The Effect of Green Tea Leaf Extract on Spatial Memory Function and Superoxide Dismutase Enzyme Activity in Mice with D-galactose Induced Dementia

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ABSTRAK

Metode: Penelitian experimental dengan "post test only control group design", menggunakan 24 ekor mencit Balb/c jantan usia 6-8 minggu, dibagi dalam 4 kelompok. Kelompok kontrol negatif (N-G) diinduksi D-galaktose 150 mg/kgBB subkutan setiap hari selama 6 minggu. Kelompok perlakuan GT-90, GT-270, dan GT-540 diinduksi D-galaktose dan diberi ekstrak daun teh hijau oral dengan dosis 90, 270, dan 540 mg/kgBB selama 6 minggu. Indikator yang diperiksa adalah fungsi memori spasial (morris water maze) dan aktivitas enzim SOD korteks cerebri dengan ELISA. Uji beda antar kelompok menggunakan One-way Anova

Hasil: Analisis statistik Mann Whitney menunjukkan bahwa rerata % waktu latensi pada kelompok GT-90 (35,29 (SD= 2.69)%), GT-270 (35,28 (SD= 2.62)%), dan GT-540 (35,62 (SD=5.05)%) lebih tinggi dari N-G (20,38 (SD = 3.21)%) dan berbeda bermakna (p<0.05). Rerata aktivitas enzim SOD kelompok GT-270 (0,78 (SD = 0,07) U/ml) lebih tinggi dari N-G (0,51 (SD=0,01) U/ml) dan berbeda bermakna (p=0,004).

Kesimpulan: Ekstrak daun teh hijau terbukti mampu memperbaiki fungsi memori spasial dan meningkatkan aktivitas enzim SOD.

Kata kunci: Ekstrak teh hijau, fungsi memori spasial, SOD, D-galaktose

ABSTRACT
Introduction: Oxidative stress and inflammation play an important role in pathogenesis of brain aging and neurodegenerative diseases such as Alzheimer. Green tea has been shown to have antioxidant, anti-inflammatory, anticancer, and neuroprotective activity. Objective: to determine the effect of green tea extract on spatial memory function and superoxide dismutase enzyme activity in mice with D-galactose induced dementia

Methods: An experimental study using “post test only control group design”. Twenty male BALB/c Mice aged 6-8 weeks were divided into 4 groups. Negative control group (NG) was induced by subcutaneous injection of D-galactose (150 mg/kg BW) once daily for 6 weeks. GT-90, GT-270, GT-540 were induced by D-galactose and orally administered with 90, 270, and 540 mg/kg BW of green tea extract once daily for 6 weeks. The spatial memory functions were assessed using Morris water maze and SOD enzyme activities were evaluated using ELISA. One-way Anova and Kruskal-Wallis were used for statistical analysis.

Results: mean percentage of latency time in the GT-90 (35.29 (SD= 2.69)%), GT-270 (35.28 (SD= 2.62)%), and GT-540 (35.62 (SD=5.05)%) were significantly higher compared to that of NG (20.38 (SD= 3.21)%), p < 0.05. SOD enzyme activity in the GT-270 (0.78 (SD = 0.07) U/ml) were significantly higher compared to that of NG (0.51 (SD = 0.01) U/ml), p = 0.004.

Conclusion: Green tea extract may improve spatial memory function and the activity of superoxide dismutase enzyme in mice with D-galactose induced dementia.

Keywords : Green tea extract, spatial memory function, SOD, D-galactose

INTRODUCTION
Dementia has been one of the biggest health problem globally. Alzheimer’s Disease International (ADI) estimated that in 2015 there were 46.8 million patients with dementia worldwide with 9.9 million of new cases annually (one case every 3 second) (Prince et al., 2015). According to World Alzheimer Report in 2009, it was estimated that by 2030 there will be 65.7
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million dementia cases worldwide and 115.4 million cases by 2050 (ADI, 2008; Pender, 2014; Wortmann, 2012). According to World Alzheimer Report in 2015 dementia cases accounted for 13.1% for disability in subjects above 60 years which is much higher than stroke, musculoskeletal, cardiovascular diseases, dan all type of cancer (Prince et al., 2015).

Some studies indicate that oxidative stress and inflammation play an important role in pathogenesis of brain aging and neurodegenerative diseases such as Alzheimer (Crews & Masliah, 2010; Gandhi & Abramov, 2012; Mandel et al., 2008; Miao et al., 2009). Excessive production of reactive oxygen compounds and/or decreased activity of antioxidant enzymes in the brain can lead to lipid peroxidation, mitochondrial DNA damage, and protein oxidation, leading to impaired neurocognitive function (Miao et al., 2009). Tests of antioxidant enzyme levels such as SOD, catalase, and GSH-PX in erythrocytes are performed to determine the health status of respondents at the cellular level. SOD suppresses oxidative damage by catalyzing superoxide anion (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$). Hydrogen peroxide is a strong oxidizer susceptible to free radicals requiring prompt neutralization (Agarwal, 2012; Gandhi & Abramov, 2012).

Spatial memory is an important indicator in assessing the neurocognitive function. Morris water maze (MWM) is the standard test used to assess laboratory animal behavior, especially memory function related to hippocampus (hippocampus-dependent memory) (Barrientos et al., 2010; Barrientos et al., 2009). Animals injected with D-galactose have been widely used in studies of brain aging and neurodegenerative diseases. D-galactose has been shown to cause impairment of spatial memory and neurocellular damage leading to cellular metabolic damage by decreasing activity of the Na$^+$, K$^+$, ATPase enzymes, and increasing oxidative stress through increased lipid peroxidation and decreased antioxidant enzyme activity (Miao et al., 2009).

Some studies show that epigallocatechin-3-gallate (EGCG), the main polyphenol in green tea, have been shown to have antioxidant, anti-inflammatory, anticancer, and neuroprotective activity (Miao et al., 2009; Mandel et al., 2008; Lee et al., 2009; Lee et al., 2013). The studies show that the oral administration of green tea EGCG (2 and 6 mg/kg BW) for 4 weeks significantly improves cognitive ability in mice induced by D-galactose (Miao et al., 2009). However, a different study showed that the administration of green tea EGCG at the dose of 50 mg/kg fail to improve spatial memory abilities as well as repair nerve cell damage in BALB/c mouse brain induced by repeated ischemia (Pu et al., 2007). This present study was aimed to determine the effect of higher and graded dose of tea leaf extract on spatial memory function and SOD enzyme activity in BALB/c mice induced by D-galactose. The use of graded doses was intended to obtain the most effective dose that can provide optimum results.

METHODS
Animal Models
This was a laboratory experimental research with a randomized, post test only control group design. In this study, 24 male BALB/c aged 6-8 weeks were obtained from the The Integrated Research and Testing Laboratory (LPPT) of Gajah Mada University.

The mice were randomly divided into 4 groups. Negative control group (NG) was subcutaneously induced by D-galactose at the dose of 150 mg/kg daily for 6 weeks. The treatment groups (GT-90, GT-270 and GT-540) were induced by D-galactose and orally administered with green tea leaf extract at the dose of 90, 270, and 540 mg/kgBW, respectively. The mean body weight of mice prior to treatment for NG, G-90, G-270, G-540 were 31.78 g (SD = 0.59), 31.92g (SD = 0.89), 32.18g (SD = 0.65) g, 31.53g (SD = 0.99) g, respectively meeting the assumptions of normality (Shapiro Wilk test p> 0.05) and homogeneity (Lavene’s test p = 0.388).

D-Galactose and Green Tea Extract
D-galactose (Gal; G0750) were from Sigma-Aldrich. The graded dose of ethanol extract of green tea (Camellia sinensis) leaf included 90, 270, and 540 mg/kgBW.

Morris Water Maze (MWM) test
At the end of week 6 of induction treatment and examination, a spatial memory function was assessed with the Morris water maze (MWM) test. MWM procedure used in this study adopted from that od studies, conducted by Bouet et al in 2010, and Bromley-Brits et al in 2011 (Bouet et al., 2010; Bromley-brits et al., 2011). In this procedure, the test was carried out through three phases, namely: (1). Exercise phase (learning phase /acquisition phase): This phase was the training process for the mice to form a spatial memory. Mice were trained to find a platform hidden underwater. a platform was placed, ± 1 cm under the water surface, in a predetermined quadrant. This phase took 4 days in a row with 4 times exercise per day. Furthermore, mice was rated for their ability to escape (out of water), marked by the success of the mice find a
hidden platform. The parameters assessed were latency time of the mice to reach the platform divided by the percentage of distance divided by total distance; (2). The retention phase (retention phase/probe trial): a test phase of long-term spatial memory function in MWM test. This phase was carried out at least 24 hours after the last exercise in the acquisition phase of the trial, and made once. Platform was omitted, and the mice were given 60 seconds to locate the previous platform. Parameters assessed included the percentage of latency time in the time taken in platform quadrant compared to the total time and distance travelled in the platform quadrant divided by total distance; (3). Sensorimotor test (visual testing) aiming to assess the ability of sensorimotor in the laboratory animals. Trial was done 3 times in 1 day. Mice were given 60 seconds per trial to be able to find the platform marked (visible platform). Time taken and distance travelled of mice were recorded (Bouet et al, 2010; Bromley-brits et al, 2011)

Termination and Supernatant of Tissue Preparation

After Morris water maze procedure was completed, the mice were terminated by cervical dislocation techniques. The brain of the terminated mice were taken. The dissection was carried out as described previously (Beaudoin et al., 2012).

The cerebral cortex tissue taken from the mice were weighed and then homogenized using of phosphate buffered saline (PBS; pH 7.4) based on 1:9 mass to volume ratio. The homogeneous solution was then centrifuged at 4500 rpm for 15 minutes at a temperature of 4°C. The supernatant of brain tissue was prepared for analysis.

Superoxide Dismutase Enzyme Examination

SOD enzyme activity were evaluated using ELISA kit for the brand bioassay system the supernatant samples of brain tissue of the terminated mice. Data was read on the wavelength of 450 nm and SOD enzyme activity was presented in U/ml.

Statistic Analysis

After collected, the data were cleaned, tabulated and coded. Spatial memory function showing a normal distribution was tested by SaphiroWilk normality test (p> 0.05). The hypothesis was tested by parametric statistical analysis using One Way ANOVA to see the differences among the three treatment groups. The differences in each treatment group were then analyzed by using post hoc least significant different (LSD). SOD enzyme activity variables showed non normal (ShapiroWilk test, p<0.05) distribution were subjected to a non-parametric statistical test of Kruskal-Wallis. Furthermore, to determine the difference in each group, posteriori test of Mann-Whitney (Dahlan, 2014) was conducted. A correlation between SOD enzyme activity and spatial memory function were tested using Pearson correlation test due to their normal distribution.

Ethical Clearance

Ethical clearance was obtained from The Ethics Committee for Health Research (KEPK) Faculty of Medicine, Diponegoro University/General Hospital prior to the commencement of the study (No 921/EC/ FK-RSDK/IX/2016).

RESULT

Probe trial phase/retention is the testing phase of spatial memory function in MWM examination. This phase is carried out at least 24 hours after the last exercise in the acquisition phase of the trial, and conducted once within 60 seconds (Bouet et al, 2010; Bromley-brits et al,2011). In this present study, the shortest time of the probe phase of mice was found in NG group (20.38, SD = 3.21)% , and the highest was found in the G-540 in (of 35.62, SD = 5 05%). The statistical analysis using One Way ANOVA found a significant difference between the experimental group (F = 2.69; p = 0.049). The results of the post hoc LSD found a significant difference between the group G-90 NG (p = 0.021), the group NG and G-270 (p = 0.02), and between the group and the G-540 NG (p = 0.018). This study showed that the administration of tea leaf extract both at 90, 270, and 540 mg/kg...
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Figure 2. Comparison mean of SOD enzyme activity (U/ml) between groups. Data were presented in mean (SD). * P <0.05

BW improves spatial memory function indicated by percentage of time latency in mice with D-galactose induced Dementia.

This study found that the lowest activity of SOD was in NG (0.51, SD = 0.01) U/ml, while the highest was in the G-270 in the amount of 0.78 (SD = 0.07) U/ml. The statistical analysis of Kruskal-Wallis test found a significant difference between the experimental groups (p = 0.007). Then, Mann-Whitney posteriori was applied showing a significant difference between the groups and the G-270 NG (p = 0.004). On the contrary, no difference was found between the NG and G-90 group (p = 0.936), and between the NG and G-540 group (p = 0.055). Thus, the present study showed that the administration of green tea leaf extract (only at 270 mg/kg BW) can increase activity of SOD of cerebral cortex of mice with D-galactose induced dementia.

Pearson correlation test resulted in no correlation between SOD enzyme activity and spatial memory function (p = 0.307, R = 0.217) indicates a weak statistical correlation with a positive positive correlation indicating that the higher the SOD enzyme activity associated with a higher spatial memory function. Diagram of transmit of enzyme activity of SOD and spatial memory function is presented in Figure 3. Pearson $r = 0.217$ $p = 0.307$

DISCUSSION

Spatial memory is an important indicator in assessing the neurocognitive function. Spatial memory impairment has been often a symptom of early dementia. Dementia is the main cause of dependence and disability in elderly. Besides aging, Alzheimer's disease is the main cause (50-75%) of dementia in the elderly (Pender, 2014).

This study was aimed to prove the effects of green tea leaf extract on spatial memory function and the activity of antioxidant enzymes (superoxide dismutase) in D-galactose induced BALB/c mice. D-galactose induced mice has been widely used in studies of brain aging and neurodegenerative diseases. D-galactose is normally reduced into sugar in the body. If it is above the normal levels in the body, D-galactose will be oxidized to aldehydes and hydrogen peroxide ($\text{H}_2\text{O}_2$) by galactose oxidase enzyme. D-galactose will change into galactitol that can not be metabolized normally, so it will accumulate in the cells, triggering osmotic stress and free radical formation (Miao et al., 2009; Kumar et al., 2011).

This study found that mice in the NG induced by D-galactose requires more time and distance longer to find hidden platform compared to G-90, G-270 and G-540 administered with green tea leaf extract at 90, 270, and 540 mg/kg BW. Mice in the control group also had difficulty in remembering the location of the platform, shown by the low percentage of time needed in the target quadrant in the retention phase of morris water maze (MWM) assay. This is consistent with the finding of the previous studies showing that subcutaneous injection of D-galactose at a 150 mg/kg daily for 6 weeks can cause memory and behavioral impairment in mice (Parameshwaran et al., 2010; Miao et al., 2009). D-galactose is also known to cause spatial memory and neurocell damage due to cellular metabolic damage by lowering the activity of Na$^+$, K$^+$ -ATPase enzymes and increasing oxidative stress through increased lipid peroxidation and decreased antioxidant enzyme activity (Miao et al., 2009). A study...
conducted by Hadzi-Petrushev et al. mentioned that the intragastric administration of D-galactose at 100 mg/kg for 6 weeks can increase levels of TNF-α and IL-1β plasma and lipid peroxidation. This study proves that the administration of D-galactose exceeding normal dose capable of triggering the production of free radicals and advanced glycation end products (AGEs). AGE binds to its receptor (RAGE) and trigger the activation of the transcription of nuclear factor -κ B (NF-κ B) that plays a role in the production of pro-inflammatory cytokines (TNF-α and IL-1β) and free radicals (Hadzi-petrushev, 2014).

Oxidative stress and inflammation have been shown to play an important role in neurodegenerative disease pathogenesis such as Alzheimer’s disease (Crews & Masliah, 2010; Gandhi & Abramov, 2012; Mandel et al., 2008; Miao et al., 2009). Some studies indicate that epigallocatechin-3-gallate (EGCG), main polyphenol in green tea; have antioxidant, anti-inflammatory, anticaner, and neuroprotective effect (Lee et al., 2013; YK Lee et al., 2009; Mandel et al., 2008; Miao et al, 2009). However, a study stated that EGCG or green tea extract at a certain dose does not have a significant effect on cognitive function, oxidative stress, or neuroinflammation status (Pu et al., 2007; Flores et al., 2014).

In this study it was found that mice treated with green tea leaf extract at 90, 270, and 540 mg/kg BW daily for 6 weeks showed an improvement in memory, with a higher percentage of time {35.29 (SD = 2.69)%; 35.28 (SD = 2.62)%; 35.62 (SD = 5.05)% respectively} in the target quadrant compared to the control group {20.38 (SD = 3.21)%}, p = 0 <0.05. These findings support previous studies stating that administration of green tea can improve or memory function in laboratory animals. Other study evaluating the effect of EGCG of green tea on aging process states that administration of green tea EGCG (2 mg/kg and 6 mg/kg BW) orally for 4 weeks significantly improve cognitive abilities (Miao et al., 2009). Another study showed that the administration of green tea EGCG 10 mg/kg daily for 4 weeks post intracerebroventricular streptozotocin-induced dementia can improve the cognitive abilities in Wistar rats assessed with MWM (Biasibetti et al., 2013). However, these results shows a contradiction to an earlier research showing that the administration of green tea EGCG at a dose of 50 mg/kg BW fail to improve spatial memory as well as repair damaged brain neurocells in BALB/c mice with recurrent induction(Pu et al., 2007). The different results could have been due to the different duration of the extract. In a study by Pu et al given EGCG green tea was administered two times, i.e 30 minutes before ischemic induction. This allows less optimum effects of EGCG green tea to repair any memory damage to the ischemic induction of ischemic. In this study, the green tea leaf extract was administrated in a small doses within a relatively longer time (6 weeks). It allowed the optimum accumulation of extracts and better effect.

Oxidative stress is an imbalance between pro-oxidant molecules and antioxidant systems (Agarwal, 2012). Excessive production of reactive oxygen compounds and/or decreased activity of antioxidant enzymes in the brain can lead to lipid peroxidation, mitochondrial DNA damage, and protein oxidation, leading to impaired neurocognitive function (Miao et al., 2009). SOD suppress oxidative damage by partitioning catalyzes superoxide anion (O_{2}^{•−}) into H_{2}O_{2}. H_{2}O_{2} is the strongest oxidizing agent that are vulnerable to free radicals, because it must be neutralized (Agarwal, 2012; Gandhi & Abramov, 2012). D-galactose induction in experimental animals is known to cause impaired cognitive function because it triggers cell oxidative stress, increases MDA levels, decreases SOD and GSH-Px enzyme activity (Miao et al., 2009). Green tea leaf extract in this study is expected to raise the antioxidant enzymes and improve memory function in mice. Indicators of antioxidant enzymes assessed in this study was the SOD enzyme activity.

This study found that green tea leaf extract administered orally at a dose of 270 mg/kgBW daily for 6 weeks can increase the activity of SOD (0.78 (SD = 0.07) U/ml) in the cerebral cortex of mice induced by D-galactose when compared to controls (0.51 (SD = 0.01) U/ml). Pearson correlation test showed no correlation between SOD enzyme activity and spatial memory function (p = 0.307) with a weak correlation (r = 0.217) and positive correlation indicating that the higher the SOD enzyme activity was associated with the higher spatial memory function. This finding is contradictory to previous studies showing antioxidant effects of green tea leaf extract on the central nervous system (Miao et al., 2009; Ejaz Ahmed et al., 2013; Assunção et al., 2011). Ejaz Ahmed et.al demonstrate that oral administration of green tea catechins hydrate at 10 and 20 mg/kgBW for 3 weeks was able to increase the activity of the enzyme superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase, as well as lower levels of malondialdehyde (MDA) in hippocampus in rats induced by intracerebroventricular streptozotocin (Ejaz Ahmed et al., 2013). The results supports the research conducted by Flores et al stating that green tea extract containing EGCG at 299.56 μg/ml for a month could not significantly change in

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antioxidant enzyme hippocampal (p> 0.05) in Wistar rats aged 3 months compared to controls (Flores et al., 2014). The difference was probably due to several factors such as age and strain of the laboratory animals, duration of the intervention, concentration of the dose and type of animal.

CONCLUSION
Green tea leaf extract at the dose of 90, 270, and 540 mg/kgBW have been shown to improve spatial memory function. While SOD enzyme elevation was observed at a dose of 270 mg/kgBW. Further research is needed with the more parameters of oxidative stress and antioxidant determine the effect of green tea leaf extract to improve neurocognitive function.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

REFERENCES


