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THE EFFECTIVENESS OF LEMON JUICE (*Citrus limon*) ON PURKINJE CELL OF WHITE MALE MICE (*Mus musculus*) CEREBELLAR CORTEX THAT EXPOSED BY MONOSODIUM GLUTAMATE (MSG)

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ABSTRACT

Introduction: *A number of studies have indicated that excessive MSG (Monosodium glutamate) consumption can lead to the formation of free radicals that can have a negative effect on purkinje cells in the cerebellar cortex. Lemon fruit is a plant that has benefits as a natural antioxidant because it contains compounds such as flavonoids, vitamin C, citric acid and other substances. This study aims to examine the effect of lemon juice (Citrus limon) to purkinje cells of the cerebellum cortex on male white mice (Mus musculus) which exposed of monosodium glutamate.*
Method: *This research is an experimental laboratory with a post test only control group design. The research subjects were 30 white male mice divided into 5 groups: normal control given standard feed, negative control given MSG dose 4mg / grBW, group of dose I , dose II , and dose III that were each given lemon juice with doses 3.33ml / kgBW, 6.67ml / kgBW, 13.33ml/ kgBW respectively. The number of purkinje cells is calculated in the cerebellar cortex by HE staining. The results of the study were analyzed by One Way ANOVA followed by the Bonferroni Post hoc test.*
Results: *The average number of purkinje cells in administration of lemon juice with dosages of 3.33 ml/kgBW, 6.67 ml/kgBW and 13.33 ml /kgBW were 14.10 cells, 16.73 cells and 17.50 cells respectively. Based on the Post Hoc test, the average number of purkinje cells at the dose of 13.33 ml / kgBW was higher than the negative control (p = 0.021)*
Conclusion: *Lemon juice could be used to reduce negative effect of MSG on purkinje cells of white male mice. Moreover, this finding could be used as reference on further research of benefit of lemon juice.*

Keyword*: Citrus limon, purkinje cell, monosodium glutamate (MSG)*

INTRODUCTION

People often add flavoring additives known as MSG (Monosodium glutamate) to enhance the taste of food. Based on the results of the 2013 Basic Indonesian Health Research, the risk of food consumption behavior in the population aged more than 10 years consumed the most seasoning (77.3%), followed by sweet foods and drinks (53.1%), and fatty foods (40.7%) [1]. FAO (The Joint Food and Agriculture Organization) of the United Nations in 1974 allocated a standard of acceptable daily intake for MSG in a range between 0 and 120 mg/kg body weight (bw)/day [2]. Excessive MSG consumption can lead to accumulation of glutamate in a very abundant amount in the synapse gap (gap between nerve cells), mentioned will be able to be excitotoxic for the brain [3]. This is a consequence of excessive calcium ion concentration in the intracellular, resulting in mitochondrial dysfunction that causes free radical formation, activation of the caspase pathway and degradation of intracellular proteins [4]. The

excitotoxic effect of MSG makes it possible to cause of negative effects on the cerebellum which play an important role in regulating motor activity and coordination. In the study of Eweka et al (2007) histological administration of MSG in rats showed damage and death of cerebellar Purkinje cells which caused changes in motor coordination function [5].

The human body can produces antioxidants (endogenous antioxidants) to fight free radicals and other harmful molecules in the body including MSG, but if free radicals and other harmful molecules are present in excessive amounts, the body needs antioxidants from outside the body (exogenous antioxidants) to fight it. Lemon fruit is rich in proanthocyaidins, flavonoids, hesperidin, eriocitrin, and vitamins E and C. Many of these phenolic compounds have been shown to be cytoprotective by scavenging superoxide anions, hydroxyl radicals, and hydrogen peroxide, thereby reducing lipid peroxidation [6]. Krisnawan et al. (2017) 's study of the testing of antioxidant potential in local and imported lemons obtained the results of qualitative testing of local and imported skin extracts and lemon juice, known to have antioxidant activity [7]. Based on previous research, this study will examine the effect of giving lemon juice *(Citrus limon)* to purkinje cells of the cerebellum cortex of male white mice *(Mus musculus)* exposed to monosodium glutamate

MATERIALS AND METHOD

This study is an experimental study with a post test only control group design using experimental animals as the subject of the study. This research was conducted at the Food and Nutrition Laboratory of the Inter-University Center (PAU) of Gajah Mada University (UGM) Yogyakarta, Anatomical Pathology Laboratory of Gadjah Mada University (UGM), and Anatomical Pathology Laboratory of Swadaya Gunung Jati University (UGJ). The experimental protocol and animal handling was approved by the Ethical Committee of the Faculty of Medicine, Swadaya Gunung Jati University (Approval number: 65/EC/FK/XI/2018).

Animal protocol

30 white male mice (2.5-3 months old) weighing 20-40 grams were randomly devided into 5 groups: Normal control (NC), negative control (C-), group of dose I, group of dose II, group of dose III. After being adapted for 7 days, each group was exposed to a 4mg / gBW MSG dose for 14 days orally, except in the NC. The dose of MSG for the mice was determined according to research conducted by Zulfiani (2013) [8]. On the 15th day MSG exposure was stopped and in each group were given lemon juice with a dose of each group of 3.33 ml / kgBW, 6.67 ml / kgBW, 13.33 ml / kgBW, except in the NC and C- group which is only given aquades and standard feed. Giving lemon juice is done once a day for 14 days using the gastric sonde.

Preparation for extraction of lemon juice.

Lemon were obtained from market in Cirebon City, West Java, Indonesia, confirmed by a taxonomist of Universitas Negeri Semarang, Central Java. Ripe lemons will change color from green to yellow, weigh around 50 - 80 grams and 5-8 cm in diameter. Lemon is cleaned with running water then cut into 2 pieces. Lemon is then squeezed using an orange squeezer that has been sterilized and separated by seeds to get the water.

Cerebellar cortex histopathology

On the 29th day, white male mice were terminated by cervical dislocation method and then necropsy was performed to take samples of cerebellar organs. Cerebellar cortical preparations are made by a process of fixating, washing, dehydrating, clearing, impregnating, embedding, cutting, staining, and mounting. Histopathological preparations were observed using a binocular light microscope with 400x magnification. The number of purkinje cells was calculated in 5 fields of view for each preparation in each treatment group.

Statistical analysis

The results of the study were analyzed by multivariate One Way ANOVA parametric test then followed by the Levene Test of homogeneity test and Bonferroni Post hoc test to find out which groups were different

RESULT

A. Number of Purkinje Cells

Observation of the number of purkinje cells was carried out using a binocular microscope with 400x magnification at 5 visual fields. Table 1 shows the results of the average number of purkinje cells calculated in each group.

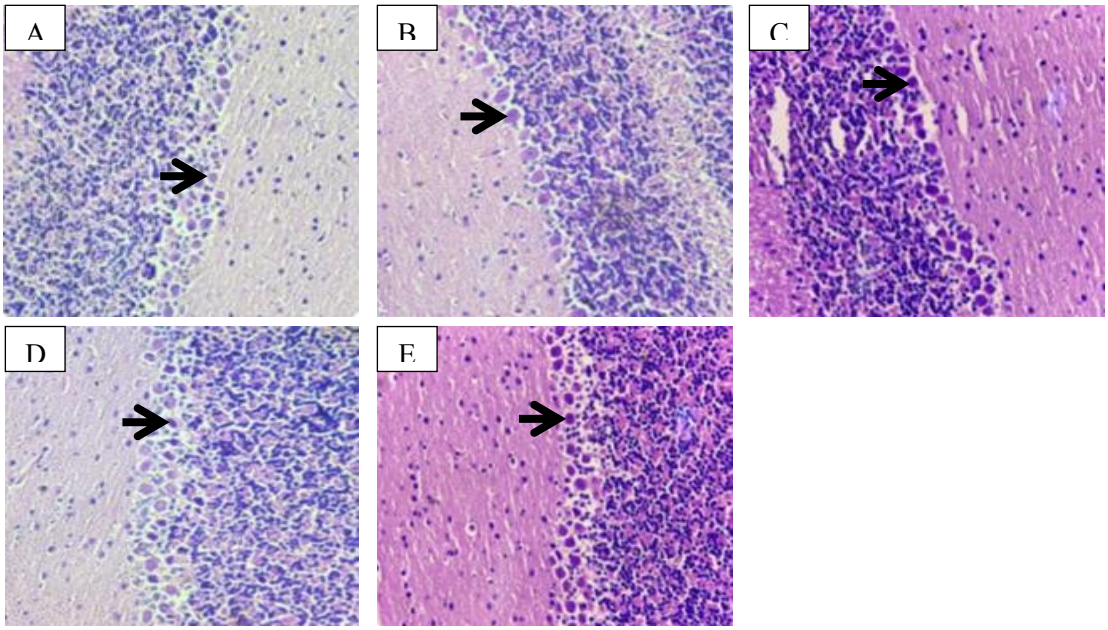

Fig 1. H&E Stained cerebellar cortex section in objective lens 400x. (A) normal control, (B) negative control, (C) group dose I, (D) group dose II, (E) group dose III. Black arrow is purkinje cells

Table 1. The results of the average number of purkinje cells calculated in each group.

Group	Average number of purkinje cells	Standar deviation	IC 95 %	
			Lower bound	Upper bound
Normal control	13,90	2,10	11,69	16,10
Negative control	12,76	2,54	10,09	15,43
Dose I	14,10	1,41	12,61	15,58
Dose II	16,73	3,76	12,78	20,67
Dose III	17,50	1,20	16,23	18,76

Based on the table 1, the results showed that the group of dose III had the highest number of purkinje cells, which has 17.50 cells, followed by the group of dose II it has 16.73 cells, then the group of dose I with an average number of 14.10 cells, normal controls in the next order with an average number of 13.90 cells and the last negative control with an average number of purkinje cells 12.76 cells.

B. One Way Anova Test

The results of the analysis of the number of purkinje cells in the cerebellar cortex in each study group obtained a significant value of 0.009 ($p < 0.05$), its mean that there were at least two groups that had

significantly different mean purkinje cells. To determine the differences in the number of purkinje cells between groups, the Post Hoc test was continued.

C. Bonferroni's Post Hoc Test

Table 2. Differences in the Number of Purkinje Cells Between Groups

Group		Average difference	p value
Normal control	Negative control	1,1	1,00
	Dose I	-0,2	1,00
	Dose II	-2,8	0,504
	Dose III	-3,6	0,15
Negative control	Normal control	-1,1	1,00
	Dose I	-1,3	1,00
	Dose II	-3,9	0,081
	Dose III	-4,7*	0,021*
Dose I	Normal control	0,2	1,00
	Negative control	1,3	1,00
	Dose II	-2,6	0,676
	Dose III	-3,4	0,208
Dose II	Normal control	2,8	0,504
	Negative control	3,9	0,081
	Dose I	2,6	0,676
	Dose III	-0,7	1,00
Dose III	Normal control	3,6	0,15
	Negative control	4,7*	0,021*
	Dose I	3,4	0,208
	Dose II	0,7	1,00

*p value < 0,05 which means significantly different

Based on the post hoc Bonferroni analysis, there was a significant difference between the negative control group and the dose of group iii who were given lemon juice with a dose of 13.33 ml / kgBW, because the value of p = 0.021 (p <0.05).

DISCUSSION

Based on the results of the study it was found that the average number of purkinje cells was lowest in the C- group compared to the normal control group, group of dose I , dose II, and dose III with the number of cells in a row of 13.90 cells, 14.10 cells , 16.73 cells, and 17.50 cells while in the C- group as many as 12.76 cells. Previous research conducted by Prastiwi (2015) showed that administration of a 3.5 mg / gBW dose of MSG could reduce the number of purkinje cells in the cerebellar cortex [2]. Research on the effects of MSG on cell damage was also carried out by Onaolapo (2016) which showed that giving MSG caused histological damage to nerve cells in cerebral organs, cerebellum and hippocampus. MSG had a major component of glutamate which might be the cause of differences in the average number of purkinje cells in C- group with the normal control group, group of dose I, dose II, and dose III [9,10].

Monosodium glutamate is one of the synthetic additives that are widely used by humans as a flavoring in food. The use of excessive amounts of MSG can lead to oxidative stress [10]. Free glutamic acid produced due to consumption of MSG will be partially bound in the intestine and the rest will be released into the blood. This free glutamic acid will spread throughout of the body, including going through the blood-brain barrier through circumventricular organs in the brain and bound to its receptors. In addition, the role of glutamate transporters found in the blood brain barrier capillary membrane helps to absorb glutamate into the brain. Purkinje cells receive 2 glutamanergic inputs, namely from climbing fiber and parallel fibers, which makes purkinje cells susceptible to glutamate exotoxicity. Excessive accumulation of glutamate in the synaptic gap causes overstimulation of the A-amino-3-hydroxy-5-methyl-4-

isoxazolepropionate (AMPA) receptor and metabotropic glutamate receptors. The overstimulation of the glutamate receptor causes an increase in influx of calcium ions (Ca2 +) into the cell. This can be caused of mitochondrial dysfunction which causes of the formation of free radicals that the trigger cell of the death [2,11].

Based on the results of this study it was found that the difference in the number of purkinje cells it was 4.73 cells between the C- group and group of dose III, that given lemon juice with a dose of 13.33 ml / KgBW. The difference in mean cells was significantly different with p value <0,05. The results of this study was indicated that by giving of lemon juice with a dose of 13.33 ml / kgBW can provide an antioxidant effect to prevent the occurrence of purkinje cell death due to exposure to MSG.

Previous of the research conducted by Anshori et al (2017) shows that lemon peel extract has antioxidant activity and prevents oxidative stress on the skin due to exposure to UV B light [12]. Similar research on the antioxidant effects of lemons by Zhou et al (2017) shows that lemon juice has a hepatoprotective effect to protect liver cells from free radicals due to alcohol consumption. Lemon fruit it has a very beneficial for the human body. The in vivo and in vitro experiments have shown that lemon has various health such as anticancer effect, antimicrobial effect, lipid-lowering effect, and protective effect against cardiovascular diseases. Lemon contains many important natural chemical components, including phenolic compounds (mainly flavonoids) and other nutrients and non-nutrients (vitamins, minerals, dietary fiber, essential oils and carotenoids [13]. Vitamin C contained in lemons can reduce lipid peroxidation, inhibit the production of reactive oxygen species, prevent mitochondrial dysfunction and DNA fragmentation, and reduce neurotoxicity, apoptosis and neuron death [14]. Vitamin C contained in lemons also has benefits for increasing the expression of genes involved in neurogenesis, maturation, and neurotransmission. Research conducted by Lee et al (2003) showed that administration of ascorbate up to 200 μM increased the differentiation of precursor cells into neurons and astrocytes for several days in culture. Ascorbate at the same of concentration also induces synaptic maturation of neurons, based on the discovery of an increase in the number of miniature postsynaptic excitatory streams in cultured neurons [15].

The beneficial effects of lemon fruit can be attributed, not only to the vitamin C, but also to the antioxidant activity of their flavonoids. Flavanoid components such as eriocitrin and hesperidin in lemon have stronger antioxidant effectiveness than other citrus flavonoids, and also eriocitrin serves to protect cells against oxidative stress by counteracting free radicals and preventing the formation of superoxide and hydroperoxide. Eriocitrin and hesperidin can increase the concentration of antioxidant enzymes, such as catalase (CAT) and glutathione (GSH). In addition, lemon juice is known as a powerful antioxidant because it contains of citric acid, vitamin E, and lemonades. Research conducted by Salam (2014) proves that administration of 1-2 g / kgBW of citric acid can reduce lipid peroxidation and increase the activity of glutathione peroxidase (GPx) in the brain and liver after injection of lipoporisaccharide (LPS). Therefore, the lemon flavonoids and citric acid might prevent cell death and tissue damage by free radicals[6,16,].

CONCLUSION

Lemon juice can inhibit the deleterious effects of MSG on the number of purkinje cells in the cerebellum cortex before male white mice. Further research is needed regarding the effect of the active ingredient of lemon juice and to reveal the precise mechanisms on how lemon juice affects the Purkinje cells and overall circuitry of cerebellum in relation to motor coordination functions

CONFLICT OF INTEREST DECLARATION

The author states that there is no conflict of interest regarding the publication of this paper.

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