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Research Article

MONOSODIUM GLUTAMATE EXPOSURE AT EARLY DEVELOPMENTAL STAGE INCREASES APOPTOSIS AND STEREOTYPIC BEHAVIOR RISKS ON ZEBRAFISH (*DANIO RERIO*) LARVAE

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ABSTRACT

Excessive glutamate may give neurotoxic effects and contribute to Autism spectrum disorder(ASD). In this study, we investigated prolonged exposure effects of 10 µg/mL Monosodium Glutamate (MSG) on intracellular calcium level, *bax*, *bcl-2*, ratio of *bax*/*bcl-2* genes expression, caspase-3, apoptosis of brain cells and stereotypic behavior of Zebrafish (*Danio rerio*) larvae at early developmental stages. Genes expression were determined by real time PCR, caspase-3 using ELISA, intracellular Ca^{2+} and apoptotic cells of brain using confocal microscopy, locomotor activity by using crossing lines assay whereas stereotypic behavior by circle swimming. The results indicated that MSG exposure increased brain *bax* and *bcl-2*; and caspase-3; intracellular Ca^{2+}; and apoptosis; stereotypic behavior; and decreased locomotor activity. Termination of MSG treatments resulted in recovery of *bax*, *bcl-2*, caspase-3 basal levels and stereotypic behavior. In conclusion, MSG exposure at early embryonic stage increased brain cell damage and risk of behavior changes.

Key words: Monosodium glutamate, early embryo, duration, apoptosis, Behavior.

INTRODUCTION

Autism spectrum disorder (ASD) is a developmental disorder in children, with increasing estimated prevalence up to 1 in every 68 children in United States (Baio, 2014). Stereotypic behavior is one of the diagnosis criteria for ASD based on the Diagnostic and Statistical Manual of Mental Disorder V (DSM V) of the American Psychiatric Association (APA, 2013). ASD is the result of many genetic (Roberts *et al.,* 2014), environmental (Jones *et al.,* 2013) or both factors (Fatemi *et al.,* 2012).

Among many of suspected ASD etiologiesis the involvement of neurotoxin substances affecting in either pre-natal and/or post-natal development. Glutamate, an amino acid and main excitatory neurotransmitter in central nervous system is a neurotoxicant when accumulated excessively (Mattson *et al.,* 2008). Monosodium glutamate (MSG), commonly consumed as a food additive enhancing savory and delicious taste, is one of the source of glutamate. There are serious neurotoxic effects from MSG when administered to animals in very large doses (Gonzales-Burgos *et al.,* 2001). Extracellular glutamate activates NMDA receptors (N-methyl-D-Aspartate) which then trigger the build up of excessive Ca^{2+}in the cell (Rizzuto *et al.,* 2003). High amount of intracellular Ca^{2+} leads to increased permeability of the mitochondrial membrane, followed by opening of its pores and activates caspase cascade resulted in apoptosis (Siniscalco, et al., 2012). Apoptosis in brain is associated with ASD as reported in post-mortem studies in which an increased of p53 transcription factor and drastic reduction of Bcl-2 anti-apoptotic protein were detected in the frontal cortex and cerebellum areas (Fatemi *et al.,* 2001). *Bax*, a pro-apoptotic gene may also have an influence although studies in ASD are still limited.

Most of the studies emphasized on acute high doses of MSG effects in brain and conducted during post-natal development (Geha *et al,* 2000). Limited data with conflicting findings have been reported on effects of prolonged exposure of MSG, during fetal or early embryonic development (Husarova *et al.,*

2013). Some human studies concluded that glutamate did not pass placental barrier into fetal circulation (Beyreuther *et al.,* 2007; Walker *et al.,* 2000). In animal studies, others showed the opposites, oral MSG 2mg/g given immediately post-natal for 10 days (Ali *et al.,* 2000) and MSG 2g/kg body weight at 17 days of pregnancy resulted in decreased learning performance and hippocampal neuronal degeneration in the neonatal mice (Zhang *et al.,* 2010).

Zebrafish is a promising animal experimental model for study of excitotoxins, for instance MSG (Mahaliyana *et al.,* 2016). Almost all of Zebrafish genome has complete sequence and has ortholog to various disease gene in human, including to explore genetic basis of autism. The rapid of embryo development, the abundance of egg production, the transparency of embryo, and the external development ease researcher to observe and roll the study in a short period (Banerjee *et al.,* 2014). So far, the study of the effects of MSG exposure on Zebrafish was only performed to evaluate the effects of different concentration (Mahaliyana *et al.,* 2016; Abdelkader *et al.,* 2012). The study that focused on the effects of low dose MSG exposure with different duration on Zebrafish embryo stage development has never been conducted before.

Thus, the aim of our present study is to clarify the behavioral effect of long term MSG on zebrafish embryos exposed at the first developmental stages (2h post-fertilization) with different duration i.e. 24, 48, and 72h. We focused on mitochondrial-dependent apoptotic pathway and stereotyped behavior effects of the larvae.

MATERIALS AND METHODS

Zebrafish maintenance

Adult wild type zebrafish both sexes were obtained from local fish supplier and species identification was validated by experts from Indonesian Institute of Sciences. Zebrafish were kept in glass aquaria with 50L capacities, at 28.5°C, with 14:10h light-dark photocycle (Westerfield *et al.,* 2000). Fish were fed twice daily (Tetramin Tropical Fish Flakes, USA). Eggs were collected in the beginning of light period (Tiedeken *et al.,* 2005). All procedures were approved by Health Research Ethics Committee of Faculty of Medicine, Brawijaya University (Registration 608/EC/ KEPK S2/11/2014 and 045/EC/KEPK/ 01/2015).

MSG preparation and exposure

Monosodium glutamate, $C_5H_8NNaO_4$ H_2O (Sigma-Aldrich, CAS 142-47-2), was prepared as a 10µg/mL solution in distilled water (Abdelkader *et al.,* 2012). Fertilized eggs were washed twice and assigned into 4 groups i.e. (i) control (distilled water) and MSG treatments for (ii) 24; (iii) 48; and (iv) 72h started at 32–64 cell stage (approximately at 2hpf). Thirty eggs were placed into each well of 6-well plate filled with 8mL of 10µg/mL MSG solution or distilled water, in three replicates, to give a total of 90 eggs per group of treatment. Thirty percent of the solution was replaced daily with fresh one. Embryos were kept in incubator, at 28.5°C, with 14:10h light-dark photocycle (Westerfield *et al.,* 2000).

Embryo Monitoring Assay

Embryos were morphologically evaluated for body defects using stereo microscope (SZ-PT, Olympus, Japan). The body length was measured as the distance of the head to tail bud. Survival and hatching rates were monitored following exposure to MSG everyday (at 24, 48 and 72hpf). Embryos, 30 from each treatment, were checked for heartbeat at 72hpf post-treatment, also prior processing for molecular and behavior assays to ensure embryos were alive (Pramulawati *et al.,* 2014).

Intracellular Ca^{2+} imaging

Intracellular Ca^{2+} level was determined in 72hpf embryos, post-treatment, incubated in 60µg/mL Fura-2AM (Sigma Aldrich, Cat# 47989) containing 2.5% DMSO in embryo medium for 30min at room temperature (Muntean *et al.,* 2010). After washings,embryos were mounted in 3% methylcellulose (Sigma Aldrich) and positioned for confocal laser scanning microscopic imaging (Olympus IX81 and FluoViewFV1000). In depth pictures were captured by scanning at 100 and 200x magnifications (approximately 30 focal planes/embryo).

Table I. Gene specific primers used for quantitative real time PCR

Gene	Forward primers (5'-3')	Reverse primers (5'-3')	Accession number
Bcl-2	TTGTGGAGAAATAC CTCAAGCAT	GAGTCTCTCTGCTGA CCGTACAT	BC133848 (Abdelkader *et al.,* 2013)
Bax	GAGCTGCACTTCTC AACAACT	CTGGTTGAAATAGCC TTGATGAC	BC055592 (Abdelkader *et al.,* 2013)
ß-actin	CGAGCAGGAGATG GGAACC	CAACGGAAACGCTCA TTGC	AF057040 (McCurley *et al.,* 2008)

Fluorescence intensity was recorded at 340/380nm extinction and 515nm emission fluorescent ratio, calibrated with [Ca^{2+}] values offline. Regions of interest were selected and analyzed using Fluoviewver 1.7a and ImageJsoftwares computed for total of fluorescence intensity =total pixel area×average pixel intensity of positive signals per μm in the brain.

Apoptosis assay

Brain cell apoptosis was identified with 5μg/mL acridine orange(AO) staining (acridinium chloride hemi-[zinc chloride], Sigma-Aldrich) for 60minutes (Parng *et al.,* 2006). Four 72hpf embryos/group were observed in depth (approximately 50 optical slices/embryo) confocal imaging with acridine orange filter setting. Positive signals of apoptotic cells were defined by fluorescence intensity and size of particle exhibited a punctate AO staining pattern in the brain.

RNA Extraction and real time PCR of *Bcl-2* and *Bax*

Brain tissue from 6 larvae from each group were collected using glass microcapillaries at 7 days after fertilization (Vargas *et al*., 2011). Brain tissue was homogenized for RNA isolation according to TRI reagent® (Sigma-Aldrich). Isolated RNA was checked for quality (A260/280nm 1.8-2.2) and quantity (NanoDrop Spectrophotometer 1000A). Real time assays were performed according to i-Green™One Step qRT-PCR Kit (Intron Biotechnology, Cat# 25107) using a LightCycler® 1.5 Instrumen (Roche Diagnostics, USA). PCR primers (Table I) were used and the thermal profile for real-time was 42°C for 15min; 95°C for 10min; followed by 35 cycles of 95°C for 15s; 60°C for 60 s; and 50°C for 60s. *ß-actin* was used to normalized the results. Each mRNA level was expressed as a ratio to *ß-actin* mRNA. Three replicates (pools of brain tissues) and three technical replicates of each RNA sample were performed. Relative mRNA expression for each gene was calculated as a fold change compared with the control group.

Enzyme Linked Immunosorbent Assays (ELISA) of Caspase-3

Brain tissues from 7 dpf zebrafish larvae, visualized under stereomicroscope (Olympus SZX16), were collected using glass microcapillaries (Vargas *et al*., 2011) and used for protein isolation (RIPA-PIC, Thermo Scientific). Caspase-3 levels were analyzed by using ELISA(MyBioSource, Cat# MBS012786). Six embryos were utilized/assay/group, repeated 3 times, totaling 18 embryos/group.

Behavior assays

Locomotor activities were tested on 24 larvae/group aged 6 dpf when spontaneous and reflective swimming competencies were mostly attained (Guo *et al*., 2004). Each well of a 12-well plate, containing one larva/well, was gridded with horizontal and vertical lining crosses each other dividing each well into 4 compartments. Larvae were video recorded for 1min twice andcounted for total number passing the lines (Ingebretson *et al.,* 2013).

Ten post-treatment larvae/group was analyzed for stereotypic behavior (Stewart *et al.,* 2013; Stewart *et al.,* 2011) on 3 and 7 dpf and repeated thrice (total 30 larvae/group). Circle swimming was monitored on larvae, placed in 54mm diameter Petridishfilled with water at 27°C, following a touch of stimulus on their tail.

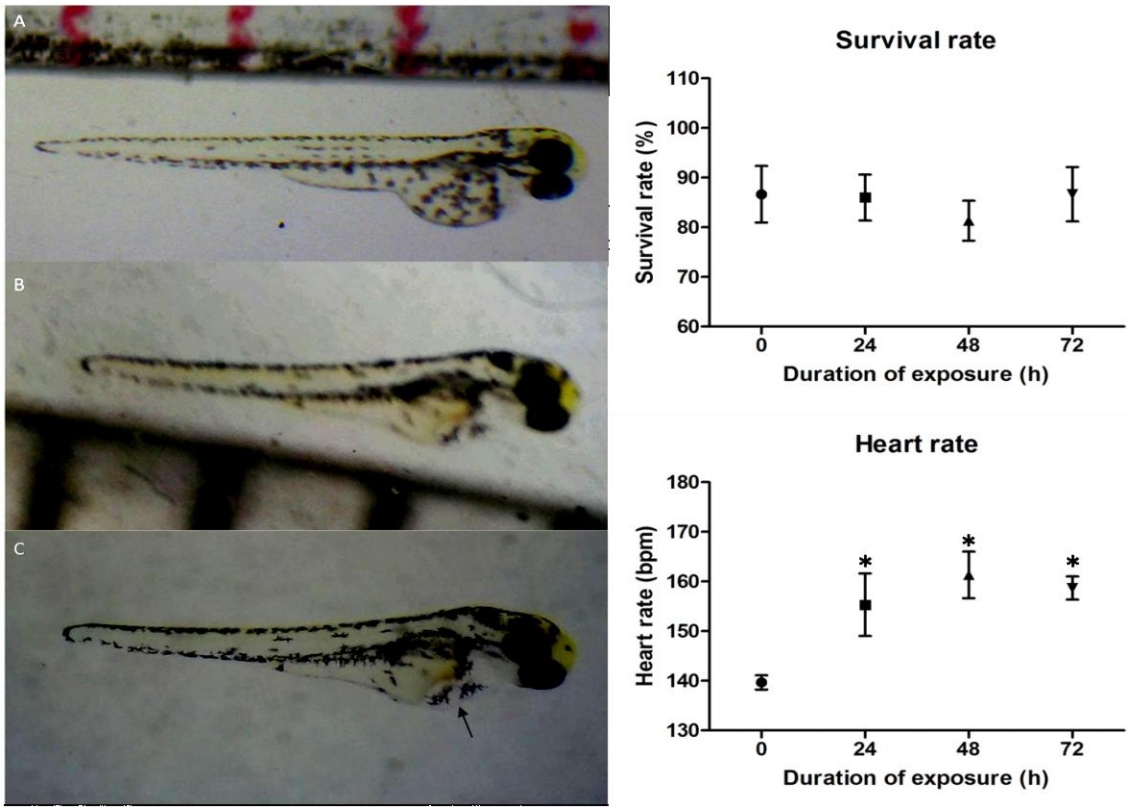

Figure 1. Morphology, survival and heart rate following MSG treatment at early developmental stage. In group treated with MSG for 72h, there were embryos exhibited shorten body length (B) and cardiac sac edema (C, arrow) which not found in control group (A). Survival rates were not affected with MSG, but heart rate increased significantly (p=0,0249) compared with that of control untreated (*).(n=3 embryos/group)

Statistical analysis

Data, shown as mean ± SEM,were screened for normality and homogeneity, and differences analyzed using ANOVA,Kruskal-Wallis or Fisher-Exact (GraphPad Prism 5 software), with $p<0.05$ considered as statistically significant.

RESULTS AND DISCUSSION

Morphological changes, heartbeat larvae and monitoring assays

We did not observed inter-group differences in survival (~86%; p=0.8495) and hatching rate (all hatched), however heartbeat rates of larvae exposed to MSG were higher than control group (Figure 1). Heartbeat rates of control group were ~140bpm, whereas in MSG groups >150bpm (p=0.0249). Morphologically, we found there were two abnormal embryos from 72h MSG treated group, one with shorten body length and one with cardiac sac edema (Figure 1).

Early exposure of MSG Increases Calcium; *Bax*; *Bcl-2*; caspase-3 levels and apoptosis of brain cells

Following MSG treatment at early embryonic stage for 24, 48, and 72h, levels of calcium, *bax*, *bcl-2*, and caspase-3 increased significantly compared with that of control untreated group. Calcium increased with MSG exposure duration ($p<0.0001$). The relative mRNA expression of *Bax* and *Bcl-2* were normalized against *ß-actin* (housekeeping gene) and expressed as ratio to the control group (Figure 2-3). Compared with control group, *Bax*; *Bcl-2*; and the ratio between *Bax*/*Bcl-2* were higher in 48 and 72h groups than control

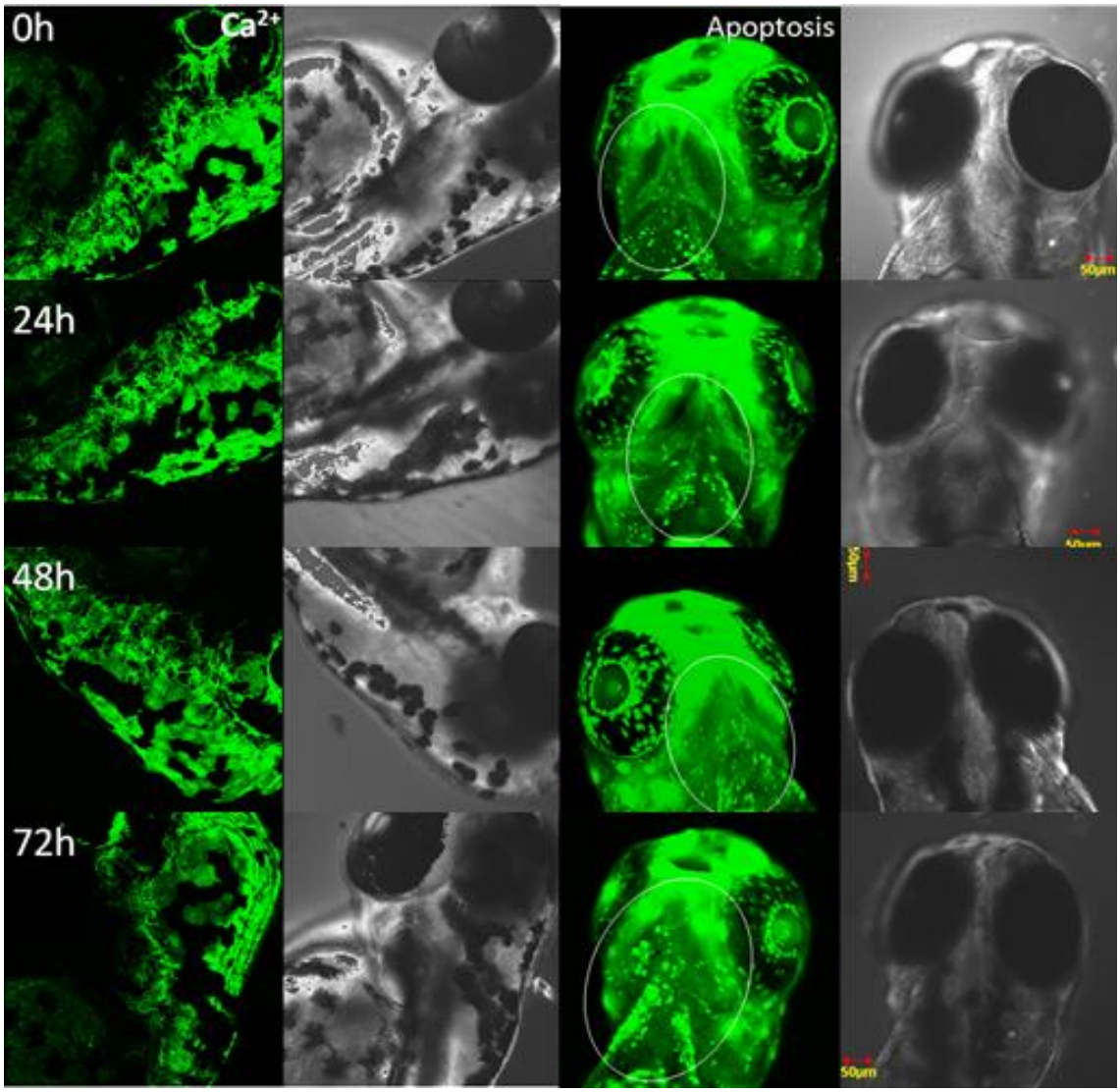

Figure 2. Confocal microscopic observation of calcium and apoptosis embryos treated with MSG. Level of intracellular calcium and apoptosis were observed in embryos 72dpf following MSG treatment for 24; 48; and 72 h and compared with control untreated (0h). Apoptosis increased in cerebellum (circled area) and other part of brain. Fluorescence images were shown as flatten z-stack images of 40-50 optical slices/embryo (n=4 embryos/group), accompanied with their corresponding bright field images.

and 24h MSG incubation groups (two-way Anova $p<0.0001$; Tukey Multiple Comparison for ratio of *Bax/Bcl-2* 48 h vs control $p=0.0170$; 72h vs control $p=0.0209$). Caspase-3, on the other hand, sharply increased at 72h incubation with MSG.

Brain apoptosis were higher in MSG treated groups than control group with ratio of apoptosis of MSG treated/control groups of total brain ranged 1.14-3.99 (One-way Anova $p=0.046$), whereas cerebellum region ranged 1.43-268.41 (Kruskal-Wallis $p=0.0092$). Apoptosis was highest in group of early embryos treated with MSG for 72h, reaching on average for total area of brain 2.5 times and for cerebellum area alone 82 times higher than control group.

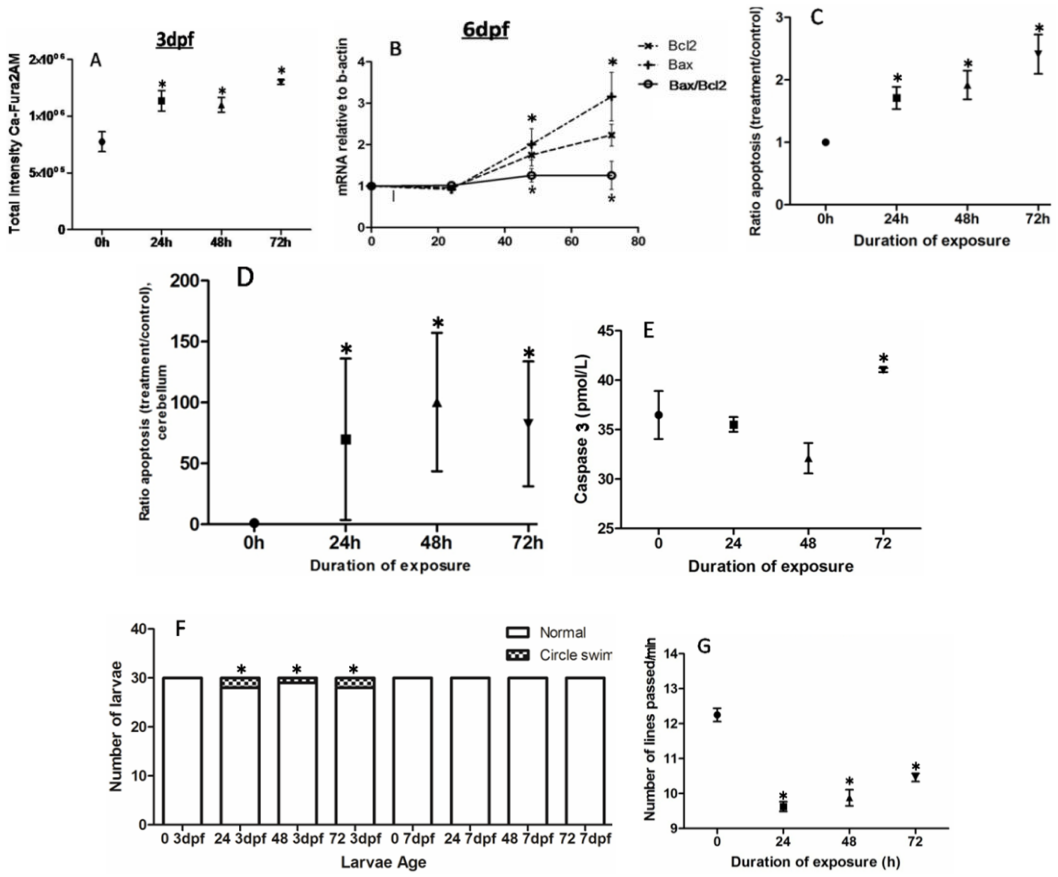

Figure 3. Molecular analysis of mitochondrial pathway apoptosis and behavioral assays. In embryos 3 dpf, levels of intracellular calcium (A); apoptosis (total brain and cerebellum, C and D, respectively); and circle swim events (F) were high following MSG exposure. On day 6 post-fertilization (6 dpf), Bax; Bcl2 and their ratio (B); as well as caspase-3 (E) were still high following MSG treatment (n=18 embryos/group), locomotor activities per minute were lower than control group (n=24 larvae/group) (G), whereas circle swim event was not detected on 7 dpf in all larvae groups (F). (*) denotes significant differences compared with the control group ($p<0.05$). Data A-E and G were represented as means ± SEM

MSG Increased Circle Swimming Events and Decreased Locomotor Activity

Tactile sensibility which measured by circle swimming assay, was affected by MSG, detected immediately following exposure (3dpf embryos) in all treated groups (Fisher Exact one tailed $p=0.039$; OR=28.11). Interestingly, further observation on 7dpf larvae, the circle swimming deviation was not detected in anygroups (Figure 3F). Early embryonic exposure of MSG starting at 1dpf for 24-72h resulted in decreased locomotor activity (one-way Anova, $p=0.0056$) observed in larvae 6dpf (Figure 3G).

Result of this study indicated that MSG exposure at a dose 10μg/mL for 24, 48 or 72h during early embryonic development increased heart rates of the embryos and risk of heart and body defects. Those observation were not the main parameters in this study, yet indicated that 10μg/mL MSG may increase risk of developmental defects.

Prolonged exposure of MSG for 24-72h during early embryonic development activated mitochondrial apoptotic pathway in the brain detected on day 3 and 6 post-fertilization larvae. Previous studies by others demonstrated similar increase of apoptosis following MSG

exposure. Chronic accumulation of MSG induced pyramidal neural loss in cortical organotypic culture (Young *et al.,* 2007) and retinal ganglion cell death (Vorwerk *et al.,* 2000). Twenty hours exposure increased cytotoxicities of 1mM or 10mM glutamate on differentiating N1E-115 neuronal cells; morphological changes observed as neurites retraction and cell shrinkage leading to apoptosis (Schelman *et al.,* 2004). Our data showed that prolonged exposure of MSG during early embryo development at dose 10µg/mL (50 µM) increased brain apoptosis. The glutamate of MSG may cause brain apoptosis and behavior changes through its agonist effects on NMDA receptors (Rizzuto, *et al.,* 2003). NMDA receptors are ionotropic receptors mediating glutamatergic neurotransmission and play a role in several basic functions in the central nervous system, from regulating neurodevelopment and synaptic plasticity, learning and memory formation, cognitive processes, rhythm generation necessary for locomotor activity and breathing, and excitotoxicity (Gonda *et al*., 2012). The longer duration of MSG exposure caused higher accumulation of glutamate in extracellular space of the brain, resulted in overstimulation of NMDA receptor.Next, calcium channel opened, allowing more Ca^{2+}influxin the cell (Rizzuto *et al*., 2003). High amount of intracellular Ca^{2+}led to increased permeability of the mitochondrial membrane, followed by opening of its pores, released cytochrome-c and activated caspase cascade resulted in apoptosis. As evidenced in our study, observed in embryos 3dpf, brain intracellularCa^{2+} increased in all groups following MSG treatment (Figure 2-3). Such high amount of intracellular $Ca^{2+}$was associated with apoptosis in the brain.

Apoptosis is an important pathogenesis of ASD. Human studies showed that number of Purkinje cells decreased in cerebellum of autistic persons postmortem (Fatemi *et al.,* 2001); the decreasing of number and increasing size of neuronal cells may cause cortex malformation which triggers electric waves resembling epileptic known as epileptiform (Casanova *et al*., 2013). Glutamate exposure in Zebrafish early embryos causes apoptosis in the brain, the developing nervous system. Apoptosis was highest in group of early embryos treated with 10µg/mL MSG for 72h, reaching on average for total area of brain 2.5 times and for cerebellum area alone 82 times higher than control group. Cerebellum involves in receiving sensory information and coordination of motoric movement, whereas its cortex is the center of Purkinje cells integrating two circuits of afferent nervous and transmitting stimuli responses into other brain structures (Heap *et al.,* 2013). Therefore, high signal of apoptosis in cerebellum and cortex area, may result in disturbance of motoric movement. Indeed, regardless of the duration of MSG exposure, our observation showed thatsuch apoptosis was associated with increased events of circle swims in 3dpf embryos, indicated that MSG increased the risk for stereotypic behavior changes.

Circle swimming is one form of stereotypic behavior, the anxiety-like phenotype,observed in zebrafish as an ASD model (Stewart *et al.,* 2013; Kyzar *et al.,* 2012).Our study showed that prolonged exposure of 10µg/mL MSG caused circle swimming in 18.33% of larvae (OR 28.11), whereas the rest 81.67% remained normal, which means that same MSG exposure did not produce same effect in all larvae. As in ASD ethiopathogenesis may involved both environment and genetics factors (Gentile *et al.,* 2013), the affected zebrafish larvae showing circle swimming may have genetic susceptibility against MSG, which needs further studies to clarify. Many mutations associated with autism have been characterized (Broek *et al.,* 2014), however, which mutation(s) involved in MSG susceptibility causing stereotypic behavior changes are to be identified.

Of note, the circle swimming behavior was not detected in all 7 dpf larvae, including those previously showed the behavior on day 3 post-fertilization. Such phenomenon may be explained first,due to the methods of observation, or secondly, by recovery mechanism(s). Firstly, the growth of zebrafish larvae changes fish's body size and shape, swimming movements and the hydrodynamics governing its propulsion (Muller *et al.,* 2004). Larvae 7dpf are longer than 3dpf and shows slower circle swim, thus, it is possible that the differences of circle swimming in normal vs

MSG treated larvae were too subtle to detect. Secondly, zebrafish larvae possess a mechanism to recover from glutamate toxicity and brain regenerative capability. An in-vitro study showed that increased Ca^{2+} detected during physiologic glutamate treatment returned to its basal level when glutamate was removed from media (Vaarmann *et al.,* 2013). Similar homeostasis may also be at work during MSG termination in our zebrafish experiments.

In MSG treatment groups, high expression of *Bax* detected in 6dpf larvae was accompanied with *Bcl-2* increased. As anti-apoptosis, *Bcl-2* inhibits apoptosis by forming complex with *Bax*,therefore the ratio of *Bax/Bcl-2* is significant. Ratio of *Bax/Bcl-2* more than 1 will turn the balance of pro/anti-apoptosis towards apoptosis, activating caspase signaling (Chan *et al.,* 2007). *Bcl-2* is regulated by brain-derived neurothropic factor (BDNF) (Sheikh *et al.,* 2010) which expressed in 24h-7dpf zebrafish embryo, including in the brain (Felice, et al., 2014). The levels of *Bax*, *Bcl-2*, ratio of *Bax/Bcl-2* and caspase-3 of 6 dpf larvae were higher in 72h MSG treatment than in 24 and 48h counterparts. These observed patterns may be associated with the time of recovery of the brain following termination of MSG treatment. On day 6, larvae treated with MSG for 24; 48; and 72h had 5; 4; and 3 days, respectively, to recover from MSG brain toxicity.

Zebrafish possesses excitatory amino acid transporter-2 (EAAT2) which removes glutamate from synapses, dissipating glutamate stimulation on NMDA receptor. Zebrafish brain was also capable synthesizing dopamine, known as protectant against toxic effect of glutamate (Vaarmann *et al.,* 2013; Holzschuh *et al.,* 2001). One or all together may operate to reverse condition back to basal, thus, *Bax*, *Bcl-2*, and caspase-3 decreased, reached normal level in 24h MSG treated embryos, 5 days post-cessation of MSG. Our observation suggested that *Bax*, *Bcl-2* and caspase-3 expressions were associated with MSG treatment. Our results were different from Fukui *et al.* (Fukui *et al.,* 2009) study which showed that glutamate induced-apoptosis was independent of *Bax*, *Bcl-2* and caspases. Such discrepancy may be due to the experimental system used, we studied in-vivo zebrafish observing the whole brain, whereas Fukui *et al.* (Fukui *et al.,* 2009) used in vitro hippocampal cells. Another mechanism to recover from apoptosis due to excessive glutamate toxicity is thatzebrafish may regenerate cells to replace the affected brain cells. Zebrafish hindbrain was demonstrated as highly plastic, facilitated by FGF signaling, able to regenerate and repattern ablated cerebellum, which resulted in no obvious locomotive malfunctions (Koster *et al.,* 2006). Successful cells regeneration and neural network repair will restore the behavior, which may explain the disappearance of circle swimming stereotypic behavior of embryos after MSG exposure ended. Further studies are warranted examining whether such regeneration ensues in all brain area affected by MSG.

In contrast with circle swimming, the locomotor activity observed as number of lines passed per minute decreased significantly in 6dpf larvae in all groups treated with MSG. The locomotor rates were slightly higher in 72h MSG treatment than in 24 and 48h counterparts.Motor behavior, the locomotion, is generated by specific neural circuits in the spinal cord (Kyriakatos *et al.,* 2011). The motor pattern is mediated by activation of ionotropic glutamate and glycine receptors in the spinal cord. Prolonged NMDA agonist such as MSG may change the shape of the synaptic drive and action potentials in motoneurons. Despite the possibility that NMDA receptor stimulation and apoptosis came to end and brain regeneration ensued when MSG exposure was ended, the recovery of brain cells and functions may not be complete. As the development of brain morphology; neuronal networks; and functions are spatial and temporal-specific, and/or some areas may need longer time to recover, successful in-time repair will restore the behavior, whereas imperfect repair of the affected brain cells may cause associated behavior deviances stay or even worsen with time. In human studies, ASD brain growth is associated with overgrowth at early-age which decelerated during later-age (Courchesne *et al.,* 2001) but with reduced coherence within frontal regions and between frontal and all other regions (Murias *et al.,* 2007). Similar scenario may operate in zebrafish following MSG-treatment-termination during early-embryo development, resulted in partial

recovery. Thus, larvae remained with decreased locomotor activity as shown in this study.

This study showed that prolonged exposure of MSG, 10µg/mL, started on day 1 post-fertilization for 24-72h, increases significantly intracellular calcium and brain apoptosis. Such apoptosis increase the risk and was associated with behavioral changes i.e. increase of circle swim events upon stimuli observed on day 3. Following termination of MSG, some of normal behavior aspects i.e. the circle swimming of 7dpf, but not locomotor activity of 6dpf larvae, may recover. Further study are required to clarify such recovery which probably through brain detoxification of glutamate and cells regeneration.The result may be relevant for human condition. Conclusion, MSG exposure at early embryonic stage increased brain cell damage and risk of behavior changes.

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