.5	Stock concentration: 1000mg/L
500	5	5	
5000	0.5	9.5	Stock concentration: 100,000mg/L
50,000	5	5	

Embryos in E3 media is the control for all experiments.

4.4 Treatment with MSG

Group 1 (Developmental period: Cleavage): Incubation time before treatment= 1.5 to 2 Hrs

Group 2 (Developmental period: End of Blastula): Incubation time before treatment = 5 hrs

After the respective time of incubation, the embryos were taken from the BOD incubator and placed in a 24-well plate with a per well density of 10 embryos. All the treatment wells were washed with appropriate working solution after removing all the E3 media. 1.5- 2ml of E3 media and the working solution was added to the control and treatment group, respectively. Every 24 hrs, all the solutions were replaced with a freshly-prepared solution. Every day, embryos in all the well were analyzed individually for mortality, hatching, and abnormality.

4.5 Hatching and mortality rate

Hatching is an important developmental process wherein the embryo comes out of the chorion layer and becomes a larva. The hatching rate was measured at different time points of 36 hrs,44 hrs, 48 hrs, and 72 hrs.

$$\text{Hatching rate} = \frac{\text{No. of hatched embryos (cumulative)}}{\text{Total no. of live embryos}} \times 100$$

Mortality in zebrafish embryos is characterized by

1. Coagulation
2. Lack of somite formation
3. Non-detachment of the tail
4. Lack/End of heartbeat.

Embryos showing all or any one of the characteristics as mentioned above were considered dead. The mortality rate was calculated for every 24 hrs upto 96hrs.

$$\text{Mortality rate} = \frac{\text{No. of embryos dead (cumulative)}}{\text{Total no. of embryos present at the start of the experiment}} \times 100$$

4.6 Imaging of the embryos

All the embryos and larvae were individually observed under an inverted bright field microscope, Olympus lX73 series with a resolution of 1280×720 (bin) and 800×600 respectively; using a 4X objective. Procam HS-10 MP camera was used to capture images from the microscope.

4.7 Thigmotactic activity

Thigmotaxis is the preference of edge/ walls. Three days old (72 hours post-treatment) treated embryos (Group I: Incubation time before treatment= 2hrs) in 500mg/L of MSG solution and control embryos in E3 media are used for this experiment. First, all the embryos from both the control and treated group were monitored under the microscope, and any embryos showing morphological abnormality were excluded from this experiment. Using a Pasteur pipette, one larva was dropped in the center of a well of the 24-well plate containing 500µl of E3 media. Videos are recorded using a digital camera to track the movement and orientation of each larva (from the control and treatment group) for 30sec. The preference of edge/walls is an indication of anxiety in zebrafish larvae.

$$\%\text{Thigmotaxis} = \frac{\text{No. of larvae that moved towards the walls}}{\text{Total no. of larvae in particular group}} \times 100$$

4.8 Measurement of oxidative stress using DCFDA ROS assay

4.8.1 Preparation of the solutions

1. 5µM of DCFDA solution:
   The total volume prepared for one experiment= 4ml
   The volume of DCFDA solution taken from 2mM stock solution= 10µl
   Volume of E3 media added= 3990µl
2. 200mg/L of tricaine solution (for immobilizing the larvae):
   The total volume prepared for one experiment =1ml
   The volume of tricaine solution taken from the 4000mg/L stock solution= 50µl

Volume of E3 media added= 950µl

4.8.2 Procedure

1. Placed 10 embryos/well from control and treatment in a 24 wells-plate (covered completely with aluminum foil).
2. The light was turned off before taking out the DCFDA solution.
3. The solution in the wells was replaced with 1ml of 5µM of DCFDA solution.
4. The plate was incubated for 30mins in BOD incubator.
5. After 30 mins, all the DCFDA solution was carefully removed, and the embryos were washed with 1ml of E3 media twice at an interval of 5mins.
6. After the second wash, new E3 media was added, and each embryo was placed on a slide (one at a time) for observation under the microscope.
7. Procam software was used to capture images at a resolution of 3366×1222 with a 4X objective and a fluorescence filter 2 (green filter). The exposure time was set at 120ms. Duration of exposure was 2mins. Both bright field and fluorescent images were taken at the start and end of 2mins. All the measurements for control and the treated group were done under the same condition.
8. Fluorescence was quantified with Image J software as described below

4.9 Fluorescence quantification using Image J

1. The images captured after oxidative stress experiment are opened in Image J software.
2. A duplicate of this image was made, and the original image was closed. The duplicate image was converted to 8-bit type using Image → Type → 8-bit
3. In the Analyze tab, Set Measurement option was chosen, and Mean, Maximum, Minimum and Integrate density options were selected.
4. After this, the Measure option from the Analyze tab was selected.
5. The results were displayed in another window. Note that the minimum and maximum value displayed in the result are 0 and 255, respectively. If not, the following steps were followed: Process → Math → Macro
6. Change the formula displayed to
   Vnew (min)= (V)- (Vmin)
   Vnew (max)= (V)* (255/Vmax)

Enter the value of Vmin and Vmax as displayed in the result table.

7. A re-measurement was done to ensure the New minimum and maximum value are set to 0 and 255, respectively.
8. In the Image tab, from the Adjust option, Threshold was selected. In the threshold dialog box, the Intermode option was selected. The upper limit for threshold was set at 255, whereas the lower limit was set according to the experimenter's discretion. (NOTE: Use the same upper and lower limit threshold for both control and treatment)
9. These changes were applied. The highlighted area was selected using the magic wand tool. The area and intensity were measured using the Measure option from the Analyze tab. The calculated area and intensity were displayed in the result window. These data were noted for statistical analysis.

4.10 Statistics

All dose-response, anxiety, and oxidative stress experiments were performed twice independently. All data were analyzed using GraphPad Prism version 5. For dose-response experiments, statistical analysis was done using one-way ANOVA test followed by Dunes post-test. Column statistics (Mean, SEM, SD) were calculated for statistical analysis of anxiety and oxidative stress data. All data are presented as mean± SEM for the indicated n numbers.

5. RESULTS:

5.1 Group I: Treatment started after 2 hrs of incubation

At 2 hrs post fertilization, the zebrafish embryos are at 2-64 cells stage of development (41). Before the start of treatment, the embryos were observed individually under the microscope. Only 2-64 cells stage embryos (shown in Fig 2.) were collected and distributed at random into the control and treatment group.

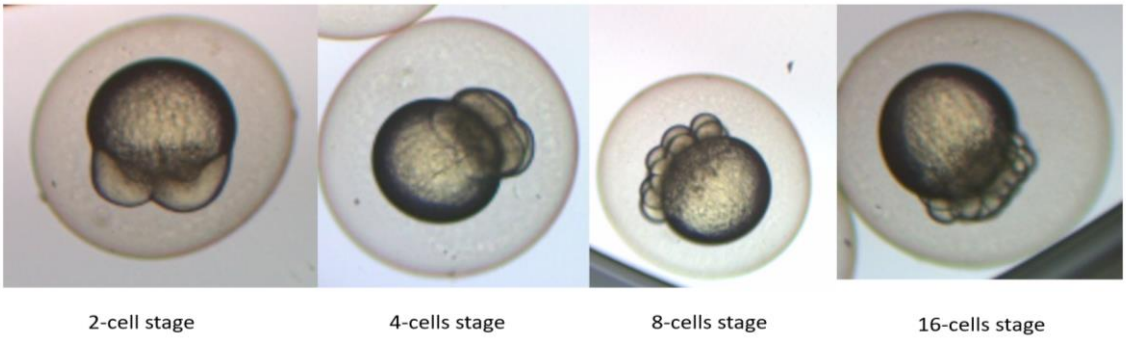

Figure 7.Different stages in the Cleavage period

5.2 Group II: Treatment started after 5 hrs of incubation

At 5 hours post fertilization, the zebrafish embryos are in the latter half of the blastula period(41). Healthy larvae were selected under the microscope and randomly distributed into control and treatment group.

Control group: Placed in E3 media

Treatment group: 50, 500, 5000 and 50,000mg/L of MSG

Duration of experiment: 4 days

Parameters studied: Mortality rate, hatching rate, and % abnormality.

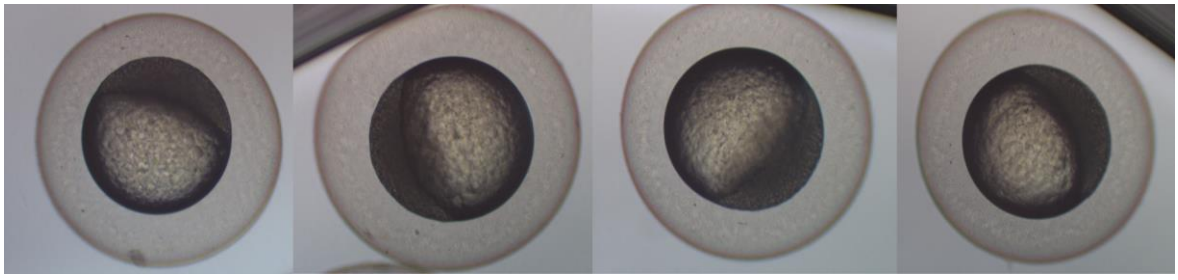

Figure 8.Embryos in the blastula period

5.3 Effect of MSG on the mortality rate

The mortality rate was measured every 24 hrs for 4 days in control as well as 50, 500, 5000, and 50,000 mg/L MSG-treated group. At a concentration of 50,000mg/L of MSG, all embryos in both group I and II show 100% mortality on day 1 (Fig.9A and 10A). Apart from this concentration, no mortality is observed in any concentration at any time point in group II (Fig.10). Compared to group II, the mortality rate in group I at 24 hrs for 500mg/L and 5000mg/L is 12.07%±12.07%and 12.38% ±9.045% higher respectively (Fig.11A). Mortality rate increased by 7.14% at 48 hrs for 5000mg/L in group I. Mortality was observed only till 48 hrs in group I (Fig.9B). No change in mortality was observed in any group at 72 and 96 hrs post-treatment (Fig.11C and 11D).

5.3.1 Effect of MSG on the mortality rate of embryos in group I

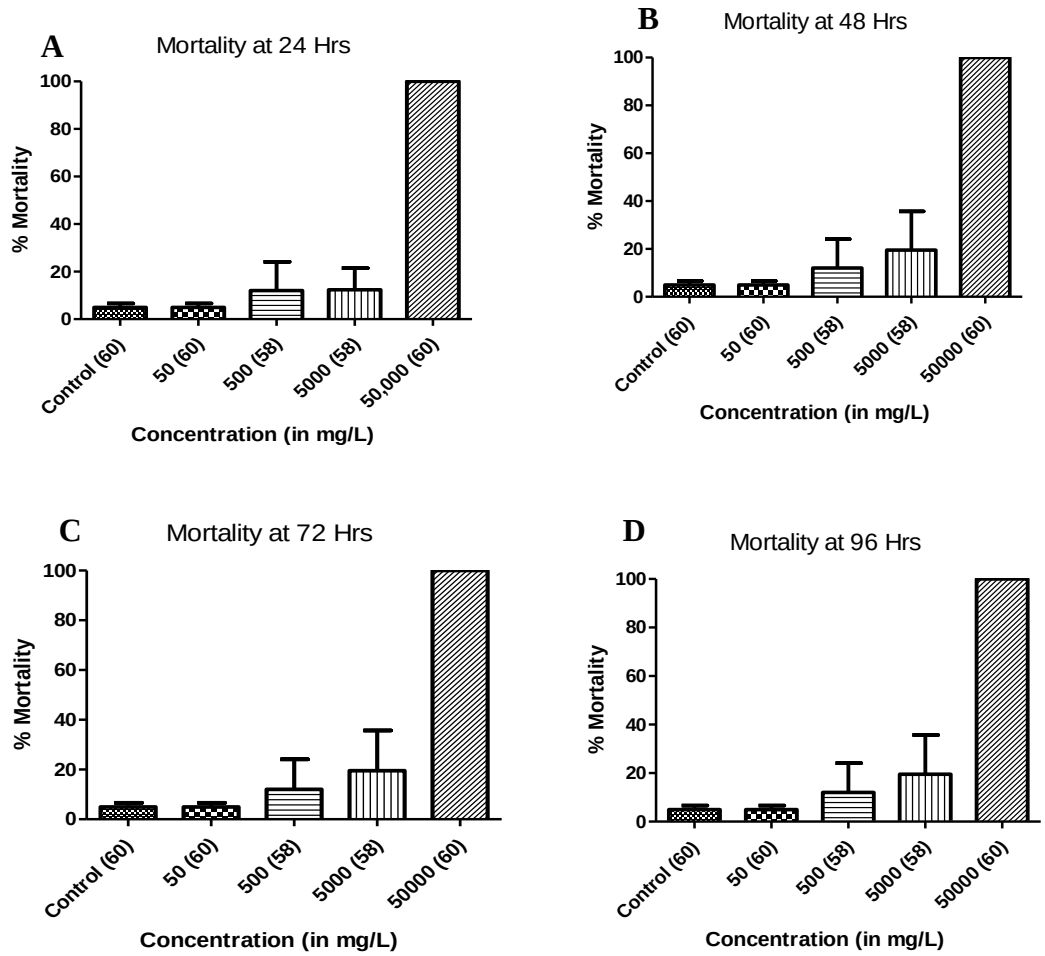

Figure 9.Effect of MSG on the mortality rate of embryos in group I: Four time points were selected, and mortality rates were plotted for control, 50mg/L, 500mg/L, 5000mg/L and

50,000mg/L MSG-treated group at A) 24 B) 48 C)72 D) 96 hours post-treatment. Data is represented as mean± SEM for 58-60 embryos in each group.

5.3.2 Effect of MSG on the mortality rate of embryos in group II

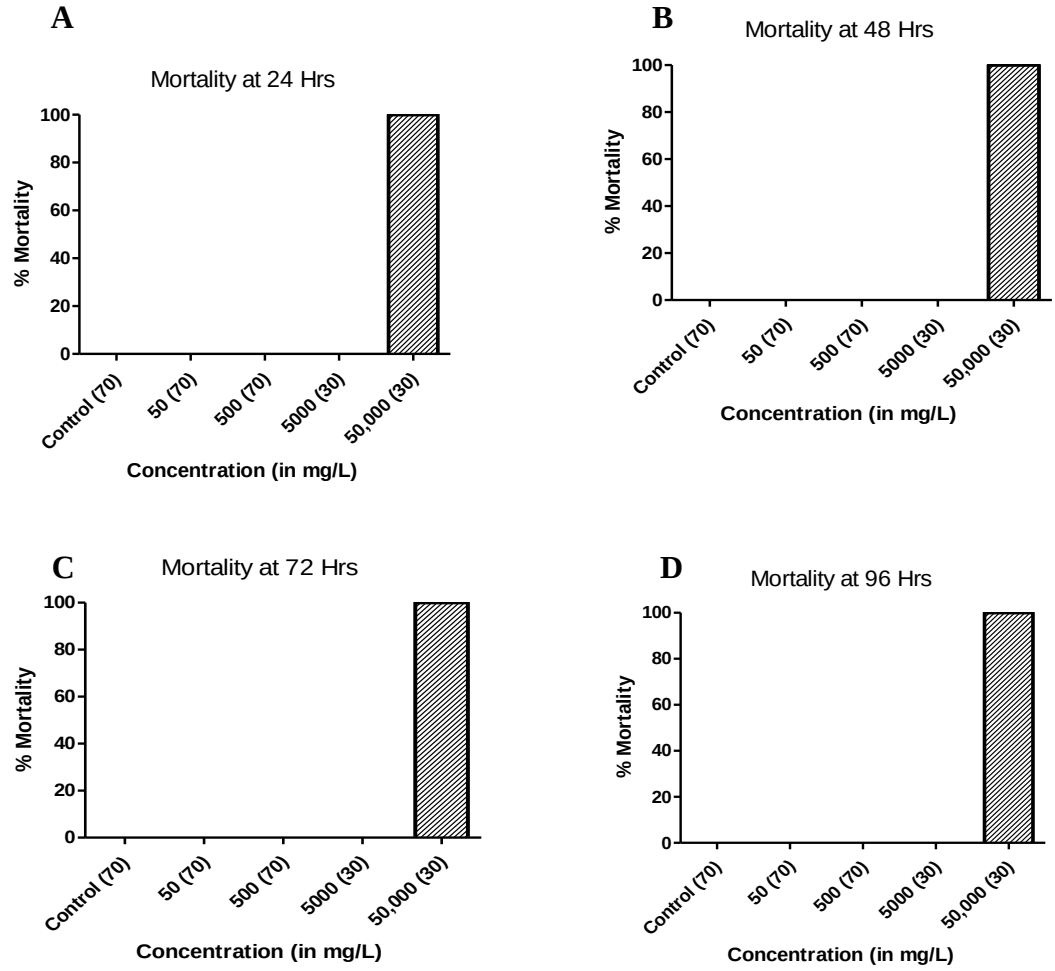

Figure 10.Effect of MSG on the mortality rate of embryos in group II: Four time points were selected, and mortality rates were plotted for control and MSG-treated group at A) 24 B) 48 C)72 D) 96 hours post-treatment. Data is represented as mean± SEM for 30 -70 embryos in each group.

5.3.3 Comparison of mortality rates from the group I and II

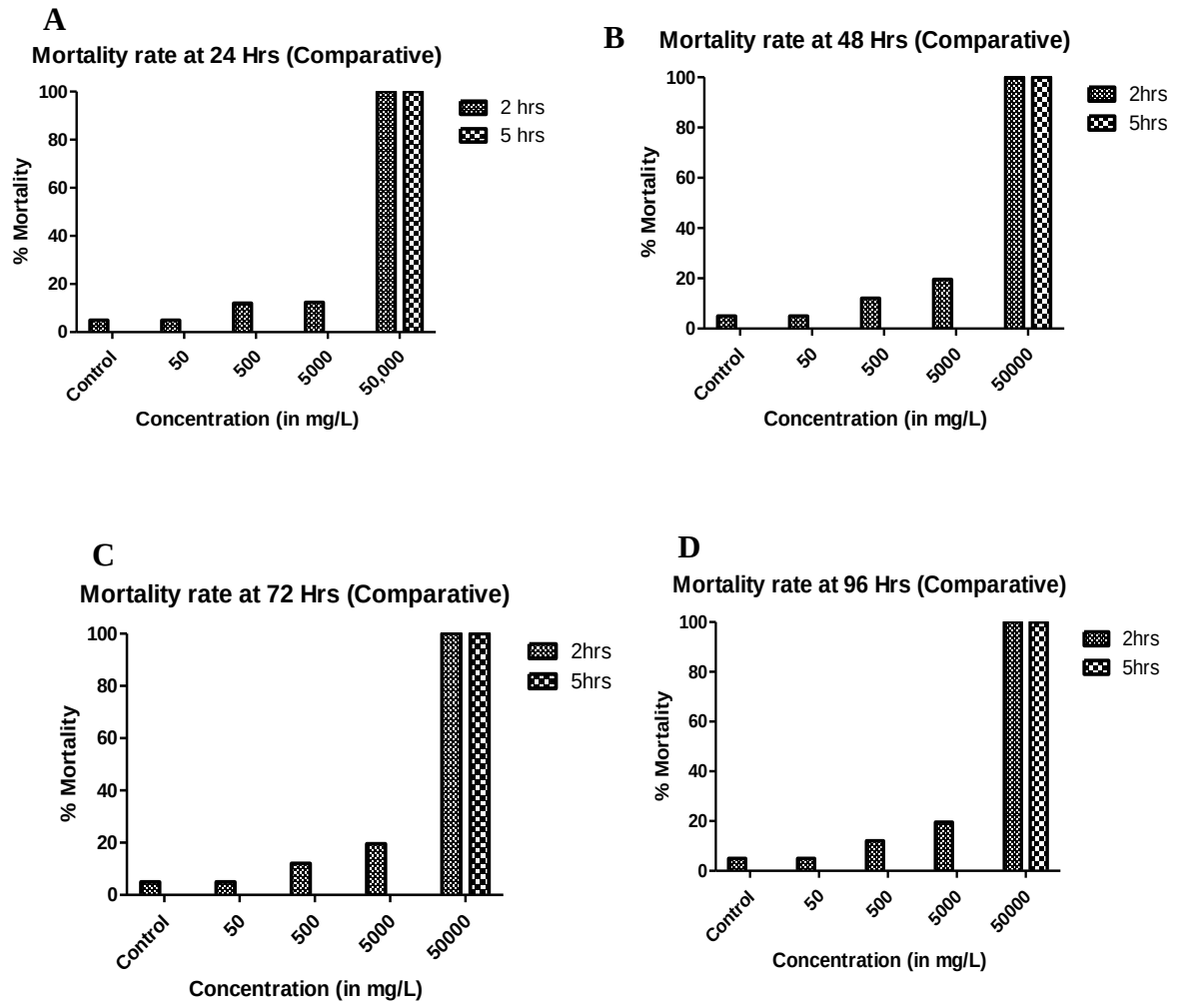

Figure 11.Comparison of mortality rates from the group I and II: Four time points were selected, and mortality rates from the group I and II were plotted together for control and MSG-treated group at A) 24 B) 48 C)72 D) 96 hours post-treatment. Data is represented as mean± SEM for 58-60 and 30 - 70 embryos in group I and II respectively.

5.4 Effect of MSG on hatching rate

Hatching marks a crucial developmental process in the zebrafish life cycle. It is characterized by the shedding of the outer layer called the chorion. Hatching was observed at different times points: 36 , 44 , 48, and 72 hrs. post-treatment for both the group I and II. No hatching in any group was observed at 36 hrs post-treatment. Hence, all the graphs are plotted, considering only three time points. As depicted in Fig.14, the hatching in group I embryos were delayed

compared to the hatching in group II embryos. In 5000mg/L concentration of group I, 30% of the embryos did not hatch even at 72 hrs post-treatment (Fig. 12C). In our lab, most of the embryos hatch by 48 hrs. In group II, the hatching rate at 48 hrs for control, 50, 500 and 5,000 mg/L of MSG is 95%±5, 89.59%±2.915, 95%±5 and 95%±5 respectively, whereas for group I, the corresponding values are 86.21%±13.80, 68.29%±7.575, 71.81%±10.95 and 24.05%±17.36 (Fig.12A-D and 13A-D). This data clearly shows that MSG induces delayed hatching when exposed during early stages of development at higher concentration.

5.4.1 Effect of MSG on hatching rate of embryos in group I

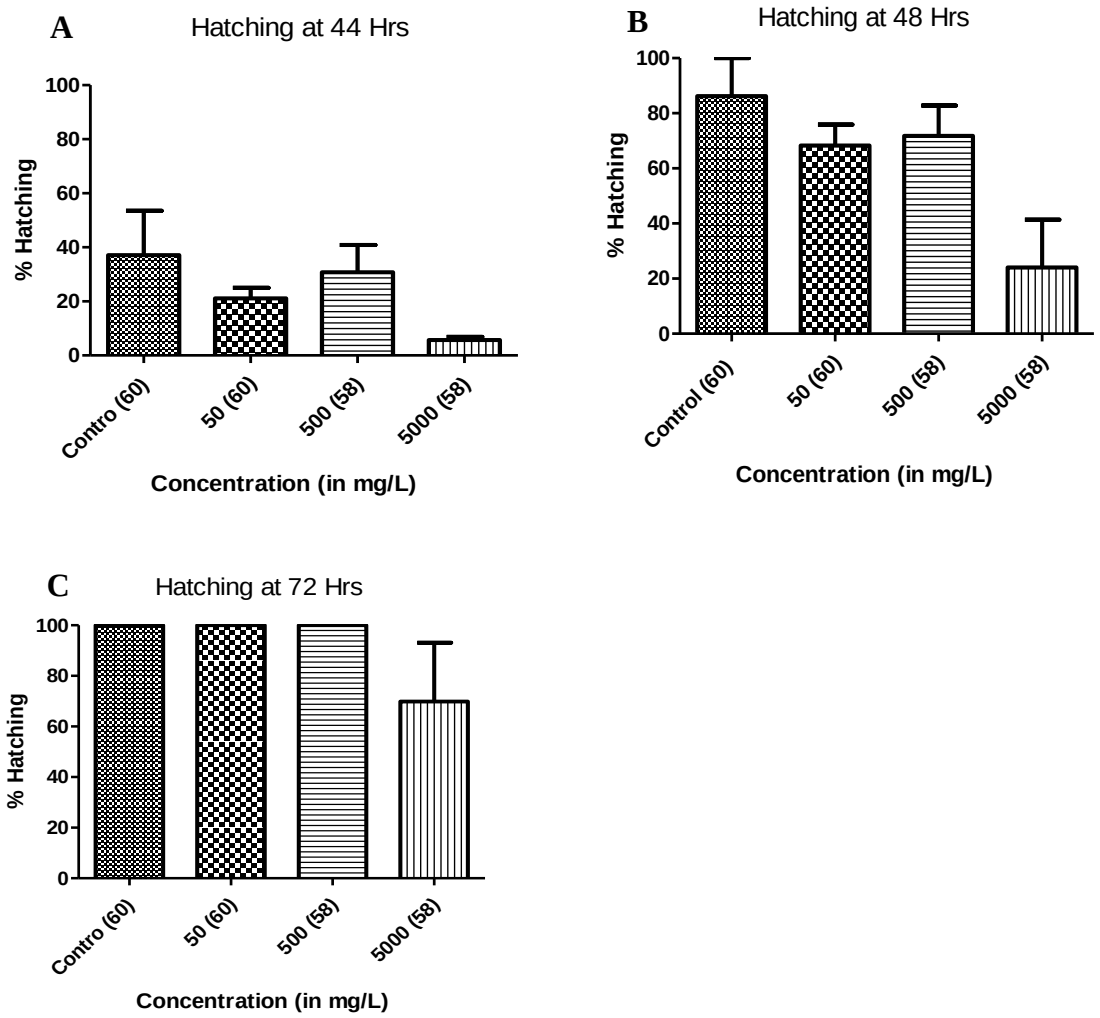

Figure 12.Effect of MSG on hatching rate of embryos in group I: Three time points were selected, and hatching rates were plotted for control, 50mg/L, 500mg/L and 5000mg/L MSG-treated group at A) 44 B) 48 C)72 hours post-treatment. Data is represented as mean± SEM for 58-60 embryos in each group.

5.4.2 Effect of MSG on hatching rate of embryos in group II

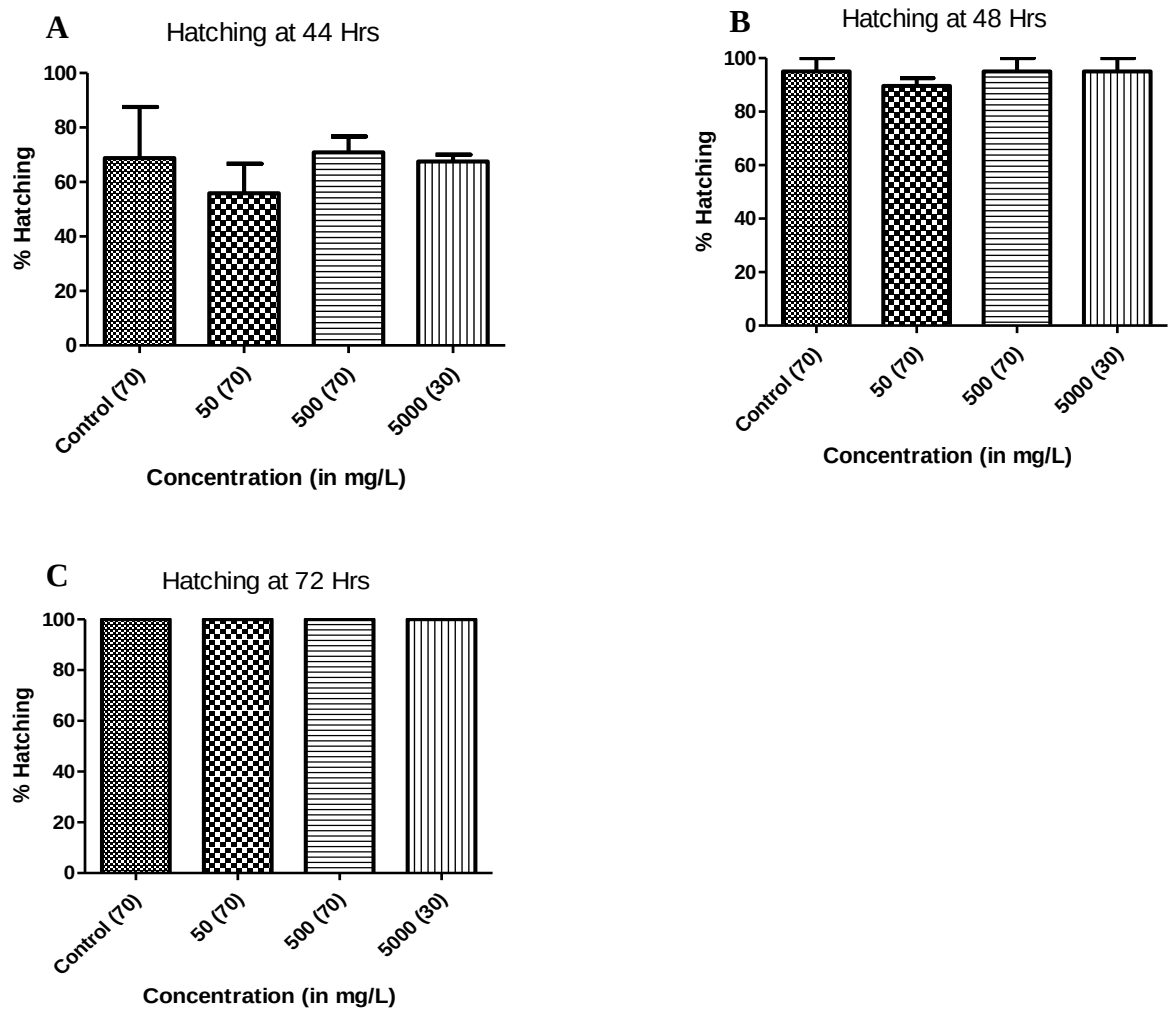

Figure 13.Effect of MSG on hatching rate of embryos in group II: Panel (A-C) represents three time points of 44, 48- and 72-hours post-treatment hatching rates plotted for control, 50mg/L, 500mg/L, and 5000mg/L MSG-treated group. Data is represented as mean± SEM for 30-70 embryos in each group.

5.4.3 Comparison of hatching rates from the group I and II

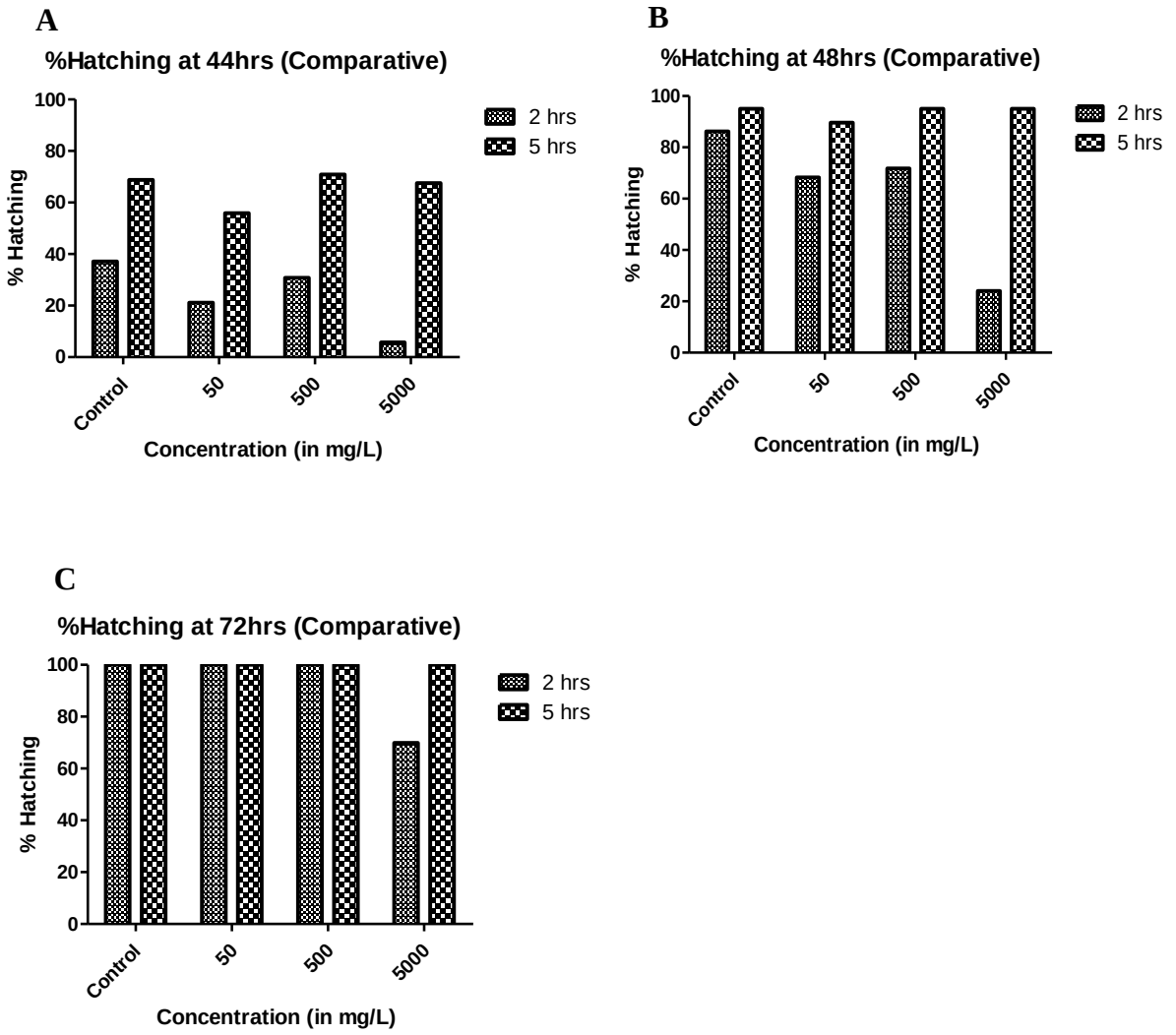

Figure 14.Comparison of hatching rates of group I and II: Three time points were selected and hatching rates of the group I and II were plotted together for control and MSG-treated group at A) 44 B) 48 C)72 hours post-treatment. Data is represented as mean± SEM for 58-60 and 30 - 70 embryos in group I and II respectively.

5.5 MSG and morphological abnormality in zebrafish embryos

For 4 days, daily, all the embryos were observed individually for morphological abnormalities, and their images were captured. With an increase in the MSG concentration from 500 to 5000mg/L in group I, the % abnormality increased from 9.09%±9.09 to 65.52%±34.39 (Fig.15A). Group II larvae displayed no signs of abnormality at any concentration, at any time point (Fig.16A-D). MSG induces abnormality in zebrafish larvae when exposed at an early developmental stage and at higher concentrations (Fig.17). The only abnormality observed in

this study was growth retardation (Fig.15D and 15E). A latency in hatching is observed at 5000mg/L concentration, which can be due to a higher number of growth-retarded embryos.

5.5.1 Effect of MSG on the morphology of embryos in group I

In group I, an abnormality was observed only on day 1. Hence, all the data on % abnormality is restricted to 24 hrs post-treatment embryos.

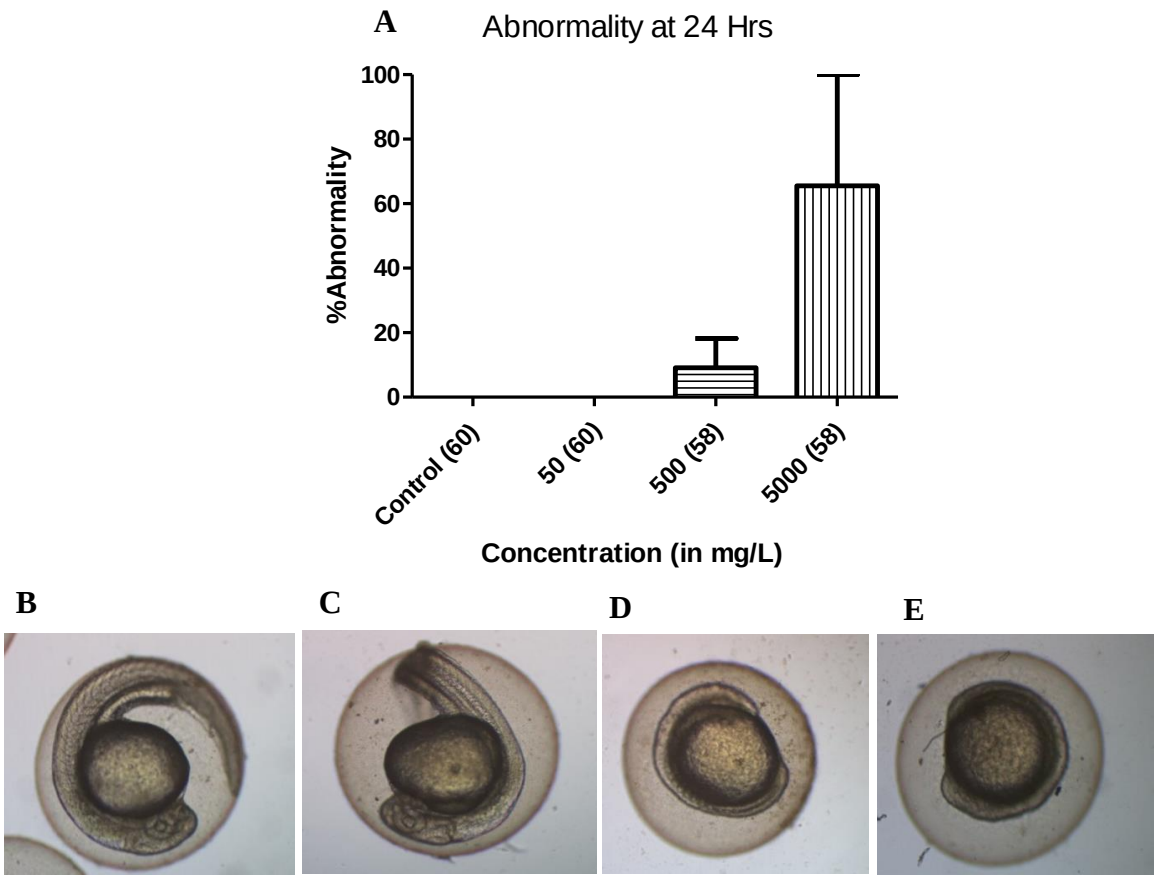

Figure 15.Effect of MSG on the morphology of embryos in group I: Abnormalities were quantified, and % abnormality data was plotted for control and treatment group at A) 24 hours post-treatment. Panel B) Representative image of control larvae; Panel C) Representative image of 50mg/L MSG-treated larvae; Panel D) Representative image of 500mg/L MSG-treated larvae Panel E) Representative image of 5000mg/L MSG-treated larvae. Data is represented as mean± SEM for 58-60 embryos in each group.

5.5.2 Effect of MSG on the morphology of embryos in group II

No morphological abnormality was observed in the group II embryos throughout the treatment period at any concentration.

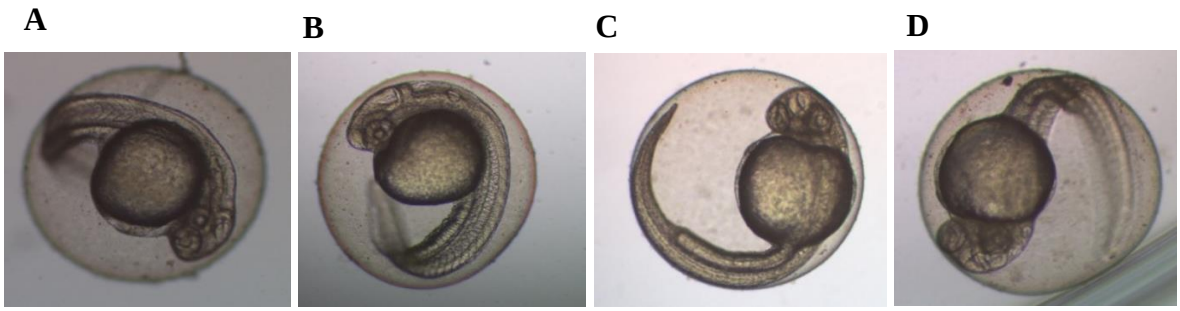

Figure 16.Effect of MSG on the morphology of embryos in group II: Panel A) Representative image of control larvae; Panel B) Representative image of 50mg/L MSG-treated larvae; Panel C) Representative image of 500mg/L MSG-treated larvae Panel D) Representative image of 5000mg/L MSG-treated larvae. All the representative images are captured at 24hrs post-treatment.

5.5.3 Comparison of %Abnormality data from the group I and II

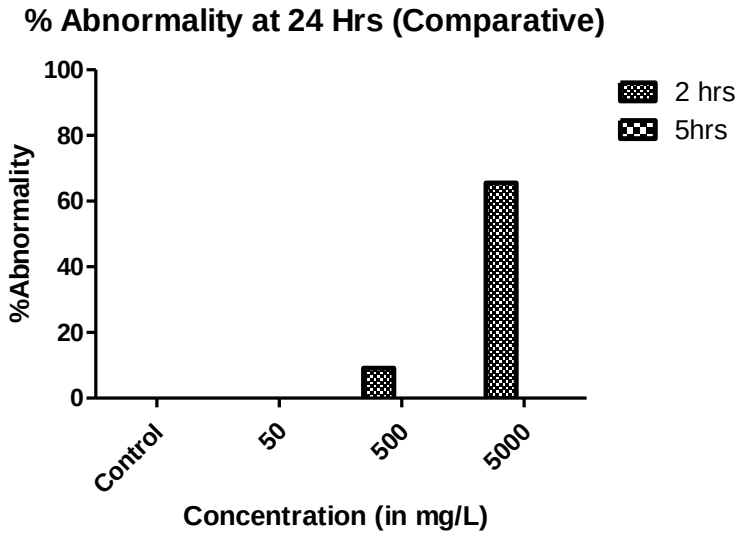

Figure 17.Comparison of %Abnormality data from the group I and II: % Abnormality data from the group I and II were plotted together for control and MSG-treated group at 24 hours post-treatment. Data is represented as mean± SEM for 58-60 and 30 - 70 embryos in group I and II respectively.

5.6 MSG and anxiety

Due to several reports claiming the involvement of MSG in inducing anxiety-like behavior, I performed a behavioral assay to assess anxiety in MSG-treated zebrafish embryos. Thigmotaxis is an indicator of anxiety-like behavior wherein the zebrafish larvae depicts a preference of edge/wall. 500 mg/L of MSG was the concentration of choice due to its relevance with the quantities consumed by humans. Also, this was the concentration of MSG in which morphological abnormality was first observed. Thigmotaxis was recorded on day 3 for control and 500 mg/L MSG-treated group. The incubation time before the start of treatment for this study was 2 hrs. The % thigmotaxis in control and 500mg/L MSG-treated group is 34.23%±4.230 and 37.50%±2.5, respectively (Fig.18). MSG did not induce anxiety in zebrafish larvae.

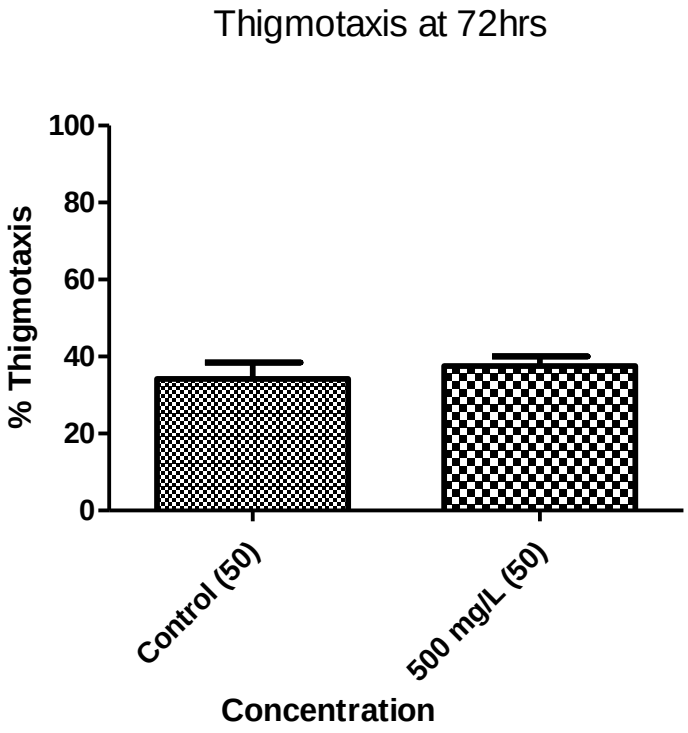

Figure 18.Effect of MSG on thigmotactic behavior of zebrafish larvae: % Thigmotaxis was quantified in control and 500mg/L MSG-treated group I larvae at 72 hours post-treatment. Data is represented as mean± SEM for 50 embryos.

5.7 MSG and oxidative stress

DCFDA ROS assay was used to measure oxidative stress in 3-days old control and treated larvae. After performing the thigmotaxis experiment, few larvae from control and 500mg/L MSG-treated group were randomly selected for oxidative stress measurement. Before the start of this experiment, the larvae were again observed under a microscope, and the ones with abnormality were excluded. The calculated fluorescence intensity is 605548 (a.u) ± 107589 and 569844 (a.u) ± 142510 for control and treated larvae respectively (Fig.19). Hence, the level of oxidative stress induced in control and treated embryos are comparably similar. This data indicates that MSG does not induce oxidative stress at a concentration of 500mg/L MSG in zebrafish larvae.

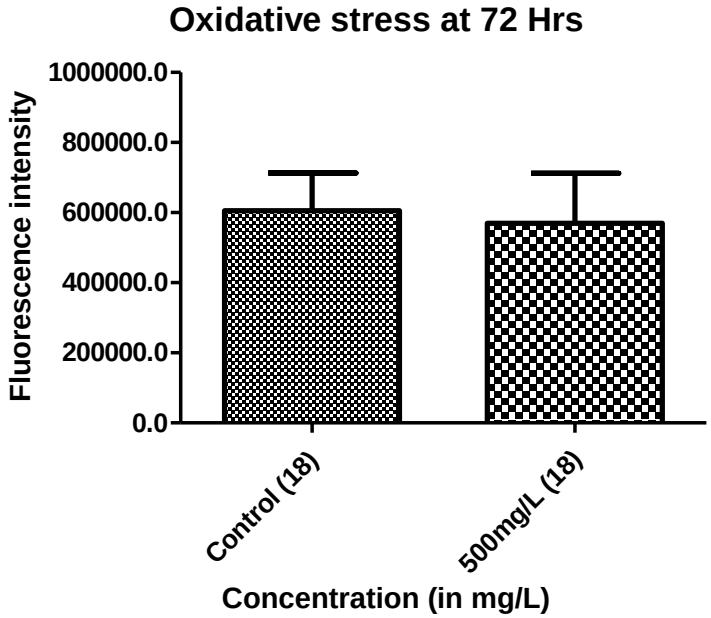

Figure 19.Effect of MSG on oxidative stress in zebrafish larvae: % Fluorescence intensity was quantified and plotted for control and 500mg/L MSG-treated group I larvae at 72 hours post-treatment. Data is represented as mean± SEM for 18 embryos.

DISCUSSION

Monosodium glutamate is a widely-used sodium salt of naturally occurring amino acid called glutamic acid. It is used abundantly and routinely as a flavor enhancer by the food industry (1). Though listed under the category of GRAS by the FDA, its increasingly undefined use in many food products might exceed the acceptable daily intake levels (3) . Studies were done to evaluate its safety using different animal models (21,23,24,26,31,33). Still, the data remains inconclusive and biased due to different study design using irrelevant concentrations. Apart from this, the mammalian models fail to provide insight into the critical developmental periods due to internal fertilization. This urged us to investigated the effect of MSG using zebrafish as an animal model. With reference to the OECD guidelines for Fish Embryo Acute Toxicity test (FET) (42) ; the hatching rate, mortality rate, and % abnormality were monitored in every 24hrs for a period of 4 days using appropriate control and 4 different treatment concentrations .

The treatment was initiated at two different developmental periods (Cleavage and Blastula) to study the temporal effect of MSG. The cleavage period in characterized by the rapid cell division to form an array of blastomeres. These blastomere undergo rapid cell rearrangements in the blastula period (41). Early start of MSG-treatment (i.e., at the time of cleavage) showed severe growth retardation at concentrations of 500mg/L and 5000mg/L (Fig. 15A). This data on MSG causing morphological abnormality in zebrafish larvae contradicts the study conducted by Mahaliyana AS *et al.* (31) who reported morphological abnormality at a low dose of 100mg/L. They observed a lack of pigmentation, growth retardation, scoliosis, yolk sac and pericardial edema at a concentration of 100-500mg/L in embryos and larvae. We speculate that this discrepancy in the data can be attributed to the different genotype of the fish, study design, and source of MSG. Most of the studies reporting toxicity has used laboratory-grade MSG, which though 99% pure, contain 1% of undefined impurities. Unlike food-grade products, these lab-produced chemicals do not undergo rigorous testing for undefined and harmful impurities like heavy metals. The product information sheet of L-Glutamic acid monosodium salt hydrate (Sigma-Aldrich, Cas number: 142-47-2) used in many studies clearly states that the product is not suitable for household or consumption purposes (43). Hence, it is critical in the study of this kind to carefully chose an appropriate food-grade source.

At 48hrs post-treatment, a delay in hatching was observed in embryos at 5000mg/L in group I (Fig. 12B). This delay in hatching could be correlated to the data on growth retardation as the embryos showing growth retardation took longer to develop and hatch.

The embryos treated at cleavage stage showed increased mortality rates compared to those treated at the blastula stage (Fig. 11). On the 3rd and 4th day of treatment, no mortality was observed in any group (Fig. 11C and 11D) indicating that the toxic effect of MSG is restricted to the early days of development when exposed at cleavage period in zebrafish. The mechanism behind this early period toxicity needs further investigation.

Previous studies on rodents have shown that MSG can induce anxiety-like behavior and other behavioral alterations (38–40). This prompted us to investigate if MSG can induce anxiety-like behavior in zebrafish. This has never been reported before. We did not observe any anxiety-like behavior induced in zebrafish larvae upon treatment with 500mg/L MSG after 3 days of treatment (measured in terms of % thigmotaxis) (Fig.18). To the best of our knowledge, this is the first behavioral study to analyze anxiety-like behavior in zebrafish larvae treated with MSG.

Many previous studies using rodent models have indicated that MSG can induce oxidative stress(34–36) and since anxiety and oxidative stress are related (44) , we wanted to know if MSG can also induce oxidative stress in zebrafish embryos without affecting their anxiety behavior. No significant induction of oxidative stress was observed upon treatment of larvae with MSG (Fig.19).

In summary, this study shows that the toxic effect of MSG is restricted to the early periods of development, particularly cleavage, and its effect can be seen in a dose-dependent manner. This study suggests caution in the consumption of high quantities of MSG-containing food; especially by children. At a relevant concentration of human consumption, food-grade MSG does not elicit anxiety-like behavior and oxidative stress in zebrafish larvae.

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