ORIGINAL ARTICLE

The Effect of Green Tea Leaf Extract on Caspase-3 Protein Level in D-Galactose Induced Balb/C Mice

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ABSTRACT

Background: Aging and dementia has become a serious problem in the global health. Green teais known to haveantioxidant, anti-inflammatory,anti-apoptotic, andneuroprotective effects which are potential as an anti-aging drug.
Aim: To prove the effect of green tea leaf extract on hippocampal caspase-3 protein level in D-galactose induced Balb/c mice.
Method: The study used post test only control group design. The samples were20 male Balb/cmices,aged 6-8weeks,divided into5groups. Normal control group (NC) was given normal salin. Negative control group(NG) was induced by subcutaneous injection of D-galactose (150mg/kg) once daily for6weeks. GT-90, GT-270,GT-540was induced byD-galactoseand orally administered by 90, 270, and 540 mg/kg green tea leaf extractonce daily for 6 weeks respectively.The indicatorof examination was caspase-3 level using ELISA. One-way Anova, post hoc LSD, and Pearson were used for statistical analysis.
Result: There was a significant difference between all groups (p=<0.001)**.** Hippocampal caspase-3 level in the NG group (12.37±1.647ng/ml) was significantly higher than GT-90 group (7.69±1.763ng/ml; p=0.001), GT-270group (7.32±1.733 ng/ml; p=0.001), and GT-540 group (3.99±2.298ng/ml; p<0.001).
Conclusion: Green tea leaf extract may reduce the level of hippocampal caspase-3 protein level in dementiainducedBalb/c mice.
Keywords: Green tea leaf extract, Caspase-3, D-galactose, Dementia

INTRODUCTION

Aging and dementia has become a serious problem in the global health. The 2016 World Report Alzheimer's estimates that 47 million people worldwide are living with dementia in 2016, with 9.9 million new cases each year (one new case every 3 seconds). This number is estimated will increase to 131 million by 2050. This estimation comes from a population-based study that examines the prevalence of dementia in different regions of the world.[1–3] 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%).[2,3]

Alzheimer's disease (AD) is the main cause of dementia (50-75%) in the elderly.[4] AD is a progressive and irreversible neurodegenerative disease, characterized by decreased of cognitive and memory function, and degeneration of cholinergic neurons.[5–7] Several studies have shown that oxidative stress, inflammatory, and apoptosis process play an important role in the pathogenesis of brain aging and neurodegenerative diseases such as Alzheimer.[8–12]Apoptosis is a form of programmed cell death and plays an important role in various physiological and pathological conditions in the aging process. Caspase-3 is the main executor caspase in the mechanism of apoptosis which plays a role in the pathogenesis of brain aging and neurodegenerative diseases.[13–15]Hippocampus is an important part of the brain in mediating spatial and contextual memory functions.[16–18]

D-galactoseis known to be widely used in animal model for brain aging and neurodegenerative diseases. D-galactoseis 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 activity.[12]

Parallel to the increase cases of dementia in elderly population, anti-aging approaches have become an important public issue. Fortunately, herbal medicines have opened new perspective as potential agents for development of new anti-aging drugs and prevention of dementia. Several studies have shown that epigallocatechin-3-gallate (EGCG) is a major polyphenol in green tea that has antioxidant, anti-inflammatory, anti-apoptotic, anticancer, and neuroprotective effects.[11,12,19–21]Singh et al.[22] said that EGCG, both in vitro and in vivo models effectively lowers neurotoxicity via the reduction in the expression of pro-apoptotic genes with no effect on anti-apoptotic genes. This study attempts to prove the effect of green tea leaf extract on hippocampal caspase-3 protein level 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 controls group design. The samples were 20Balb/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 4 groups by simple randomization. Normal control group (NC) was given normal salin. Negative control group(NG) was induced by subcutaneous injection of D-galactose (150mg/kg) once daily for6weeks. GT-90, GT-270,GT-540was induced byD-galactoseand orally administered by 90, 270, and 540 mg/kg green tea leaf extractonce daily for 6 weeks respectively.

D-galactose and green tea leaf extract: D-galactose reagent obtained from the Sigma-Aldrich with G-0750 catalog code.The graded dose of ethanol extract of green tea *(Camellia sinensis)* leaf included 90, 270, and 540 mg/kgBW.

Termination and preparation of sample: Mice subsequently terminated by cervical dislocation techniques at the end of week 6. The hippocampal dissection procedure was based on the protocol of previous studies by Beaudoin, *et al*.[23]

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 4000 rpm for 15 min at 4° C,thus obtained supernatant of hippocampal tissue.

Measurement of caspase-3 protein level: The supernatants were used for measurements of hippocampal caspase-3 protein level using mouse caspase-3 ELISAkit (Elabscience, USA, E-EL-MO238 catalog code). Data were read at 450 nm wavelength and caspase-3 protein levels were expressed as ng/ ml protein.

Statistical analysis: *One-way Anova*and *post hoc LSD* were used for statistical analysis.Statistical analyses were done using SPSS version 21.0 for Windows.

Ethical clearance: The study protocol had received ethical approval from the Medical Research Ethics Committee of Faculty of Medicine, Diponegoro University/ Dr.Kariadi Semarang with ethical clearance no.061/EC/FK-RSDK/VIII/2017.

RESULTS

This study found that the highest hippocampal caspase-3 protein levels was in the NG group (12.37±1.647ng/ml), while the lowest was in the GT-540 group (3.99±2.298ng/ml).Hippocampal caspase-3 level in the NG group (12.37±1.647ng/ml)was significantly higher than NC group (7.31±0.708ng/ml).Hippocampal caspase-3 level in the GT-540 group (3.99±2.298ng/ml) was lower than GT-270 (7.32±1.733 ng/ml), and GT-90 group (7.69±1.763ng/ml) (Table 1, Figure 1).

One Way Anova test showed a significant difference between all groups (p=<0.001). The *post hoc LSD* test showed a significant difference between the NG and GT-90 groups (p=0.001), NG and GT-270 groups (p=0.001), and between the NG and GT-540 groups (p<0.001) (Table 2).Thus, it was proven that the administrationof green tea leaf extract could affect the reduction of hippocampal mice caspase-3 protein levels induced by D-galactose. There was no difference between GT-90 and GT-270 (p=0.734), but there was significant difference between GT-90 and GT-540 (p=0.003) and between GT-270 and GT-540 (p=0.006).

Table 1{ Hippocampal Caspase-3 Level in All Groups (ng/ml)

Groups	Mean ± SD	Median (min-max)
NC	7.31 ±0.708	7.25 (6.62-8.05)
NG	12.37±1.647	12.38 (10.28-14.53)
GT-90	7.69±1.763	7.26 (5.48-9.53)
GT-270	7.32±1.733	7.26 (5.32-9.94)
GT-540	3.99±2.298	4.76 (0.03-5.61)

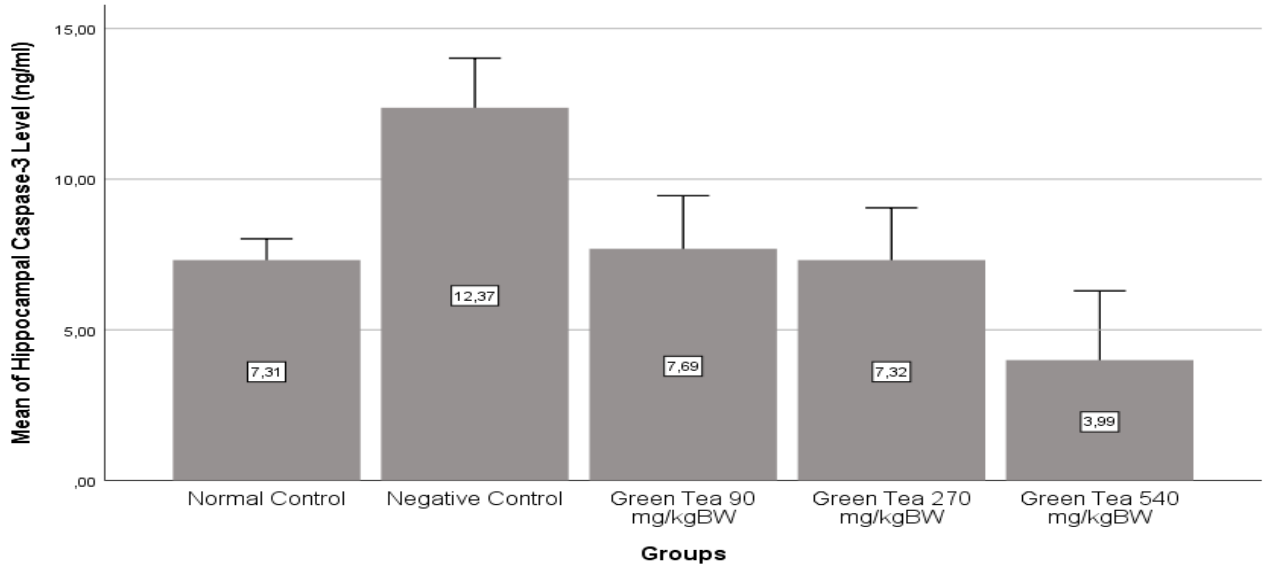
One Way Anova, p <0.001

Table 2: Comparison of Hippocampal Caspase-3 Level Between Groups

	NC	NG	GT-90	GT-270	GT-540
NC	-	*p*<0.001*	*p*=0.731	*p*=0.997	*p*=0.006*
NG		-	*p*<0,001*	*p*<0,001*	*p*<0,001*
GT-90			-	*p*=0,734	*p*=0,003*
GT-270				-	*p*=0,006*
GT-540					-

Post Hoc LSD

Figure1: Mean Comparison of Hippocampal Caspase-3 Level (ng/ml)Between Groups



DISCUSSION

In this study, it was found that mice in the GT-90, GT-270, and GT-540 which were given extract once daily for 6 weeks showed lower hippocampal caspase-3 protein levels compared to the control group. This is consistent with previous studies which stated that the administration of green tea extract can provide anti-inflammatory and antiapoptotic effects in experimental animals.[12,14,20,22,24–26]

Apoptosis is defined as programmed cell death, can occur physiologically and pathologically. Apoptosis is a mechanism characterized by morphological and biochemical changes, including an increase in the number of free radicals, activation of caspase proteins, cell shrinkage, chromatin condensation and nucleus degradation. Apoptosis can be triggered through several stimuli from outside or inside the cell, can be signals to cell membrane receptors, DNA damage, chemotherapy, and radiation. Excessive production of free radicals contributes to the process of apoptosis through the release of several apoptogenic factors such as cytochrome C, apoptosis-induction factor (AIF), and activation of caspase proteins.[14]

There are 2 apoptotic pathways: the intrinsic and the extrinsic pathway. The intrinsic pathway is affected by the permeability of the mitochondrial membrane. Internal stimuli such as hypoxia, genetic damage, oxidative stress are some of the triggers. This pathway is regulated by the Bcl-2 protein (B-cell lymphoma 2). There are 2 groups of proteins, pro-apoptotic proteins (Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, HRK), and antiapoptotic proteins (Bcl-2, Bcl-Xl). If there is a disturbance of the balance between pro-apoptosis and anti-apoptosis, then the mitochondria will release cytochrome-C and apoptotic induction factor (AIF). The release of cytochrome-C will activates caspase-3 through the formation of a complex known as apoptosome which consists of cytochrome-C, APAF-1, and caspase 9. The extrinsic pathway begins when the death ligand binds to its receptor. The main death ligands are TNF and Fas Ligand (FasL), with their receptors TNF receptor 1 (TNFR1) and Fas (CD95). TNF works by binding to two types of receptors: TNFR1 and TNFR2. TNF that binds to TNFR1 can trigger apoptosis through 2 pathways: TNFR1-associated death domain (TRADD) and the Fas-associated death domain (FADD). TNFR1 is composed of cytoplasmic death domain (DD) that binds to the TRADD adapter protein, which is indirectly able to activate the caspase pathway and cause apoptosis. TRADD and FADD will form a complex called death-inducing signaling complex (DISC), which then activates caspase-8 (caspase initiator) and caspase-3. Both intrinsic and extrinsic pathways will join to caspase-3, which is responsible for apoptosis of the cell nucleus. Caspase-3 is the main executor/ effector caspase that plays a role in both intrinsic and extrinsic pathways[13–15,27]

Several studies showed that apoptosis leads to neuronal death in neurodegenerative disease, such as Alzheimer. It is known that caspase-3 plays a significant role in the apoptosis and its level is markedly increased in diseases where such phenomenon occurs e.g. AD.[28,29]Singh *et al.*[22] said that EGCG, both in vitro and in vivo models effectively lowers neurotoxicity via the reduction in the expression of pro-apoptotic genes (bax, bad, mdm1, caspase 1, caspase 3, caspase 6, TRAIL, p21, gadd45 and fas ligand) with no effect on anti-apoptotic genes (Bcl-w, Bcl-2 and Bcl-xL).

CONCLUSIONS

Green tea leaf extract may reduce the level of hippocampal caspase-3 protein level in miceinduceddementia.

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