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Allium Ascalonicum L. Ethanolic Extract Inhibited the Decrease of eNOS but did not Inhibit the Decrease of Spatial Memory in D-Galactose-Induced Balb/c Male Mice

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Abstract: ***Background: Currently, the focus of health services has shifted to maintaining cognitive function in old age. Impaired cognitive function can be caused by aging of blood vessels, characterized by decreased endothelial function and nitric oxide (NO). Shallots have been proven to contain flavonoids which have a neuroprotective effect by increasing cerebral blood flow. This study evaluates the effectiveness of the ethanolic extract of shallots in inhibiting the decrease of spatial memory and eNOS levels. Method: The randomized pre-posttest control group design was carried out on 26 healthy three-month-old Balb/C male mice with a 20-30 grams bodyweight, divided equally into two random groups. The control group was given 2 ml of aquadest orally, while the treatment group was given ethanolic extract of shallots at a dose of 16.8mg/20gBW/day dissolved in 2 mL of aquadest. After two hours of administration, both groups were induced with D-Galactose orally 0.3 mg/gr BW/day once a day. Treatments were given daily for four weeks. Measurement of eNOS levels and spatial memory using Morris Water Maze test was carried out before the intervention (pretest) and after (posttest). Comparative analysis was conducted to evaluate the differences between the two groups and a correlation test between eNOS levels and spatial memory was performed. Results: The pre-posttest comparison resultsof eNOS showed a significant decrease (P<0.05) in both groups, however the difference between posttest-pretest (Δ post-pretest) showed that the decrease was significantly higher in control compared to the treatment group (p=0.031).This indicates that shallot ethanolic extract was able to inhibit the decrease of eNOS level. The pre-posttest comparison results showed a significant decrease (P<0.05) in spatial memory and Δ post-pretest did not differ (p>0.05) between control and treatment groups. This indicates shallot ethanolic extract did not inhibit the decrease of spatial memory. No significant correlation was foundbetween eNOS and spatial memory. Conclusion: Administration of oral ethanolic shallots extractinhibited the decreaseofeNOS level but did not inhibit the decrease of spatial memory. The potential of shallots needs to be studied furtherto get an effective period of administration and doses.***

Keywords: *Allium Ascalonicum L.*, D-Galactose, eNOS, Shallots, Spatial memory

1. Introduction

Decreased cerebral blood flow often occurs in the elderly and caused by aging of the blood vessels [1]. Blood vessels aging characterized by decrease in endothelial function and nitric oxide (NO). NO is produced by the NO synthase (NOS) group. Endothelial NOS (eNOS) expressed on vascular endothelial cells[2]. NO readily reacts with molecular oxygen to produce peroxynitrite. The increase in peroxynitrite will reduce NO through the formation of uncoupling eNOS which causes an increase in oxidative stress and endothelial damage[3].

Decreased blood flow to the brain is associated with decreased cognitive function. Cognitive abilities include levels of learning and memory abilities, all of which are regulated in the cerebrum centered on the hippocampus. The facts show that elderly people suffer from cognitive impairment as a result of natural aging, without necessarily having neurodegenerative diseases. Memory is one of the earliest cognitive functions to show decline during aging[4]. One of the research methods for aging models can be done by giving D-Galactose [5].

Scientists are experimenting a lot to find ways to prevent memory loss associated with aging[6]. There is increasing attention to the potential of phytochemical compounds to improve memory, learning and cognitive abilities in general. Many studies have shown that regular consumption of flavonoids has a positive effect on neurocognitive performance. Flavonoids have the ability as neuroprotection, suppress inflammation in the brain and also increase cerebral blood flow thereby inducing angiogenesis and the growth of new cells in the hippocampus area[7, 8].

The use of plants as herbs in Indonesia has been used since ancient times. Shallot (*Allium ascalonicum L*) is a type of plant that can be used for treatment. Shallots are multipurpose spices that are easily available and widely found in Indonesia. Shallots contain phytochemical compounds including flavanols, flavonoids, quercetin, saponins and others[9]. Shallot peel extract is included in the group of very active antioxidants[10].

The research was initiated by the interest in knowing the benefits of shallots to see the potential of the extract and its flavonoid content to prevent further brain aging. The use of shallot extract in research to prevent and treat aging has not been widely carried out. In addition, there has been no

research on the effect of shallots on brain aging, mainly to prevent a decrease in spatial memory and maintain eNOS levels. Based on this, the research is carried out to evaluate the effectiveness of shallots (*Allium ascalonicum L.*) ethanolic extract to prevent spatial memory decline and maintain eNOS levels in male Balb/c D-galactose induced mice (*Mus musculus*).

2. Methodology

Design and Sample Studies

The experimental research design was carried out on a population of male mice (*Mus musculus*) BALB/C strain using a randomized pretestand posttest control group design. A simple random sampling method was carried out on the population that met the inclusion criteria for the study, namely male mice aged three months and having a bodyweight range of 20-30 grams. The Pocock formula with a drop-out tolerance of 10% is used to calculate the required number of samples. The total sample required in this study was 26 male mice which were then randomly divided into two groups (control group and study group). Both groups (13 individuals each) followed the adaptation procedure for one week before the intervention began.

Extract Preparation

The ethanolic extract of shallot (*Allium ascalonicum L.*) was made using shallots are obtained from Denpasar, Bali. Collected shallots were rinsed, dried and milled to obtain powder form. Extraction was conducted with maceration method using ethanol 96%. Filtrate and dregs were separated. Filtrate was evaporated in 45^0C using vacuum rotary evaporator until a crude powder was obtained.

Subject Intervention

Before the intervention started, the examination of eNOS levels and the Morris Water Maze test were carried out and recorded as pretest. The control group was given 2 ml of aquadest orally every day. The treatment group was given the ethanolic extract of shallots (*Allium ascalonicum L.*) at a dose of 16.8mg/20gr BW/day dissolved in 2 mL of aquadest orally every day. After 2 hours, mice in both groups were induced using D-galactose given orally, once per day at a dose of 0.3 mg/gr BW/day (6-9mg/mice/day). The intervention was carried for four weeks. After the treatment was completed, the examination of eNOS levels and the Morris Water Maze test were carried out simultaneously and recorded as posttest data.

eNOS Level Test

eNOS is a nitric oxide-forming substrate in the endothelium of blood vessels, used as a reference for measuring the level of oxidative stress in blood vessels. It measured using ELISA method from mice blood serum before and after treatment was completed. This study used the Mouse Endothelial Nitric Oxide Synthase (eNOS/NOS3) ELISA Kit.

Spatial Memory Test

Morris water maze (MWM) is widely used to study spatial memory and learning. It was a circular drum-shaped pool with a hidden platform under the surface of the water. The pool is divided into 4 quadrants. The pond is placed in a large room with visual cues that can be used as navigational aids, images of different geometric shapes and striking colors mounted on the walls of the pool to mark the quadrants of the pool. Platforms can be hidden by making the water opaque by adding ingredients such as milk or harmless dyes.

Assessment of spatial memory using Morris water maze is done by calculating the number of platform crossings and probe tests during the probe trial phase. Prior to the probe trial, spatial acquisition or spatial learning is carried out, in which experimental animals must learn to use cues to guide them to a hidden platform. This experiment was carried out 4 times every day, for 5 consecutive days.

A probe trial was conducted to assess the spatial memory function of experimental animals, which was carried out 24 hours after completing spatial acquisition. In the trial probe, the experimental animals were allowed to swim for 60 seconds without a platform, recording the number of times the rats crossed the quadrant of the platform in 60 seconds (number of platform crossings) and the length of time (seconds) the experimental animals were in the quadrant of the platform in 60 seconds (probe test).

Data analysis

Statistical analysis was performed using SPSS Ver.26 for windows. Appropriate parametric comparison tests were performed on normally distributed variables, while non-parametric tests were performed on data that were not normally distributed to compare the two groups. In addition, a correlation test was conducted to examine the relationship between eNOS levels and spatial memory.

3. Results

At the end of the study, 2 mice could not cross platform quadrant at alland 2 mice died, leaving 11 mice in each group. The comparison results are presented in Table 1.The comparison test showed that the pretest data on the number of platform crossings and probe test variables had the same baseline ($p>0.05$) but not on eNOS levels ($p<0.05$). The comparison of pre and posttest on eNOS levels showed a significant decrease in both groups ($p<0.05$) but the difference between posttest-pretest (Δ post-pretest) in the control group was significantly bigger from the treatment group ($p<0.05$). This indicates that administration of shallot extract to D-galactose-induced Balb/c male mice was able to inhibit the decrease of eNOS levels.

The pre-posttest comparison results of on number of platform crossings and probe tests showed a significant decrease in both groups ($p<0.05$). The difference between posttest-prettest (Δpost-pretest) was not different ($p>0.05$). This indicates that the administration of shallot extract to male Balb/C D-Galactose induced mice did not inhibit the decrease of spatial memory.

Table 1: Comparative Analysis Pretest and Posttest between Groups

Variable	Group	Pretest	Posttest	Δ Post-pretest	P
eNOS Level (ng/ml)	Control	1.55±0.15	1.24±0.19	-0.31±0.13	**<0.001[a]**
	Treatment	1.30±0.28	1.26±0.45	-0.04±0.36	**<0.001[a]**
	P*	**0.011[b]**	**0.868[b]**	**0.031[b]**	
Number of Platform Crossings (times/60secs)	Control	4.0(3.0-7.0)	2.0(2.0-4.0)	-2.0((-5.0)-0.0)	**0.007[c]**
	Treatment	7.0(2.0-9.0)	4.0(2.0-7.0)	-3.0((-5.0)-1.0)	**0.015[c]**
	P**	**0.170[d]**	**0.010[d]**	**0.340[d]**	
Probe Test (secs)	Control	13.53±3.68	3.09±1.25	-10.42±4.36	**<0.001[a]**
	Treatment	12.71±5.57	4.86±2.54	-8.03±4.86	**<0.001[a]**
	P*	**0.693[b]**	**0.085[b]**	**0.239[b]**	

*Data tested by parametric test is presented in the form of mean±SD
**Non-parametric test data are presented in the median form (minimum-maximum)
a significance test using t-test dependent
b significance test using independent t test
c significance test using the Wilcoxon pair rank test
d significance test using Mann Whitney test

The correlation test between eNOS levels and the number of platform crossings and probe tests did not significantly correlate between the two. These results indicate that eNOS levels have no relationship with spatial memory in this study. The results of the Spearman correlation test are presented in Table 2.

Table 2: Spearman Correlation on eNOS level and Spatial Memory

Variable	Correlation Coefficient	P
eNOS- *Number of crossing platform*	-0.015	0.947
eNOS-*Probe test*	0.265	0.234

r=correlation coefficient

4. Discussion

Effect of Shallot Ethanolic Extract on eNOS Levels

The results of the comparison test showed that the mean eNOS levels showed a significant decrease ($p<0.05$) pre-posttest both in the control group (1.55±0.15 ng/ml vs. 1.24±0.19 ng/ml) and treatment (1.30±0.28 ng/ml vs. 1.26±0.45 ng/ml).Even though the eNOS level still decreased, but the decrease (Δpost-pretest) was significantly higher in the control group compared to the group given the shallot ethanolic extract ((-0.31±0.13) ng/ml vs. (–0.04±0.36) ng/ml). These results indicate that the administration of shallot ethanolic was able to inhibit the decrease of eNOS level compared to the control group.

Even though in this study shown that shallot ethanolic extract had the capability to inhibit the decrease of eNOS level, however this result was different compared to the effect of Buni fruit extract (*Antidesma bunius L.*) that able to maintain eNOS levels in male wistar that exposed to cigarette smoke[11]. The results of this study also different from study of eNOS levels using garlic and onion extracts which showed that administration for 8 weeks was able to increase eNOS activity [12]. This difference can be caused by differences in the length of the study, due to the short period of administration in this study the benefits of giving the extract had not been maximized. Similar statements regarding the effect of giving cocoa flavanols on endothelial function where the effect of acute administration is not as strong as that of long-term administration [13].

The ability of shallot ethanolic extract to inhibitthe decrease, or maintain or increasing eNOS depends on its flavonoid contents. The final effect of flavonoids on NO levels will depend on the concentration used[14]. The different concentration of flavonoids can be resulted from the difference solvent used in the extract making process. Flavonoid content was more found in onion extraction using 96% ethanol compared to 70% ethanol [15]. The extraction method can also cause differences in the flavonoid content possessed, the Microwave Assisted Extraction method is greater than the maceration method[16]. In addition, as stated by Vu et al. (2013) ; Sukasih and Musadad (2018) differences in varieties can cause differences in the content of the ingredients contained in them [17, 18].

Effect of Shallot Ethanolic Extract on Spatial Memory

The spatial memory assessment was carried out using a trial probe which aims to determine whether the experimental animals can remember where the platform is located. Assessment of the probe trial can be done by counting the number of times the animal tries to swim across the platform (number of platform crossings) or how long it spends in the quadrant of the platform (probe test). In this study, both measurements were carried out.

The results of the comparative test in this study showed a significant decrease ($p<0.05$) in the number of platform crossing pre-posttest control group (4.0(3.0-7.0) times/60secs vs. 2.0(2.0-4 ,0) times/60secs) and treatment (7.0(2.0-9.0) times/60secs vs 4.0(2.0-7.0) times/60secs). The results of the comparison of the pre-posttest probe test also showed a significant decrease ($p<0.05$) in the control group (13.53±3.68sec vs 3.09±1.25sec) and the treatment group (12.71±5.57sec vs 4, 86±2.54sec). The decrease (Δpost-pretest) in the number of platform crossings and probe test in the control group compared to the treatment was not significantly different. This indicated that the administration of shallot extract did not inhibit the decline of spatial memory in D-Galactose-induced Balb/c male mice.

The results of this study were different from research ashitaba leaf extract which contain flavonoids can prevent a decrease in probe test and number of platform crossing compared to the control group (Wijaya, 2019). The difference in results might be due to differences in the

content and antioxidant activity of ashitaba leaves and shallots. Ashitaba leaves IC50% was 80.16 ppm while shallot used in this study IC50% was 694.19 ppm, based on this comparison, where shallots have antioxidant activity 8.66 times less than ashitaba leaves, then the dose of onion extract should be given in this study at least 8 times the dose of ashitaba leaf extract (4mg/20gBW/day). However, in this study, the dose used was only 16.8mg/20gBW which refers to the research of Nisa and Rosita (2010), the effective dose of shallot extract to lower cholesterol in rats was 120mg/200gBW which when used in mice was converted to 16.8mg/20gBB [8, 19].In addition to the differences in dosage used, the difference offlavonoid contents in shallot can be caused by the solvents used to make extracts[15] , the extraction method[16] also the differences in varieties[17, 18].

The period of administration of the shallot ethanolic extract in this study was only carried out for 4 weeks, so that the maximum yield of the shallot ethanolic extract on spatial memory may not have been achieved. This is confirmed by the study of the effect of green tea extract on the spatial memory of mice, administration with a larger dose but only done 2 times was not able to improve spatial memory when compared to giving green tea with a smaller dose for 6 weeks which was proven to be able to improve cognitive function. The difference in this study could be due to the non-optimal effect of green tea when given for 2 times, while giving it for 6 weeks caused a more optimal accumulation of extract so that a better effect was obtained[20].

Although there was significant decrease either on number of platform crossings or probe test, there were a tendency ($p>0.05$) that the the decrease was higher in the control group compared to the treatment group. This tendency suggests the administration of shallot ethanolic extract might had its effect. As mentioned by De La Torre (2005) the disruption of eNOS bioactivity that causes spatial memory impairment begins to occur at 8 weeks after vascular occlusion but has not been fully achieved [21]. This statement showed the reason why the shallot ethanolic extract already begin to show its effect on eNOS level but not in spatial memory.

From the results of this study, it shows that the potential of shallots still needs to be investigated further. Not achieving the optimal dose in this study and the short period of administration can cause the optimal benefits of shallots had not achieved yet, further research is needed using several different doses, with different sources or varieties of shallots and different extraction methods as well as longer period of administration.

Correlation of Spatial Memory and eNOS Level

The results of the correlation test between spatial memory calculated using the number of platform crossings and probe test with eNOS found that there was no significant correlation between spatial memory and eNOS.

Research on the potential role of NOS in learning and memory by genetically or pharmacologically inactivating NOS has shown inconsistent results. Several studies have shown no effect due to NOS inhibition on learning and memory[22]. Frisch et al (2000) found that mice with eNOS deficiency showed good performance in the water maze, this could be due to the effects of physiological parameters, such as decreased reactivity of GABAergic neurotransmission or changes in vascular function and effects on behavioral processes[23]. Similar results were found in the study of Blokland et al (1999) where local inhibition of NOS in the rat hippocampus did not interfere with place learning with the Morris Water Maze, while the performance of rats in solving the Morris Water Maze could be influenced by several factors including thermoregulation and stress [24].

The difference in the time of the study could be the cause of not achieving the maximum effect of memory decline and eNOS levels, so that the results of the comparison test were not statistically significant. In addition, other factors that can affect this study include the performance of rats which are influenced by thermoregulation and stress[24]. Memory function is influenced by other factors such as stress or anxiety, diet or nutrition, neurotoxic substances[25], stimulation or stimulation of the brain[26], physical activity and the environment[27].

5. Conclusion

As conclusion, administration of oral ethanolic shallots extract inhibited the decrease of eNOS level but did not inhibit the decrease of spatial memory. No significant correlation was found between spatial memory and eNOS level. The ability of shallot ethanolic extract to inhibit the decrease of eNOS level and for its high flavonoid contents showed that potentiality of shallots still needs to be explored, further study needs to be done with a longer period of administration and various doses to find the effective duration of administration and dose.

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