ORIGINAL ARTICLE

Subchronic Administration of High-dose Monosodium Glutamate Causes Spatial Memory Dysfunction and Structural Deficits of Rat Hippocampus

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ABSTRACT

Introduction: The toxicity of high concentration monosodium glutamate (MSG) has become a controversial issue because of its inconsistent results in human and animal studies. This present study aims to evaluate the effect of subchronic high-doses oral administration of MSG on spatial memory performance and hippocampal pyramidal cells number. **Methods:** This study involved twenty-eight male Wistar rats, which were divided into a control group of NaCl 0.9% and three intervention groups of MSG 1.0 mg/g bodyweight (M1), 2.0 mg/g bodyweight (M2), and 4.0 mg/g bodyweight (M3) for 30 days. Statistical analysis used a One-way ANOVA test. **Results:** The result showed significant differences in spatial memory on the Morris Water Maze (MWM) test, including path length ($p = 0.020$) and escape latency ($p = 0.011$) according to general linear model repeated measurement analysis. The mean difference of estimated hippocampal pyramidal cells total number among the groups showed volume ($p = 0.001$), numerical density ($p = 0.590$), and cells number ($p = 0.004$). Furthermore, Post-Hoc analysis in both spatial memory and hippocampal pyramidal cells showed that the increasing MSG dose from 1.0 to 4.0 mg/g bodyweight led to a decrease in the results of spatial memory performance on the MWM test and a decrease in hippocampal cells. **Conclusion:** The present study has provided novel quantitative data that subchronic administration of high-dose MSG caused deleterious effects on the spatial memory function and the volume and number of hippocampal pyramidal cells.
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Keywords: Cognitive, Hippocampus, Monosodium glutamate, Spatial memory, Stereology

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INTRODUCTION

Monosodium glutamate (MSG) is an L-glutamate salt well-known as a taste enhancer in instant foods (1). The average MSG consumption in Asia is 1.4-2.3 g/day, in Europe 1.0 g/day, in developed countries 0.3-1.0 g/day (2). As the central nervous system's main excitatory neurotransmitter, glutamate contributes a normal functional role in nerve conductivity, learning process, synapses plasticity, and memory formation (3). However, a high glutamate concentration seems to bring about neurotoxicity leading to severe nerve damage (4). The Expert Committee on Food Additives of WHO in 1971, 1974, and 1988, the European Scientific Committee for Food, and the Federation of American Societies for Experimental Biology concluded that MSG was safe for humans (5). The acceptable daily intake of MSG based on Food and Agriculture Organization recommendations ranged from 0 – 120 mg/kg bodyweight each day. In contrast, several animal studies have shown that MSG is possibly toxic to the kidney (15 mg/kg bodyweight), liver (5 mg/kg bodyweight), ovaries (0,08 mg/kg bodyweight), brain (3 g/kg bodyweight) and orofacial structures (500 mM) (6–11). Nevertheless, the toxic effects of MSG have been a controversial issue due to inconsistent results in human and animal studies.

Several studies emphasizes the processing of scene or spatial memory as distinct roles performed by hippocampus. External stimuli such as spatial and scenes environments are almost perfectly recognized by the

interconnection between hippocampal pyramidal cells and neuron within primary cortical visual area of the brain (12). The distribution of functional γ-Aminobutyric acid (GABA) receptors is an essential factor of neuronal information processing on individual hippocampal neurons (13). However, studies showed that several exogenous substance can disturb this information processing particularly pyramidal cells in the CA1, CA2, and CA3 of Ammon's horn (14–16).

Several studies evaluated the effect of the oral administration of MSG on brain structures (17). A previous study showed that intraperitoneal injections of MSG for ten consecutive days caused deficits in Purkinje and pyramidal cells within hippocampus and cerebellum, respectively. However, those deficit did not occur in the prefrontal cortex of Wistar rats (18). Another animal study showed that administration of 2 g/kg/day intraperitoneal injection of MSG caused pyramidal cell degeneration and shrunken glial cells in the hippocampus (19). However, those studies used a parenteral administration route, which was not a common route for MSG as a food additives substance. Hence, this present study aims to determine the effects of subchronic high-doses oral administration of MSG on the total number of hippocampal pyramidal cells and the spatial memory of rats.

MATERIALS AND METHODS

Study design

This post-test only with a control study was performed in the Physiology Department Laboratory of Gadjah Mada University (UGM), Indonesia. A total of 28 male healthy Wistar rats, aged 6-8 weeks (100-150 g), were taken from the UGM Pharmaceutical animal laboratory house (Approved by Health Research Ethics Committee of UGM No: KE/FK/66/EC/2016). All rats were adapted in the experimental room for seven days before the intervention. They were kept under natural daily cycle of 12-hour light-dark. Based on Federer's sample size formula, the rats were divided into seven rats for each four groups. The control group was given an oral solution of NaCl 0.9%, and three intervention groups were given oral solution of MSG with doses of 1.0, 2.0, and 4.0 mg/g bodyweight (M1, M2, and M3, respectively) according to laboratory protocol. The MSG solution was made fresh daily by dissolving 99% MSG powder in 2 ml 0.9% NaCl solution. The total volume for each oral administration is 3 ml. It is administered once daily using a gastric tube for 30 consecutive days. The rats might consume food and water ad libitum during intervention. The sick or dead rat was excluded from the study (Figure 1).

Morris Water Maze

Morris Water Maze (MWM) was used to test the spatial memory of the rats. This procedure includes escape acquisition and memory persistence tests. It

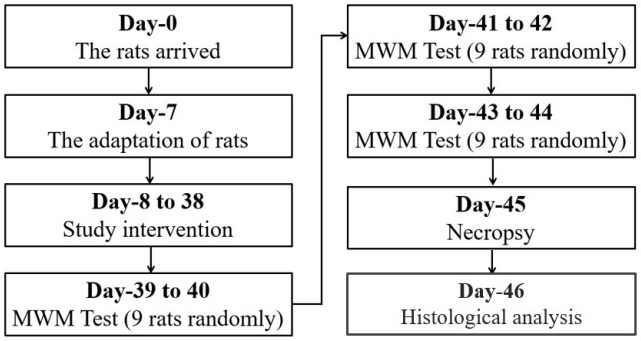

Figure 1: The rats' intervention schedule arrangement of the research. MWM, Morris Water Maze test

was started one day after 30 days of NaCl 0.9% and MSG intervention. The escape acquisition test was conducted for three consecutive days (eight trials each day). Because of its repeated measurement, we used general linear model repeated measurement analysis. The memory persistence test was performed 3, 6, and 9 days after the last day of the escape acquisition phase (Figure 1). The rat had to undergo one trial only on each day of the memory persistence phase (20).

The MWM test apparatus consists of a 150 cm diameter circular pool with 40 cm height. The pool is 18 cm in depth to avoid the tail or legs from reaching the pool's floor. Its temperature is 25±2°C water (20). The MWM tank has four quadrants (A, B, C, D). A white tin container circular platform with a diameter of 13 cm and height of 16.5 cm was placed in the middle of one randomly selected quadrant, whose top surface was 1.5–2.5 cm below the water's surface. Milk was added to the tank to hide the platform from the sight of the rats (21). The platform position was maintained constant throughout the test. There were eight starting points around the perimeter of the pool of each quadrant which was equally spaced. A video camera was set over the approximate center of the pool, and it was connected to a computer software (BIOBSERVE GmbH, Bonn, Germany) to observe the rats' behavior.

The test began by placing any given rat in a randomly selected starting point. Its head faced the pool's wall. The rat had to swim to escape from the water. It was expected that the rat would accidentally strike on the platform and climb onto it, marking the test's end. The duration taken by each rat from the beginning to the end was recorded as the escape latency. The length of the track was measured by using a curvimeter (Comcurve10; KoizumiSokkiMfg, Nagaoka-shi, Niigata, Japan) on the screen of a laptop computer.

Necropsy

One day after the last day of memory persistence test of MWM test, the rats were killed by trans-cardiac perfusion using 4% PBS-formaldehyde after the intramuscular injection of 0.15 cc/100 g bodyweight Ketamine HCl. Subsequently, the rat skulls were opened, their brains

were dissected, and hippocampus was isolated.

Histological and stereological procedures of the hippocampus

The histological and stereological procedures (Figure 2) of the hippocampus referred to previous studies (22). The extracted hippocampus was immersed with 4% paraformaldehyde for 24 hours. They were dehydrated in graded concentrations of ethanol, cleared in solution of toluene , infiltrated with embedded in paraffin blocks. The obtained hippocampal images from pre-dehydration and post-clearing procedures were collected to calculate shrinkage of the volume. A regular array grid spaces and points from ImageJ® was set on the images with the pre-dehydration and post-clearing interpoint distance of 3 µm and 22.5 µm, respectively. Those point within the images were calculate to estimate the volume shrinkage:

$$\text{Volume shrinkage} = \left[1 - \left(\frac{\text{post clearing area}}{\text{predehydration area}}\right)\right]^{1.5}$$

This strategy yielded an average number of points of 80, 166, and 267 counted for the hippocampus (CA1) and hippocampus (CA2, CA3).

Blocks that contained hippocampus were sectioned into 3 µm thickness using LeicaRM 2235 microtome. The first section, which showed the cutting tissue, was considered the first section, and the following sections were numbered consecutively. For the hippocampus of each rat, a section from number 1 to 150 was randomly selected, and this number pointed to the number of sections to be sampled together with its adjacent section. The following 148 serial sections were discarded, and

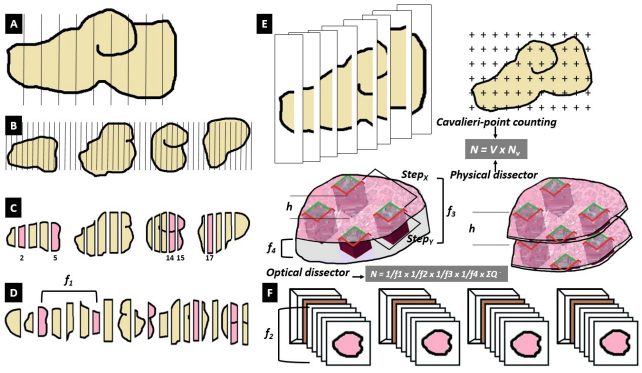

Figure 2: Stereological sampling cascade process that performed on the hippocampus: (A) The tissue is cut into slices, (B) That are cut into small fragments, (C) The slice pieces can be selected by simple random sampling method as illustrated five random numbers are selected from 1 to 19, (D) Systematic uniform random is selected randomly and systematic sampling is performed (every fourth fragment). Process to know the total number of the cell (N). (E) Ratio-technique, Cavalieri point-counting exemplified the tissue sample and the physical dissector. (F) Optical fractionator with hippocampal fragments (f_1), the paraffin blocks are sectioned exhaustively (thick slices) and a sample of fraction of these are selected (f_2). They are analyzed and the stage conduct step movements in x, y, thus a fraction of this area is selected (f_3). In the last step, onl a fraction (f_4) of hippocampal tissue thickness is analyzed (f_4 = h/thickness of section) in the optical dissector. Later, all the cells are calculated (ΣQ^-).

the subsequent pairs of the section were sampled. These procedures were continued until the whole hippocampal tissue was sectioned exhaustively. The average number of pairs of the section was nine pairs.

Hippocampal volume estimation

The Cavalieri principle was used to calculate the hippocampal pyramidal cells layer. The hippocampal images from each section pair were observed by Olympus-CX21FS1 at 40x magnification and captured by Optilab CX-21. Adobe Photoshop® CS6 software was used to combine those images to construct a complete hippocampal image. Then, the complete image was viewed using ImageJ® software and superimposed with a regularly spaced array of test points at a distance of 8 mm (for hippocampal pyramidal cells layer of CA1 region) and 9.5 mm (for hippocampal pyramidal cells layer of CA2-CA3 regions) between points. All points which hit the hippocampal pyramidal cells layer of CA1 and CA2-CA3 regions were counted.

Estimated density and the total number of hippocampal pyramidal cells

The numerical densities of the hippocampal pyramidal cells were determined using a physical dissector probe and following the forbidden line rule (23). The hippocampal image was captured at 400x magnification. Counting frames of 75 x 35 mm2 and 100 x 100 mm2 were used to count hippocampal pyramidal cells' profiles, respectively. These frames represented areas of 0.1 x 0.046 mm^2 (1000x magnification) and 0.1 x 0.1mm^2 (1000 x magnification), respectively. The frames were blinded, and the counting was done randomly. The calculation of numerical density (Nv) of the cells used the following formula:

$$\text{Nv} = \frac{\Sigma Q^-}{\Sigma P.\,a.\,h.\,(1 + \text{Volume shrinkage})}$$

ΣQ^- is the number of nucleoli, a is the counting frames area (µm2), h is the dissectors' height equal to the section thickness (3 µm). ΣP is the counting frames total number. The hippocampal pyramidal cells number was counted by the volume and numerical density multiplication (Figure 3).

Statistical analysis

The bodyweight gains and the approximated hippocampal pyramidal cells number of CA1 and CA2-CA3 regions were compared by One-Way ANOVA. The normally distributed data of MWM were statistically tested by One-way ANOVA, meanwhile the non-normally distributed data were analyzed by Kruskal-Wallis. Post-Hoc Tukey and Mann-Whitney tests were conducted for the parametric and non-parametric tests, respectively. The precision of stereological procedures was evaluated using $CV^2_{total} = CE^2 + CV^2_{biol}$. CV_{total} is the total coefficient of variation. CE is the coefficient of error. CV_{biol} is the biological coefficient of variation. The stereological procedure is considered optimal if

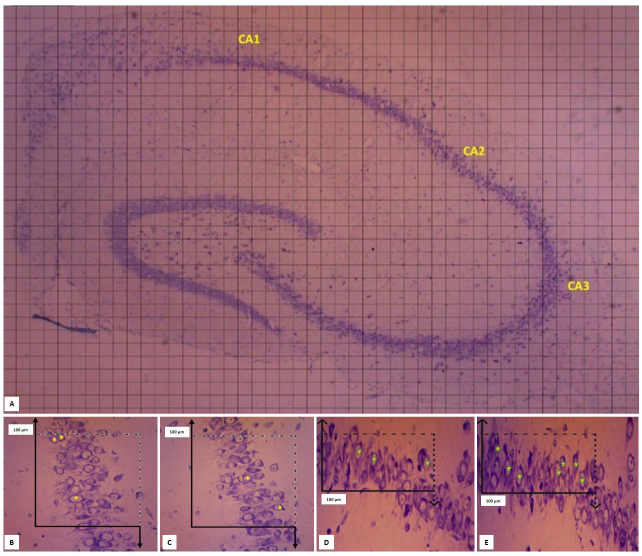

Figure 3: (A) Hippocampal tissue sections superimposed by grids of regularly spaced points for the volume estimation using the Cavalieri principle. CA, Cornu Ammonis. (X40, Hematoxylin and Eosin staining). (B-E) Selected hippocampus sections from each groups were superimposed by counting frames: (B) NaCl 0.9% intervention group; (C) M1, group of 1.0 mg/g body weight MSG; (D) M2, group of 2.0 mg/g body weight MSG; (E) M4, group of 4.0 mg/g body weight MSG. Dashed lines represent inclusion lines, whereas full drawn lines represent exclusion lines. Yellow stars mark neurons being counted. (X400, Hematoxylin and Eosin staining).

$0.2 < CE^2/(CV_{total})^2 < 0.5$. The IBM SPSS 22 was used to perform the statistical analysis with a significance level of $p < 0.05$.

Ethical consideration

This study protocol was approved by the Health Research Ethics Committee of UGM (No: KE/FK/66/EC/2016). The written study report used the ARRIVE (Animal Research: Reporting of In Vivo Experiments) to maximize the quality and reliability of this study.

RESULTS

Before and after the intervention, the rats' bodyweight data did not show significant differences among the study groups (Table I). The latency and the path length of the persistent memory test of the M3 group were significantly higher than in other groups. The increasing score trend from the control to M3 groups indicated that the more MSG dosage was given, the longer latency and path length measurement was (Table II and Figure 4). The volume, numerical density (Nv), and estimated total number of hippocampal CA1 region pyramidal cells are shown in Table III. Further analysis in hippocampal pyramidal volume and cells number showed that increasing MSG dose from 1.0 to 4.0 mg/g bodyweight led to hippocampal cells volume and number decrease. The analysis for the precision of the stereological procedures showed that estimating the hippocampus volume of the CA1 region ($CE^2/ CV_{total}^2 = 0.08$) was inefficient, but the estimation of pyramidal cells number

Table I: The mean (SD) of rats body weight before and after the intervention

Body weight (g)	Intervention				
	NaCl 0.9%	M1	M2	M4	P[a]
Pre intervention	195.1 (21.32)	191.7 (29.54)	180.4 (35.87)	180.6 (21.34)	0.613
Post intervention	243.7 (28.63)	253.1 (18.16)	257.3 (43.39)	251.6 (21.69)	0.791
Body weight gain	48.57 (15.5)	61.43 (26.36)	76.85 (18.55)	71.00 (21.84)	0.148

M1, group of 1.0 mg/g body weight MSG; M2, group of 2.0 mg/g body weight MSG; M4, group of 4.0 mg/g body weight MSG.
[a] One-way ANOVA, $P \leq 0.05$ is considered statistically significant.

Table II: Mean ± SD of escape latency (seconds) of the persistence memory phase of the Morris Water Maze test.

Intervention	N	Day-3	Day-10	Day-17
NaCl 0.9%	7	8.86 ± 5.84	8.71 ± 2.43	5.86 ± 6.97
M1	7	11.29 ± 6.55	16.57 ± 12.27	14.29 ± 6.84
M2	7	17.86 ± 13.70	20.14 ± 8.40	21.86 ± 10.43
M4	7	64.57 ± 5.27	22.29 ± 14.58	31.57 ±3 0.95
P		0.277[a]	0.031[b]	0.001[b]

M1, group of 1.0 mg/g body weight MSG; M2, group of 2.0 mg/g body weight MSG; M4, group of 4.0 mg/g body weight MSG.
[a] Kruskal Wallis, $P \leq 0.05$ is considered statistically significant.
[b] One-way ANOVA, $P \leq 0.05$ is considered statistically significant.

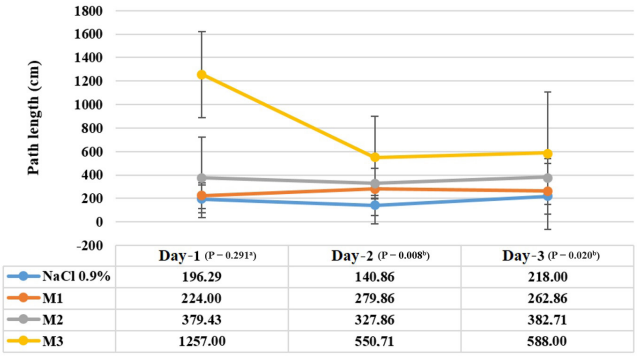

Figure 4: Mean ± SD path length (cm) of persistence memory Morris Water Maze test based on the group. M1, group of 1.0 mg/g body weight MSG; M2, group of 2.0 mg/g body weight MSG; M4, group of 4.0 mg/g body weight MSG. a Kruskal Wallis, b One-way ANOVA, $P \leq 0.05$ is considered statistically significant.

Table III: Mean ± SD of hippocampal volume, numerical density (Nv), and number of pyramidal cells of the CA1 region. The precision and effectiveness of the procedures in estimating the hippocampus volume and the number of pyramidal cells on CA1 region were also displayed

Intervention	N	Volume ± SD (mm^3); CV; CE	Nv ± SD ($/mm^3$)($x10^3$)	Cells number $x10^3$ ± SD; CV; CE
NaCl 0.9%	7	0.65 ± 0.14; 0.21; 0.05	279.559 ± 67.41	178.586 ± 17.794; 0.24; 0.09
M1	7	0.52 ± 0.11; 0.21; 0.05	264.524 ± 58.396	133.333 ± 23.206; 0.17; 0.09
M2	7	0.42 ± 0.03; 0.06; 0.04	281.156 ± 76.493	115.37 ± 26.456; 0.23; 0.11
M4	7	0.41 ± 0.08; 0.21; 0.06	266.097 ± 55.682	105.039 ± 16.678; 0.16; 0.10
P		0.001[a]	0.590[a]	0.004[a]

M1, group of 1.0 mg/g body weight MSG; M2, group of 2.0 mg/g body weight MSG; M4, group of 4.0 mg/g body weight MSG; CV, coefficient of variation; CE, coefficient of error.
[a] One-way ANOVA, $P \leq 0.05$ is considered statistically significant.

was efficient (CE^2/ CV_{total}^2 = 0.49). The pyramidal cells of the hippocampal CA2-CA3 region showed no significant main effect among the groups. The analysis for the precision of the stereological procedures showed that the estimation of the hippocampal volume of the CA2-CA3 region was less efficient than optimum (CE^2/ CV_{total}^2 = 0.18), but the estimation of the pyramidal cells number was efficient (CE^2/ CV_{total}^2 = 0.34) (Table III).

DISCUSSION

This study showed that the oral administration of 4 mg/g bodyweight of MSG for 30 consecutive days caused a significant decrease in the CA1 region of hippocampal pyramidal cells, but not the CA2-CA3 regions pyramidal cells, and a significant decrease in spatial memory retention. The MSG doses used in the present study were in a range of low to high toxicity levels. The MSG toxicity to the hippocampus of humans and laboratory animals increases Ca^{2+} influx through glutamate-gated channels. The Ca^{2+} influx causes downstream consequences such as triggering catabolic processes and generating reactive oxygen species (ROS) that eventually cause neuronal injury (24). However, the toxic dose estimation to humans requires consideration about the variation in pharmacodynamics and pharmacokinetics between humans and rats (25).

The medial portion of temporal lobe has been regarded to play role in the long-term memory formation and allocentric viewpoint space information storage. Thus, damage to the hippocampus causes in spatial memory deficit (26). A systematic review showed that MSG, aluminum chloride, high salt-cholesterol diet caused toxic-substance-induced hippocampal degeneration. Most of those reviewed-studies (52 studies) evaluated neurodegenerative markers including synaptic damage, cytoplasmic swelling, pyknotic nuclei, vacuolization, and hippocampal neuronal shrinkage (27). It also confirmed the presences of apoptosis molecular markers such as Caspase-3, B-cell associated X-protein, B-cell lymphoma protein-2, fluoro jade, and terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL), and neuroglial responses (microtubule associated protein-2, glial fibrillary acidic protein, neuronal nuclei, ionized calcium-binding adaptor molecule-1) (27).

The MSG-induced excitotoxicity and selective neuronal vulnerability caused a decreased number of pyramidal cells in the hippocampal CA1 region but not in the CA2-CA3 regions (28). An increase of glutamate level in the central nervous system may cause an overstimulation of ionotropic glutamate receptors, including N-methyl-D-aspartate (NMDA), Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kinase receptors, which in turn leads to the increase of the influx of Ca^{2+} and Na^{+} to post-synaptic neurons (29). The accumulation of intracellular Ca^{2+} disrupts calcium homeostasis, damages organelles functions, and increases nitric oxide (NO) and free radical production (30). Several studies about the effects of MSG on hippocampal pyramidal cells showed that MSG administration caused the death of dentate gyrus granular and CA1 hippocampal pyramidal cells, but not the CA2-CA3 pyramidal cells (31).

At the MWM test on rats, the learning ability and memory consolidation were evaluated by escape acquisition and memory persistence tests (32). The M3 group traveled at longer latency and trajectory than rats of control, M1, and M2 groups. This difference in the memory persistence test implied interference in memory consolidation ability. Correspondingly, there was also a decrease in the estimated total number of hippocampal CA1 pyramidal cells. Several studies on rats reported that the activity found in the CA1 region was essential for retrieving memory, encoding, and storing or consolidating spatial information (33). Memory consolidation, which was an essential role of the CA1 region, was impaired due to decreasing number of pyramidal cells in the CA2-CA3 region, which supplied the CA1 regions' function (28). However, CA2-CA3 do not have mossy fibers' innervation as the CA1 region has. In the CA1 region, the density of pyramidal cells is denser than the CA2-CA3 regions have (34,35).

Consumption of MSG orally has been shown to increase blood glutamate levels (2). However, it does not directly cause neuronal damage in the brain. After 30 minutes of MSG administration, glutamate levels in blood plasma increased 395% at a dose of 75 mg/kg bodyweight and increased to 556% at a dose of 150 mg/kg bodyweight (36). The presence of a clearance mechanism for increased glutamate levels in blood plasma (extracellular levels) does not cause an excitotoxicity reaction, in which extracellular glutamate levels are maintained at 5-10 μM levels (37). However, if high levels of glutamate occur continuously, there may be a failure of the clearance mechanism triggered by endogenous excitotoxic conditions. If the clearance mechanism fails, the glutamate levels will increase and trigger extracellular excitotoxic conditions that eventually cause neuronal damage (38).

CONCLUSION

The administration of high-dose of MSG for 30 days caused deleterious effects on the spatial memory function, hippocampal pyramidal cells volume and number of rats. The increase of MSG dose from 1.0 to 4.0 mg/g bodyweight led to a decrease in the MWM spatial memory performance test and a decrease in hippocampal cells volume and number in rats. Thus, limiting consumption of MSG is necessary and further studies regarding the effect of MSG on human spatial memory are needed.

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