THE EFFECTS OF MONOSODIUM GLUTAMATE ON WORKING MEMORY AND ESTIMATED TOTAL NUMBER OF MEDIAL PREFRONTAL CORTEX PYRAMIDAL CELLS OF JUVENILE RATS

Pengaruh Monosodium Glutamat pada Memori Kerja dan Perkiraan Jumlah Total Sel Piramidal Korteks Prefrontal Medial Tikus Remaja

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ABSTRACT
Monosodium glutamate (MSG) is a food additive substance that is widely used as seasoning and flavor enhancer throughout the world. However, MSG at certain dosages is also thought to cause damage to many organs, including prefrontal cortex. The aim of this study was to examine the effects of a range of doses of MSG on the working memory and estimated total number of pyramidal neurons in the medial prefrontal cortex of juvenile rats. A total of 24 male rats aged 4 to 5 weeks were divided into four groups, namely, control (C), T2.5, T3, and T3.5 groups, which received intraperitoneal injection of 0.9% sodium chloride solution, 2.5 mg/g body weight (bw) of MSG, 3.0 mg/g bw of MSG, and 3.5 mg/g bw of MSG, respectively, for 10 consecutive days. The working memory of the rats was examined by Y-maze procedure after the treatment by calculating the alternation percentage. The number of prefrontal cortex pyramidal cells was estimated using stereology physical fractionator method. It has been found that there was no significant difference in the percentage of alternation between C group and all MSG-treated groups, while the estimation of the number of pyramidal cells had a significant difference between C group and T2.5 and T3.5 groups, but not with T3 group. The conclusion of this study was that the administration of MSG at every dosage are not affect the working memory and there was an effect of MSG administration at doses of 2.5 and 3.5 mg/g body weight on the total number of pyramidal cells in the medial prefrontal cortex of adolescent rats.

KEYWORDS:
MSG, Working Memory, Pyramidal Cell, Stereology

INTRODUCTION
Monosodium glutamate (MSG) is a food additive substance that is widely used as seasoning and flavor enhancer throughout the world (Livingstone, 1981; Eweka et al., 2011). It gives the fifth basic taste, that is described as savoury flavour...
(Ault, 2004). In 1958, American Food and Drug Administration (FDA) classified MSG as "Generally Recognized as Safe" (GRAS) substance. However, ten years later, there was a report about the emergence of a syndrome (the so-called "Chinese Restaurant Syndrome") which encompasses symptoms such as numbness at the back of the neck, general weakness, and palpitation. MSG was suspected as the cause of this syndrome due to its allegedly significant proportion in cuisine, but scientific evidence was lacking (Geha et al., 2000a; Geha et al., 2000b; Mosby, 2009). In 1995, the Federation of American Societies for Experimental Biology (FASEB) reported that MSG is generally safe for consumption (Department of Human and Health Services, Food and Drug Administration, 1996). However, ongoing experiments on animals demonstrated the deleterious effects of MSG on various organs (Moreno et al., 2005; Farombi & Onyema, 2006; Pavlovic et al., 2007).

Glutamate is known as the primary excitatory neurotransmitter in human brain. Glutamate plays an important role in synaptic plasticity, learning, and neural development. Over the past four decades, a direct relationship between neuroexcitatory and neurotoxic characteristics of glutamate has been associated with the activations of excitatory receptors. This stimulation causes enzymatic cascades resulting in cells deaths (Ankarcrona et al., 1995; Martin et al., 2000; Maragakis & Rothstein, 2001).

The neurotoxic properties of glutamate have been demonstrated for the first time in rat pups, in which systemic administration of glutamate (2.2 g/kg bw) for 14 days caused retinal degeneration (Stegink et al., 1973). A study evaluating the long-term behavior of rats due to administration of 3 mg/g bw MSG during the neonatal period resulted in the decrease of locomotor movement and level of habituation (Hlinak et al., 2005). Furthermore, other recent studies also showed neurotoxic effects of MSG at high doses. The administration of 4 mg/g bw MSG via different routes at various ages caused damage to neurons of the hypothalamic arcuate nucleus and disorders of memory storage in adult rats (Park et al., 2000), the death of pyramidal neurons and proliferation of glial cell of the prefrontal cortex in neonatal rats (Gonzales-Burgos et al., 2001), and the increase of reactivity of astrocytes and glial cells in the fronto-parietal cortex of adult rats (Martinez-Contreras et al., 2002). Lesions in the prefrontal cortex usually interfere with executive functions, including inhibition response, organization of the temporal behaviour, and working memory of rats (Cerqueira et al., 2005; Han et al., 2013).

A series of studies in our laboratory, however, found that the administration of 4 mg/g bw MSG caused lethal effects on juvenile rats (Aminuddin et
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We therefore lowered the dose of MSG down to 2 mg/g bw (Hermawati et al., 2014; Aminuddin et al., 2015) and found that the MSG-treated rats demonstrated deficits in working memory but without discernible loss of pyramidal cells of the medial prefrontal cortex (unpublished results). Whilst our previous study found that MSG caused deficits in the number of Purkinje cells of cerebella and motor coordination performances of rats at a particularly high dose (Prastiwi et al., 2015), it remained unclear whether such deficits occurred in the medial prefrontal cortex as a result of MSG exposure. It was the aim of the present study to examine the effects of a range of doses of MSG on the working memory and estimated total number of pyramidal neurons in the medial prefrontal cortex of juvenile rats.

MATERIALS AND METHODS

Animals

Twenty-four male Wistar rats aged 4-5 weeks with the body weights of 100-150 grams were used in the present study. The animals were obtained from the Department of Pharmacology, Faculty of Medicine, Universitas Muhammadiyah Surakarta. The rats were habituated to the experimental room for seven days prior to experimental treatments. The rats were randomly assigned into four groups consisting of six rats per group. C group was the negative control group, while T2.5, T3.0, and T3.5 groups served as treatment groups. They were housed in separate well-ventilated cages (3 rats per cage) under standard conditions of naturally 12 h light/dark cycles and free access to pellets and water throughout the experiment. The experimental procedure and animal handling were approved by the Ethics Committee of the Faculty of Medicine, Universitas Gadjah Mada (approval number KE/FK/81/EC).

MSG treatment

Manufactured products of monosodium glutamate (MSG) powder containing 99%+ MSG was obtained from the market. The powder was dissolved in 2 ml of 0.9% NaCl. This solution was prepared fresh daily (prior to the treatment) in order to prevent the MSG from crystallization. The rats in C group were injected intra-peritoneally with 2 ml normal saline. The rats of T2.5, T3.0, and T3.5 groups were injected intra-peritoneally with 2.5 mg/g bw, 3.0 mg/g bw, and 3.5 mg/g bw of MSG, respectively. The treatments for all groups were carried out for 10 consecutive days.

Y-maze test

Y-maze test was performed after treatment (day 18) to assess the working

![Figure 1. Y-maze. Red arrow: the rat in the Y-maze](image-url)
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memory of the rats. The Y-maze apparatus consisted of three arms of identical size forming angles of 120° between the arms and thus constructing a “Y” shape (see Figure 1). Each arm has a length of 50 cm, a height of 20 cm, and a width of 10 cm.

The Y-maze procedure was carried out according to the protocol of previous studies (Tamura et al., 2006; Detrait et al., 2010). The Y-maze equipment was cleaned to remove any odor cues prior to the test for each rat. Each rat was placed in the distal part of a randomly selected arm and allowed to move freely to explore the maze for 8 minutes. The number and sequence of entries of the rat into any given arm was recorded. The rat was judged of entering an arm when the hind legs of the rats perfectly entered the arm. The number of alternations was calculated from the sequence of arm entries of the rat. One alternation was counted as a sequence of entry of the rat into three different arms. For example: Suppose there are three arms (A, B, and C). From the testing, entrance arm sequences obtained as follows: ABCABACABC. The alternations are: ABC, BCA, CAB, BAC, CAB and ABC (ABA and ACA are not counted). Thus obtained 6 alternation (6 sequences 3 arms). Percentage of alternation was estimated as the ratio of the actual number of alternations to the number of possible alternations multiplied by 100. The possible alternation was defined as the total arms entries minus two. The equation of percentage of alternation is as follows: (Tamura et al., 2006)

\[
\% \text{ alternation} = \frac{\text{number of alternations}}{\text{total arms entries} - 2} \times 100
\]

Histological study

Following behavioural tests, all rats were sacrificed. The rats were anesthetized using ketamine 0.15 cc/100 g bw (PT. Guardian Pharmatama, Jakarta, Indonesia) prior to transcardiac perfusion with 4% formaldehyde dissolved in phosphate buffer solution. The cerebrums of the rats were removed from the skulls and weighed. The prefrontal cortices were subsequently separated from the rest of the cerebrums. The prefrontal cortices were delineated as 4.7 mm until 1.6 mm anterior from the bregma of juvenile rat cerebrums. These prefrontal cortices were then immersed in 4% formaldehyde solution for one day.

The brain tissues containing prefrontal cortex were dehydrated in ethanol with ascending concentrations, cleared in xylene, and embedded in paraffin wax. The tissues were then cut according to physical fractionator design as was described in previous studies (Boyce et al., 2010). Serial sections of 3 \( \mu \text{m} \) thick of any given tissue were obtained using a rotatory microtome (Leica, RM 2235, Biosystems Nussloch GmbH, Germany). A number between 1–80 was selected randomly by lottery for each rat. This number was used to determine the first section of the tissue to be selected for stereological analysis. Afterwards, every 80\(^{th} \) sections were sampled until
the tissue was exhaustively sectioned ($f_1 = 80$). The sections were deparaffinised and stained with 0.1% toluidine blue.

These stained sections were subsequently viewed under photographic light microscope (Olympus, CX21FS1, Olympus Singapore PTE Ltd., Singapore) with 400x magnification. Photomicrographs of the specimens were obtained using microscope digital camera (Olympus, CX41, DP20-5E, Olympus Singapore PTE Ltd., Singapore). All pyramidal neurons of all sections of the medial prefrontal cortex of any given rat which showed nucleoli were counted ($n$) (see Figure 2). Estimates of the total number of the medial prefrontal cortex pyramidal cells ($N$) were calculated using the formula: (Boyce et al., 2010)

$$N = f_1 \times n$$

**Statistical analyses**

The data of the percentages of alternation and total number of pyramidal neurons were analysed using one-way ANOVA procedure. Post-hoc least significant difference (LSD) test was performed whenever necessary. The correlation between the working memory (as represented by the percentages of alternation) and the medial prefrontal cortex pyramidal cells number was determined using Pearson correlation test. Statistical significance was set at a probability value of $p < 0.05$. All statistical analyses were carried out using SPSS statistical software version 19 (IBM company, USA).

### RESULTS AND DISCUSSION

**Percentage of alternation in the working memory test**

Table 1 presents the data of the percentage of alternation of the four groups of rats in the Y-maze task. One way ANOVA of these data showed there was a significant main effect of groups in the percentage of alternation.

<table>
<thead>
<tr>
<th>Group (n= @6)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>69.55 ± 5.62</td>
</tr>
<tr>
<td>T2.5</td>
<td>72.03 ± 6.85</td>
</tr>
<tr>
<td>T3.0</td>
<td>62.26 ± 5.01</td>
</tr>
<tr>
<td>T3.5</td>
<td>78.12 ± 10.51</td>
</tr>
</tbody>
</table>

Results of one-way ANOVA:

Groups: df = 23; F = 4.830; $p = 0.011$

C = 2 ml NaCl 0.9% (ip); T2.5 = 2.5 mg/g bw MSG + 2 ml NaCl 0.9% (ip); T3.0 = 3.0 mg/g bw MSG + 2 ml NaCl 0.9% (ip); T3.5 = 3.5 mg/g bw MSG + 2 ml NaCl 0.9% (ip).

ANOVA: analysis of variance; n: number of rats; df: degree of freedom; F: F value; C: control; NaCl: sodium chloride; MSG: monosodium glutamate; ip: intraperitoneal; bw: body weight.

Table 2. Multiple Comparisons Post-hoc LSD Test of the Alternation Percentage

<table>
<thead>
<tr>
<th>Group (n= @6)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C vs T2.5</td>
<td>0.563</td>
</tr>
<tr>
<td>C vs T3</td>
<td>0.100</td>
</tr>
<tr>
<td>C vs T3.5</td>
<td>0.056</td>
</tr>
<tr>
<td>T2.5 vs T3</td>
<td>0.031*</td>
</tr>
<tr>
<td>T2.5 vs T3.5</td>
<td>0.165</td>
</tr>
<tr>
<td>T3 vs T3.5</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

* $p < 0.05$

Multiple comparisons post-hoc LSD test of these data showed that the percentage of alternation of T3.0 group was significantly lower than that of T2.5 group ($p = 0.031$) and that of T3.5 group ($p = 0.001$). On the other hand, there was no significant difference in the percentage of alternation between C group with all MSG-treated groups.

The result showed that MSG with doses between 2.5 - 3.5 mg/g bw didn’t affect working memory, which means that MSG with doses in this
range yet raises neurotoxic effect on the medial prefrontal cortex. This may be due to the concentration of glutamate in the brain extracellular fluid is kept low for optimal brain function.

The brain is isolated from blood plasma due to the blood-brain barrier (BBB) which surrounds the entire central nervous system. Blood-brain barrier is composed of several layers, including capillary endothelial cells, the basal membrane that encloses the capillaries, pericytes contained in the basal membrane and astrocytes processes surrounding basal membrane. Each layer has the potential to restrict the movement of solutes (Smith, 2000; Hawkins, 2009; Saunders et al., 2012; Hawkins et al., 2013).

Capillary endothelial cells are a major part of the BBB. Cerebral capillary endothelial cells different from the other capillary endothelial cells. The differences are in the fewer amounts of cytoplasmic vesicles, more mitochondria, and a large number of tight junctions between cells that overlap. Close links hinder the movement of molecules between cells, preventing the membrane molecules move from one cell to another, and divides the endothelial cell membrane into two different sides. Therefore, the population of lipid and intrinsic protein (transporter) are different on either side of the membrane (luminal and abluminal), so the type and speed of molecules across the membrane are limited (Hawkins, 2009; Hawkins et al., 2013).

The study of transport through BBB indicates that the facilitated transport and active transport plays a role in brain metabolism. Facilitated transport of glutamate is only found in the luminal membrane, which allows the release of glutamate from endothelial cells into plasma, while at the abluminal membrane, there are glutamate Na+-dependent transporters. Transporter in abluminal membrane are excitatory amino acid transporters (EAAT) 1, 2, and 3 (Hawkins et al., 2013). In addition to the abluminal membrane of endothelial cells, EAAT also present in the plasma membrane of astrocytes and nerve cells. Transporters of this type have the ability to clean glutamate from the synaptic cleft and extracellular fluid (ECF) (Chao et al., 2010).

When the concentration of glutamate in the ECF exceeds the optimal level, then EAAT in the abluminal membrane pump glutamate into the endothelial cells, and facilitated transport system in the luminal membrane allows glutamate out into the blood circulation. Glutamate can enter the endothelial cells, but the circulation of glutamate from the endothelial cells to the brain is almost impossible. This is because the energy used by EAAT, derived from the difference Na+ gradient concentration between ECF and endothelial cells. Organization of the BBB causes the circulation of glutamate from the blood circulation into the brain may not occur (Hawkins, 2009).
The medial prefrontal cortex pyramidal cells number

The data of the estimated total number of the medial prefrontal cortex pyramidal are shown in Table 2. One-way ANOVA of these data revealed that there was a significant main effect of groups in the estimated total number of pyramidal cells. Multiple comparisons post-hoc LSD test of these data showed that the number of pyramidal cells of C group was significantly lower than that of T2.5 group ($p = 0.007$) and T3.5 group ($p = 0.008$). On the other hand, there was no significant difference in the number of the neurons between C group and T3 group.

Beside as an excitatory neurotransmitter, glutamate is also a potent neurotoxin when present in large amounts in the synaptic cleft. Excessive glutamate in the synaptic cleft will give a toxic effect (excitotoxicity) as a result of excessive activation of glutamate receptors, thereby causing neuron cells death (Dalcin et al., 2007; Wang & Qin, 2010). This study showed that MSG with doses between 2.5 – 3.5 mg/g bw not to cause damage to the medial prefrontal cortex pyramidal cells. These results are consistent with our research before (unpublished results), that the administration of 2 mg/g bw MSG for 10 days in juvenile rats, have not led to a decrease in prefrontal cortex pyramidal cells number. This maybe because of the MSG-treated in short duration.

Glutamate concentration in brain extracellular fluid is kept in a very low rate (0.5–2 µmol/L). Because when glutamate exists in excessive concentration, it can cause excitotoxicity resulting in nerve cell death. One of these tight regulations can
be seen from the presence of glutamate transporters in the luminal membrane which can only transport glutamate from endothelial cells to blood plasma, so the number of pyramidal cells in the control and treated group are not significantly different. Research by administering acute treatment performed by Brown et al. (2005), adult male rats were given mild stress for 7 days has undergone remodeling apical dendrites, with atrophy in the distal branches.

Another study examines the number of the prefrontal cortex pyramidal cells after acute treatment performed by Gonzales-Burgos et al. (2001), cell death caused by excitotoxicity and dendritic hypotrophy occurs in rat neonates that exposed with 4 mg/g bw MSG for 5 days. Excitotoxicity in this study occurred because of glutamate transporter expressed in large amounts in the brains of neonates who are still growing, so that there are excessive amount of glutamate in the brain. Transport mechanisms in the developing brain barrier to affect the composition of the environment in the brain and provide nutrients and other molecules necessary for the growth and differentiation of the brain. Excessive amount of glutamate gives neurotoxic effects, so there are many cells death (Saunders et al., 2012).

CONCLUSION

It has been found that the administration of MSG at every dosage are not affect the working memory and the estimated total number of pyramidal cells of juvenile rats.

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