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The Effect of Green Tea Epigallocatechin-3-Gallate on Spatial Memory Function, Malondialdehyde and TNF-α Level in D-Galactose-Induced BALB/C Mice

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ABSTRACT

Introduction: Neuroinflammatory process and oxidative stress play an important role in the mechanism of brain aging and neurodegenerative diseases such as Alzheimer. Epigallocatechin-3-gallate (EGCG) have antioxidant, anti-inflammatory, and neuroprotective effects. This study aims to prove the effect of green tea EGCG on spatial memory function, malondialdehyde (MDA), and TNF-α level in Balb/c mice induced by Dgalactose. Method : An experimental study using "post test only control group design". The samples were 18 male Balb/c mices, aged 6-8 weeks, divided into 3 groups. Negative control group (N-C) was induced by subcutaneous injection of D-galactose (150 mg/kg) once daily for 6 weeks. EGCG-2 and EGCG-6 were induced by D-galactose and orally administered by 2 and 6 mg/kg EGCG once daily for 5 weeks. The indicator of examination were spatial memory function using morris water maze, MDA and TNF-α level using Elisa. One-way Anova, Kruskal-Wallis, and Pearson were used for statistical analysis. Results : Means of % escape latency time and path length in the EGCG-2 {42,02(SD=5,9);43,47(SD=5,97)%} and EGCG-6 {40,45(SD=6,5); 41,78(SD=6,77)%} were significantly higher than N-C {28,68(SD=9,15), p=0,013; 32,98(SD=7,75)%,p=0,04}. MDA level in the EGCG-2 {587,79(SD=76,04)ng/ml} significantly than N-C was smaller{722,64(SD=134,78)ng/ml,p=0,037}. TNF-α level in all groups was not different (p=0,786). There was a significant and strong correlation between MDA level and spatial memory function (r=-0.551; p=0,018). Conclusion : EGCG may improve spatial memory function and oxidative stress in mice induced dementia, but it may not improve the status of neuroinflammation.

Keywords: Green tea epigallocatechin-3-gallate, spatial memory function, MDA, TNFa, D- galactose

Dementia has become a serious problem in the global health. The 2015 World Report Alzheimer's estimates that 46.8 million people worldwide are living with dementia in 2015, with 9.9 million new cases each year (one new case every 3 seconds). This number is estimated will increase to 74.7 million cases by 2030 and 131.5 million by 2050. This estimation comes from a population-based study that examines the prevalence of dementia in different regions of 42 the world^{1,28,33,34}. According to The 2016 World Health Report, dementia contributed 11.2% causing disability cases in subjects aged over 60 years, greater than stroke (9.5%), musculoskeletal disorders (8.9%), cardiovascular disease (5%), and all types of cancer $(2.4\%)^{33,34}$.

Alzheimer's disease (AD) is the main cause of dementia (50-75%) in the elderly²⁷). AD is and irreversible progressive neurodegenerative disease, characterized by decreased of cognitive and memory function, and degeneration • of cholinergic neurons^{3,12,15)}. Several studies have shown that oxidative stress and inflammatory process play an important role in the pathogenesis of brain aging and neurodegenerative diseases such as Alzheimer^{10,14,23,24,30}).

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36 Oxidative stress is a condition characterized by an imbalance between prooxidant molecules and the antioxidant system²). MDA is formed by the degradation of free radicals OH from unsaturated fatty acids, converted to highly reactive free radicals^{2,14}). In this study, MDA is examined because it is the final product of lipid peroxidation process that can³⁵ represent oxidative stress processes in the central nervous system.

Spatial memory is one of the important indicators for assessing neurocognitive function. Hippocampus is an important part of the brain in mediating spatial and contextual memory functions. Morris water maze (MWM) is a standardized examination used to assess the hippocampal-dependent memory in experimental animal. The MWM plays an important role in the validation of rodent models for neurocognitive disorders such as AD^{8,9,31}.

D-galactose are known to be widely used in animal model for brain aging and neurodegenerative diseases. D-galactose is known to cause aging-related changes including the spatial memory impairment and destruction of nerve cells. D-galactose causes cellular metabolic damage by decreasing the activity of Na⁺,K⁺,ATPase enzymes and increasing oxidative stress through increased lipid peroxidation and decreased antioxidant enzyme activity²⁴).

Several studies have shown that epigallocatechin-3-gallate (EGCG) is a major polyphenol in green tea that has antioxidant, anti-inflammatory, anticancer, and neuroprotective effects²⁰⁻²⁴⁾. In a crosssectional study in Japan that examined the association between green tea consumption and cognitive function, it was mentioned that the consumption of green tea 2 cups or more per day (100 ml/ cups) was associated with a decreased prevalence of cognitive impairment¹⁹⁾. However, in other study showed that the administration of EGCG with a dose of 50 mg/kgBW was not able to improve spatial memory function or repair brain nerve cells damage in repeated ischemic induced Balb/c mice²⁹⁾. The exact effect of EGCG on cognitive remains unclear. This study attempts to prove the effect of EGCG green tea on spatial memory function, oxidative stress, and neuroinflammatory status in D-galactose induced Balb/c mice. The use of multilevel doses is intended to obtain the most effective dose that can provide optimal results.

MATERIALS AND METHODS

Experimental animals and study design

This research was an experimental study with randomized, post test only control group design. The samples were 18 Balb/c males mices, aged 6-8 weeks obtained from the Integrated Research and Testing Laboratory, Gajah Mada University, Jogjakarta, Indonesia.

The sample was divided into 3 groups by simple randomization. N-C group was induced by subcutaneous injection of Dgalactose (150 mg/kg) once daily for 6 weeks. EGCG-2 and EGCG-6 were induced by Dgalactose and orally administered by 2 and 6 mg/kgBW EGCG once daily for 5 weeks. The mean of mice body weight before treatment in N-C group was 29,60 (SD = 0,99) gram, EGCG-2 group was 27,87 (SD = 1,21) gram, and EGCG-6 group was 28.52 (SD = 0.75) gram, thus qualify the normality assumption (Shapiro Wilk p > 0.05) and homogenity (Lavene's test p = 0.412).

Reagents and chemicals

The pure epigallocatechin-3-gallate (EGCG) green tea compound was obtained from the Sigma-Aldrich with E-4143 catalog code, as well as the D-galactose reagent obtained from the Sigma-Aldrich with G-0750 catalog code.

Morris water maze test

At the end of the 6th week of treatment and induction, spatial memory function was examined with morris water maze (MWM). The MWM is a circular pool filled with water (100 cm in diameter and 50 cm in height). Mices were trained to use extra-maze visual cues to locate an escape platform hidden just below the surface of the opaque water. In this procedure, the test was done through 3 phases: (1) Learning/ acquisition phase: a training process 3 for mices to form their spatial memory. Mice were trained to find a hidden platform. The platform was placed in \pm 1 cm below the surface of the water, in a predetermined quadrant. This phase was done for 4 days in a row with 4 times per day. Mice tested their ability to escape, characterized by the success of mice to find a hidden platform. The path length and escape latencies time to find the terminal platform for all mice in 6 days were measured and calculated; (2) Retention phase (probe trial): a test phase of spatial memory function. This

phase is carried out at least 24 hours after the last exercise in the acquisition phase, and made just 1 time. Platform removed, and the mice were given 60 seconds to locate the previous platform. The parameter measurement was % time and % distance in target quadrant; (3) Visual testing: to assess the sensorimotor and visual functions of the animals. Trial was done 3 times in 1 day. Mice were given 60 seconds per trial to find a visible platform. The path length and escape latencies were recorded^{8,9,31}).

Termination and preparation of sample

After MWM examination, mice subsequently terminated by cervical dislocation techniques. The hippocampal dissection procedure was based on the protocol of previous studies by Beaudoin, et al, 2012⁶.

The mice hippocampal tissues were weighed and then homogenized together with phosphate buffered saline (PBS; pH 7.4) with a ratio of tissue weight and volume of PBS is 1:9. Then homogeneous solution was centrifuged at 8000 rpm for 15 min at 4° C, thus obtained supernatant of hippocampal tissue.

Measurement of malondialdehyde level

The supernatants were used for measurements of MDA level using MDA assay kits (Elabscience, USA). Data was read at 450 nm wavelength and MDA levels were expressed as ng/ ml protein.

Measurement of TNF-a level

The supernatants were used for measurements of TNF- α level using TNF- α assay kits 25 (BioLegend, LEGEND MAX, California USA). Cytokines in tissue were expressed as pg/ ml protein.

Statistical analysis

One-way Anova, Kruskal-Wallis, and Pearson were used for statistical analysis. Statistical analyses were done using SPSS version 21.0 for Windows.

Ethical clearance

The study protocol has received ethical approval from the Medical Research Ethics Committee of Faculty of Medicine, Diponegoro University/ Dr. Kariadi Semarang with ethical clearance no. 462 / EC / FK-RSDK / 2015.

RESULTS

The visual testing phase of MWM showed that all mice can reach the platform before the time runs out (<60 seconds). The slowest escape latency time was in the EGCG-2 group (6.49 (SD=3.85)) seconds, while the fastest was in the EGCG-6 group (6.21 (SD=4.42)) seconds. The longest path length was in the N-C group (0.92 (SD=0.25)) meters, while the shortest was in the EGCG-6 group (0.83 (SD=0.41)) meters. The Kruskal-Wallis test showed that there was no differences between N-C, EGCG-2, and EGCG-6 group for escape latency time (p = 0.960) and path length (p = 0.587). This result showed that there was no difference of sensorimotor function of the mice in the N-C, EGCG-2, and EGCG-6 groups, so the acquisition trial and probe trial can be done.

The learning phase (acquisition trial) of MWM showed that mice in the N-C group took longer time and longer distance to find hidden platform on day 1, 2, 3, or 4. Mice on EGCG-2 and EGCG groups showed better results with faster escape latency time recording and shorter path length when compared to the N-C group especially at days 2, 3, and 4 (p <0.05). While between EGCG-2 and EGCG-6 group, the results were not different from the first day until the fourth day (p > 0.05).

In the probe trial phase it was found that the lowest % time was in the N-C group (28.68 (SD=9,15))%, while the highest was in the EGCG-2 group (42.02 (SD=5.90))%. The One Way Anova test showed a significant difference between the experimental groups (F = 5,593; p=0.015). The post hoc LSD test showed a significant difference between the N-C group and the EGCG-2 group (p = 0.007), and between the N-C group and the EGCG-6 group (p = 0.014). But there was no difference between EGCG-2 and EGCG-6 group (p =0,716) (Figure 1). Mean of % path length in probe trial phase was lowest in the N-C group (32.98 (SD = 7.75))%, while the highest was in the EGCG-2 group (43.47 (SD = 5.97))%. One Way Anova test showed a significant differences between experimental groups (F = 4.028, p = 0.040). The post hoc LSD test showed a significant difference between the N-C group and the EGCG-2 group (p = 0.018), and between the N-C group and the EGCG-6 group (p = 0.042). But there was no difference e between EGCG-2 and EGCG-6 (p = 0.677) (Figure 2). This result proves the hypothesis that EGCG in both 2 and 6 mg / kgBW doses

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has an effect on the improvement of spatial memory function in dementia induced Balb/c mice using D-galactose.

In this study it was found that the lowest MDA levels were in the EGCG-2 group (587.79 (SD = 76.04)) ng / ml, while the highest was in the N-C group (722.64 (SD = 134.78)) ng/ ml. *Kruskal-Wallis* test showed no differences between experimental groups (p=0.141). The *Mann-Whitney* test showed a significant difference between the N-C and EGCG-2 groups (p=0.037). In contrast, no differences were found between the N-C and EGCG-6 groups (p=0.470), and between the EGCG-2 and EGCG-6 groups (p=0.336) (Figure 3). Thus, it can be concluded that EGCG 2 mg/ kgBW can affect decreased

levels of dementia-induced hippocampus MDA mice. *Pearson* correlation test showed a significant correlation between MDA level and spatial memory function (p=0,018; r=0,551). Negative correlation, indicating that higher MDA levels are associated with lower spatial memory function. The correlation diagrams of MDA level and spatial memory function are presented in Figure 4.

In this study it was found that EGCG orally at a dose of 2 and 6 mg/kgBW daily for 5 weeks did not provide changing levels of TNF- α (*p*=0.786). *Pearson* correlation test showed no correlation between TNF- α levels with spatial memory function (*p* = 0.313).

FIGURES



Figure 1. Mean comparison of % time in target quadrant on probe trial phase MWM; mean (SD)



Figure 2. Mean Comparison of % path length in target quadrant on probe trial phase mean (SD)

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Figure 3. Mean comparison of MDA level (ng / ml) levels between groups; mean (SD)



Pearson r = -0,551 p = 0,018

Figure 4. Correlation between MDA level (ng/ml) and spatial memory function

DISCUSSION

The result of this study indicate that mice in EGCG-2 and 6 group showed better ability with faster escape latency time recording and shorter distance compared to control in learning phase of MWM. In addition, the mice in EGCG-2 and 6 group also showed better memory ability, with high percentage of time and path length in target quadrant compared with control group (p=0,015; p=0,040) in probe trial phase of MWM. This is in consistant with previous studies which stated that EGCG green tea can improve memory spatial function in experimental animals²⁴). Other studies have suggested that administering EGCG 10 mg/kgBW daily for 4 weeks can improve the cognitive abilities of rats Wistar induced dementia bv intracerebroventrikular streptozotocin⁷⁾. However, the results of this study did not match the previous study which states that the administration of EGCG with a dose of 50 mg/kgBW is not able to increase spatial memory function or repair brain nerve cells damaged in repeated ischemic induced Balb/c mice²⁹⁾. These different results are likely due different duration of EGCG to administration. In the study by Pu et al,

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EGCG was given only 2 times, ie 30 minutes before ischemic induction was done. This allows less optimum effects of EGCG to repair memory impairment due to ischemic induction. In this study, EGCG was administered in small doses for a relatively long time (5 weeks). This causes the accumulation of EGCG optimally and give better effect.

Spatial memory is an important indicator in assessing neurocognitive function of an individual. Spatial memory impairment is often an early symptom found in early dementia. Dementia is a major cause of disability in elderly. In the aging process, Alzheimer's disease is the main cause (50-75%) of dementia in the elderly²⁷.

The experimental animals induced by Dgalactose are known to be widely used in brain aging and neurodegenerative diseases. The results of this study indicate that mice in N-C group take longer time and longer distance to find hidden platform when compared with EGCG-2 and 6 group. Mice in the N-C group also get difficulties in recalling the location of the platform, indicated by the low percentage of time and path length passed in the target quadrant of MWM. This is consistent with previous studies in which 150 mg/kgBW subcutaneous injections of Dgalactose daily for 6 weeks may cause behavioral and memory impairment in mice18,24,26). D-galactose is also known to cause spatial memory impairment and nerve cell damage due to cellular metabolic damage by decreasing the activity of Na⁺, K⁺, ATPase enzymes and increasing oxidative stress through increased lipid peroxidation and decreased antioxidant enzyme activity24). Hadzi-Petrushev et al mentioned that administration of D-galactose intragaster 100 mg/kgBW for 6 weeks was able to increase levels of TNF-a and IL-18 plasma and lipid peroxidation. This study proves that administration of D-galactose at concentrations exceeding normal can lead to the production of free radicals and advanced glycation end products (AGEs). AGE will bind to its receptors (RAGE) and trigger activation of the nuclear factor-kB (NF-kB) transcription factor that plays a role in the production of proinflammatory cytokines (TNF-α and IL-1β) and free radicals¹⁶).

2 Oxidative stress and inflammatory processes are known to play an important role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease^{10,14,17,23,24,30,32}). Several studies have shown that epigallocatechin-3-gallate (EGCG), a major polyphenol in green tea, has antioxidant, anti-inflammatory, anticancer, and neuroprotective effects²⁰⁻²⁴⁾. However, other studies suggest that EGCG or green tea extract with a certain dose does not have a significant effect on cognitive function. oxidative stress, or neuroinflammatory status^{13,29)}. Excess production of reactive oxygen species and or decreased activity of antioxidant enzymes in the brain can lead to lipid peroxidation processes, mitochondrial DNA damage, and protein oxidation, leading to impaired neurocognitive function²⁴⁾. Malondialdehyde (MDA) is a dialdehyde compound which is the final product of lipid peroxidation in the body^{2,14,17,25}). D-galactose induction in experimental animals is known to cause impaired cognitive functioning because it triggers cellular oxidative stress, increases MDA levels, decreases SOD and GSH-Px enzyme activity¹¹).

The results of this study indicate that EGCG 2 and 6 mg/kgBW given daily for 5 weeks can decrease MDA levels in Dgalactose-induced mice hippocampus. Pearson correlation test showed that there was a significant correlation between MDA level and spatial memory function (p=0.018), with strong correlation strength (r=0.551), and negative correlation direction indicating that higher MDA level was associated with lower spatial memory function. This is in consistant with previous studies that mention the antioxidant effects of EGCG on the central nervous system^{4,12,24}). Ejaz Ahmed et.al proved that 10 and 20 mg/ kgBW of green tea catechin hydrate orally for 3 weeks was able to increase the activity of SOD, GSH-Px, and catalase enzymes, also decrease MDA level of hippocampus Wistar rats induced by intracerebroventricular streptozotocin¹²⁾. Other studies have suggested that administration of green tea extract can significantly lower MDA levels when compared with controls⁴⁾. However, these results differ from research of Flores et al mention that green tea extracts containing EGCG 299.56 ug/ml administered over one month can not change hippocampus MDA levels in 10 months Wistar rats¹³⁾. This difference is likely to be influenced by the age of the experimental animal, the duration of the intervention, the dose concentration, and the type of the experimental animal.

In this study it was found that EGCG 2 and 6 mg/kgBW did not provide changes in TNF- α levels. This is due to the use of young adult animals in this study. Older experimental animals show an exaggerated and more prolonged neuroinflammatory response when compared to young adults. In a study conducted by Barrientos et.al who studied the proportionate responses of proinflammatory cytokines in mice (3 months) and old age (24 months) infected with Escherichia coli, it was found that both groups had an elevated IL -18 hippocampus at 2 h post infection. IL-18 levels in mouse hippocampus decreased significantly after 24 h post-infection in adult rats group, whereas in old age rats group IL-18 levels continued to increase until the 8th day post infection. IL-18 levels decreased as baseline after the 14th post infection day. This suggests a more durable neuroinflammatory response to the aging process.⁵⁾ In this study, researchers used young adult rats (6-8 weeks) in which new TNF- α levels were tested on the 7th day after the last D-galactose injection. This allows the decrease of TNF- α levels to initial conditions. This became one of the limitations in this study. Further research on the effects of green tea EGCG on neuroinflammatory status should be performed using effective timing of TNF-α assay levels post-dementia induction.

From this research we can conclude that EGCG may improve spatial memory function and oxidative stress in mice induced dementia, but it may not improve the status of neuroinflammation.

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