Chlorogenic acid ameliorates memory dysfunction via attenuating frontal lobe oxidative stress and apoptosis in diabetic rat model

Ramadhea Laila Afifa An-Nur Willya SAPUTRI[1,2], Nur ARFIAN[1,*], Junaedy YUNUS[1], Fauziyatul MUNAWAROH[2,3], Sagita Mega Sekar KENCANA[2], Dwi Cahyani Ratna SARI[1]

[1]Department of Anatomy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

[2]Master Program on Biomedical Sciences, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

[3]Faculty of Medicine, Institut Pertanian Bogor, Bogor, Indonesia

***Correspondence:** nur_arfian@ugm.ac.id

ORCIDs:

Ramadhea Laila Afifa An-Nur Willya SAPUTRI: https://orcid.org/0009-0001-6295-0402

Nur ARFIAN: https://orcid.org/0000-0003-1694-2054

Junaedy YUNUS: https://orcid.org/0000-0002-1500-3015

Fauziyatul MUNAWAROH: https://orcid.org/0009-0004-7772-7221

Sagita Mega Sekar KENCANA: https://orcid.org/0009-0002-7834-5669

Dwi Cahyani Ratna SARI: https://orcid.org/0000-0002-1126-4939

Acknowledgment/disclaimers/conflict of interest

This work was funded by Academic Excellence UGM and Dana Masyarakat of Prof. Dwi Cahyani Ratna Sari, MD. Some of the data were used for completing master program of Ramadhea Laila Afifa An-Nur Willya Saputri. The authors also thank Mr. Mulyana for maintaining the animal and laboratory assistant.

The authors declare that there is no conflict of interest regarding the publication of this study.

Informed consent

(-)

Chlorogenic acid ameliorates memory dysfunction via attenuating frontal lobe oxidative stress and apoptosis in diabetic rat model

Abstract

Background/aim: Diabetes mellitus, characterized by hyperglycemia, causes various complications, one of which is memory dysfunction. The frontal lobe is known to be responsible for impaired memory function due to hyperglycemia and is associated with oxidative stress-mediated neuronal cell apoptosis. Chlorogenic acid (CGA) is reported to have neuroprotective effects. However, its effect on the frontal lobe in DM rats is not widely known. This research aimed to elucidate the effect of CGA on the mRNA expressions of SOD1, SOD2, p53, and Bcl-2 in the frontal lobe of DM rats.

Materials and methods: Thirty male Wistar rats (2-month-old, 150-200 gBW) were randomly divided into six groups: C (control), DM1.5 (1.5-month DM), DM2 (2-month DM), CGA12.5, CGA25 and CGA50 (DM+CGA 12.5, 25 and 50 mg/kgBW, respectively). A single dose of streptozotocin 60 mg/kgBW was intraperitoneally injected. Intraperitoneal CGA injection was administered daily for DM1.5 rats for 14 days. Path length was measured in the Morris water maze (MWM) probe test. After termination, the frontal lobes were carefully harvested for RNA extraction. Reverse transcriptase PCR was performed to examine the mRNA expression of SOD1, SOD2, p53, and Bcl-2.

Results: DM2 group demonstrated significant shorter path length on the MWM probe test and significant lower mRNA expression of SOD1 and Bcl-2, compared to C group. After CGA administration, the CGA25 group showed a significantly shorter path length than the C group. CGA12.5 and CGA25 had significantly higher mRNA expression of SOD1 than the DM1.5 group. Compared to the DM1.5 and DM2 groups, SOD2 mRNA

expression of the administration of all three CGA doses increased markedly. Furthermore, Bcl-2 mRNA expression was significantly increased in the CGA12.5 and CGA50 groups, compared with the DM2 group.

Conclusion: Chlorogenic acid might improve memory function through upregulation of frontal lobes' SOD1, SOD2, and Bcl-2 mRNA expression in DM rats.

Key words: Chlorogenic acid, morris water maze, oxidative stress, apoptosis, diabetes

1. Introduction

Diabetes mellitus (DM) is a metabolic disease that is commonly found in the population and is often associated with several complications such as kidney injury, heart disease, and brain abnormalities [1,2]. Previous studies reported that brain injury in DM patients were shown by micro- and macrostructure changes of the brain manifested by progressive cognitive deterioration, cerebral infarction, cerebral atrophy, and neurodegeneration [3]. Perantie *et al*. [4] and Yonguc *et al*. [5] showed that the brain, specifically the hippocampus (region CA1 and CA3) and frontal cortex, underwent changes and contributed to acquisition and retention memory function impairment after hyperglycaemic conditions. The retention memory function in rodents can be assessed by Morris water maze (MWM) probe test [6].

Oxidative stress has been widely accepted to be a key mediator of the development of diabetes and its complications, due to increased free radicals and the failure of antioxidant defenses [2,5-6]. Hyperglycemia causes tissue and endothelial damage through the polyol pathway, formation of advance glycation end-products (AGE), activation of protein kinase C (PKC), and hexosamine pathway which further generate reactive oxidative species (ROS) [9]. Under hyperglycemia conditions, the antioxidant superoxide dismutase (SOD) enzyme is unable to eliminate the accumulation of ROS [10]. The ROS play an important role in the pathogenesis of diabetic complications, depending on its effect on the target tissues [9]. The target cells, including the glomerular mesangial cells, the capillary endothelial cells of the retina, and the neuronal cells, are incapable of adequately regulating the concentrations of intracellular glucose in the diabetic ambience [9]. Excessive quantities of ROS oxidize various biomolecules like DNA, proteins, carbohydrates, and lipids, producing oxidative stress [9]. DNA damage due to stress

condition can activate the p53 protein, which is a regulator of the pro-apoptosis Bax protein because it is reported that there is a p53-binding site on the Bax gene promoter [11]. As a response to cell death, p53 protein also rapidly translocates to mitochondria and physically interacts with the anti-apoptosis Bcl-2 protein [10-11]. Therefore, p53 plays a role in the regulation of cell fate through the Bax/Bcl-2 ratio [13]. Memory dysfunction due to hyperglycemia is often associated with oxidative stress-mediated neuronal cell apoptosis [7].

Chlorogenic acid (CGA) can be found in green coffee and tea extracts and contains phenolic acids which are known to play roles as antioxidant, anti-inflammatory, and central nervous system stimulators [14]. An in vivo study using rats reported that CGA could improve diabetic nephropathy by reducing oxidative stress and the response to inflammation in the kidney [15]. The neuroprotective effect of CGA was found to improve memory function of the rat model of transient global ischemia through upregulation of antioxidant and antiapoptotic compounds [16]. However, the effect of CGA on memory dysfunction due to progressive DM is still unknown. This study aimed to examine the effect of CGA administration on the mRNA expression of SOD1, SOD2, p53, and Bcl-2 in the frontal lobe of rats with DM.

2. Materials and Methods

2.1. Experimental Designs

Thirty male Wistar rats (aged 2 months, body weighted 150-200 gram) were kept in cages belonging to the Anatomy Laboratory of the Faculty of Medicine, UGM, Yogyakarta, with a light-dark cycle of 12:12 hours, room temperature 26-31° and humidity 70-90%. Rats were placed in separate cages between groups and fed standard AIN-93A and drinking boiled water ad libitum. This study has received approval from the Ethics

Committee of the UGM FK-KMK on 7th June 2022 with the number KE/FK/0700/EC/2022.

Rats were randomly divided into six groups, namely, group C (control), DM1.5 (1.5-month DM), DM2 (2-month DM), CGA12.5, CGA25 and CGA50 (DM+CGA dose 12.5, 25 and 50 mg/kgBW, respectively). A single dose intraperitoneal injection of streptozotocin (STZ) (Cayman Chemical, Item No. 13104) 60 mg/kgBW was administered to induce DM [17]. Intraperitoneal CGA (Sigma-Aldrich, USA, Cat. #3878-1G) injection was administered at 1.5 months after DM induction, daily for 14 days. Intraperitoneal saline injection for group C was administered in equivalent amounts. Glucose level was measured with a portable glucometer from the tail vein. Blood glucose greater than 250 mg/dl is considered diabetes. Morris water maze test was conducted before termination to find out the amelioration of function memory.

2.2. Morris Water Maze (MWM) Test

The Morris water maze (MWM) test consists of a circular tank 1.8 meters in diameter and 0.5 meters high filled with water (20-24°C) and a platform (13 cm diameter and 16.5 cm high) placed 1.5-2.5 cm below the water surface to escape from the water. The surface of the water is made opaque using milk. The water tank is divided into 4 virtual quadrants. The platform was placed in the same position for each rat in each experiment. MWM was performed for 6 consecutive days by each rat. For the first 5 days, the rats were allowed to swim until they finally found and climbed the platform. On day 6, the platform was retrieved, and a probe test was performed to assess the spatial memory retention ability [6]. The probe test was carried out for 120 seconds. The path length in the target quadrant was recorded using a video camera placed above the tank and measured using a computer.

2.3. Termination & Tissue Preparation

Rats were terminated at one-and-a-half months (groups C and DM1.5) and two months (groups DM2, CGA12.5, CGA25, and CGA50) after animal model making. Termination was carried out using intraperitoneal injection of ketamine 100 mg/kgBW. Next, an incision was made from the abdominal region to the thorax until the heart was visible. Perfusion was performed using a 0.9% NaCl solution through the left ventricle of the heart, until the blood was run out. The calvaria cranii of the rats were opened. The cerebral hemispheres were separated, the meninges were opened, and then the anterior third was cut to isolate the frontal lobe. The left frontal lobes were placed in RNA Later® Stabilization Solution (Ambion, AM7021). The right frontal lobes were fixed in paraformaldehyde 4% in PBS for 24 hours and embedded in paraffin for immunohistochemical staining examination.

2.4. RNA Extraction & Reverse transcriptase-PCR (RT-PCR)

The frontal lobes were extracted using Genezol RNA Solution (GENEzol™, GZR100) according to the manufacturer's protocol. The RNA concentration was calculated using a nanodrop (Maestrogen, MN-913A). RNA was synthesized into cDNA using a cDNA synthesis kit (SMOBio, RP1400) under PCR conditions of 30°C for 10 minutes, 42°C for 60 minutes, and 85°C for 5 minutes. Then, the cDNA was stored in a refrigerator at -20°C. Reverse transcriptase-PCR was performed to amplify specific target genes, namely, SOD1, SOD2, p53, Bcl-2, and β-actin with the primer sequences as shown in the Table 1.

Table 1. Primer sequences for reverse-transcriptase PCR

Gene	Primer sequence
SOD1	Forward: 5'-GCGGTGAACCAGTTGTGGTG-3' Reverse: 3'-AGCCACATTGCCCAGGTCTC-5'

SOD2	Forward: 5'-ATGTTGTGTCGGGCGGCGTGCAGC-3' Reverse: 3'-GCGCCTCGTGGTACTTCTCCTCGGTG-5'
p53	Forward: 5'-CCCCTGAAGACTGGATAACTGT-3' Reverse: 3'-ATTAGGTGACCCTGTCGCTG-5'
Bcl-2	Forward: 5'-GCGTCAACAGGGAGATGTCA-3' Reverse: 3'-TTCCACAAAGGCATCCCAGC-5'
β-actin	Forward: 5'-TTCCACAAAGGCATCCCAGC-3' Reverse: 3'-TTCCACAAAGGCATCCCAGC-5'

The cDNA was mixed with primers and Taq Master Mix (GoTaq®Green, M7122), then incubated in 94°C denaturation for 10 seconds, annealing in 55°C for SOD1, p53, and Bcl-2, in 58°C for SOD2, and in 54°C for β-actin for 30 seconds, extension phase in 72°C for 1 minutes, and final extension phase with the conditions of 72°C for 10 minutes for 35 cycles. The PCR products were separated using 2% agarose gel with 100bp DNA ladder (SMOBio, DM2400). Gene expressions were quantified with densitometry analysis using ImageJ software. β-actin mRNA expression was used as the housekeeping gene.

2.5. Immunohistochemical (IHC) Staining

Immunohistochemical staining was done using p53 antibody (p53 Rabbit pAb, ABclonal A5761, 400x dilution) after heated in Citrate Buffer and inhibited for endogenous peroxide using 3% H_2O_2 in PBS. Antibodies were incubated overnight. Secondary antibody incubation was done for 1 hour using appropriate secondary antibody. Positive p53 IHC staining was indicated as brown color in frontal lobe cell nucleus under the microscope in 100x and 400x magnification.

2.6. Statistical Analysis

The obtained data were analyzed using SPSS 25.0 software (IBM Corp., Chicago). Normality test with Saphiro-Wilk was conducted to determine the distribution of the data. Data that were normally distributed ($p > 0.05$) were subjected to a one-way ANOVA test, followed by a post-hoc Fisher's LSD test. Data that were not normally distributed ($p < 0.05$) were subjected to the Kruskal-Wallis H test, followed by a post-hoc test using the Kruskal-Wallis one-way ANOVA. The data is statistically significant if the probability value of $p < 0.05$. The simple regression test and the Spearman test were drawn upon to test for correlation. Value of $p < 0.05$ were considered statistically significant.

3. Results

3.1. CGA affected blood glucose level

Blood glucose measurement was undergone before termination. The comparison of blood glucose means $\pm$ SD between the 6 groups is shown in Figure 1a [18]. The mean $\pm$ SD of blood glucose in all groups, except the control group, were greater than 250 mg/dl. We demonstrated that streptozotocin injection showed higher blood glucose compared to the control group ($p < 0.05$). According to Furman B.L., the blood glucose of rats given STZ which is greater than 250 mg/dl and/or is statistically significantly higher than that of the control indicates that the experimental animal model had hyperglycemia and could be used as a further study of the DM model [17]. After chlorogenic acid administration, blood glucose of CGA50 group showed a higher level compared to the control group. However, blood glucose was significantly lowered after CGA doses of 12.5 and 25 mg/kgBW compared to the DM2 group ($p < 0.05$). Nevertheless, there were no difference between CGA-treated groups.

3.2. CGA might restore spatial memory deficit

Total path length in millimeters (mm) was measured while the rats were in the target quadrant to examine the memory retention. The comparison of the path length of the memory retention test means ± SD between the 6 groups is shown in Figure 1b [18]. The path length between the DM1.5 group did not show any differences from that of the C group. However, the DM2 group showed a statistically significant shorter path length compared to group C (p = 0.003) and group DM1,5 (p = 0.02). After chlorogenic acid administration, the CGA25 group showed a statistically significant shorter path length compared to the C group (p = 0.036). Although not statistically significant, the path lengths in the target quadrant of the CGA12.5 and CGA50 groups were longer than that of the DM2 group but were not different than that of the C group. There was no difference between the three doses of chlorogenic acid administration.

The trajectory of the probe test of the DM rats is shown in Figure 1c [18]. The target quadrant was located on right-bottom quadrant. From the figure, the control and DM1.5 groups concentrated on the target quadrant, whereas the DM2 groups did not concentrate on the target quadrant. After chlorogenic acid administration, the CGA12.5 and CGA50 groups concentrated in the target quadrant, while the CGA25 group did not concentrate in the target quadrant.

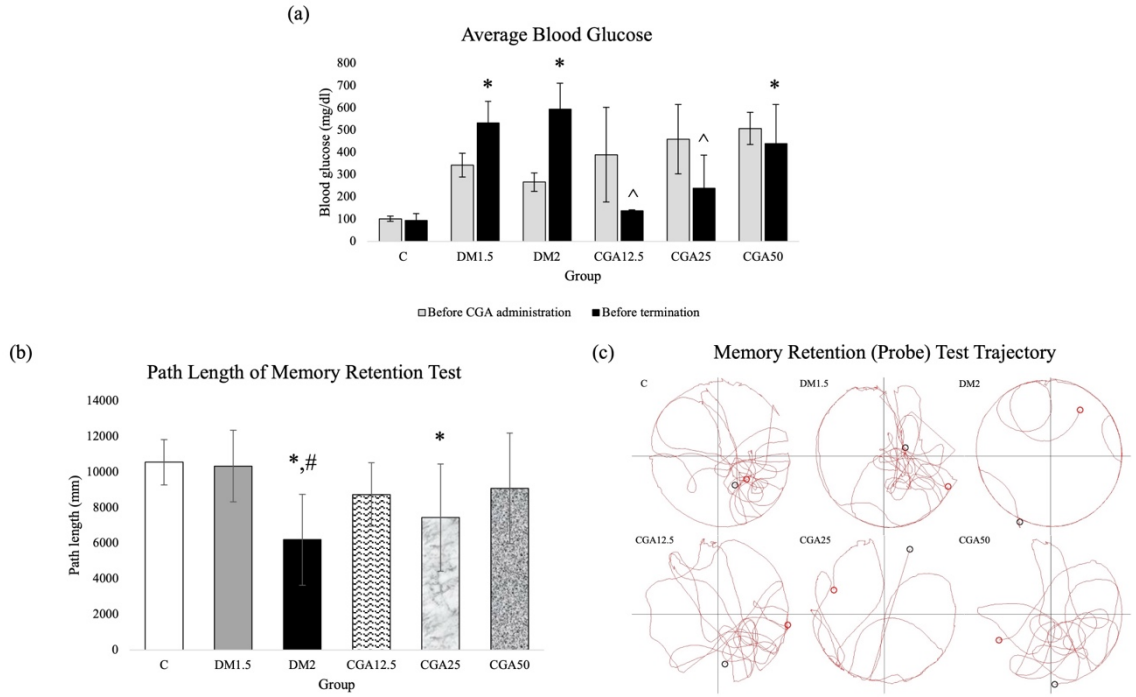

Figure 1. (a) Average blood glucose level before CGA administration vs. before termination, (b) CGA effects on path length of memory retention test and (c) theirs probe test trajectory. Data were represented as mean ± SD. (*$p < 0.05$ vs C; #$p < 0.05$ vs DM1.5; ^$p < 0.05$ vs DM2)

3.3. CGA elevated anti-apoptosis Bcl-2 expression

The mRNA expression of p53/β-actin means ± SD between the six groups are shown in Figure 2a [18]. Kruskal-Wallis H test demonstrated that there was no significant difference between the groups in the pro-apoptosis p53 mRNA expression.

The mRNA expression of Bcl-2/β-actin means ± SD among the six groups is shown in Figure 2b [18]. The Bcl-2 mRNA expression in the DM1.5 and DM2 groups were lower than that in the C group, but only the DM2 group showed a statistically significant difference ($p = 0.019$). In comparison to the DM2 group, CGA administration showed that the Bcl-2 mRNA expressions were higher in the CGA12.5 ($p = 0.043$) and CGA50 ($p = 0.005$) groups, which was statistically significant. Furthermore, the Bcl-2 mRNA

expression of the CGA50 group was significantly higher than in the DM1.5 group (p = 0.034).

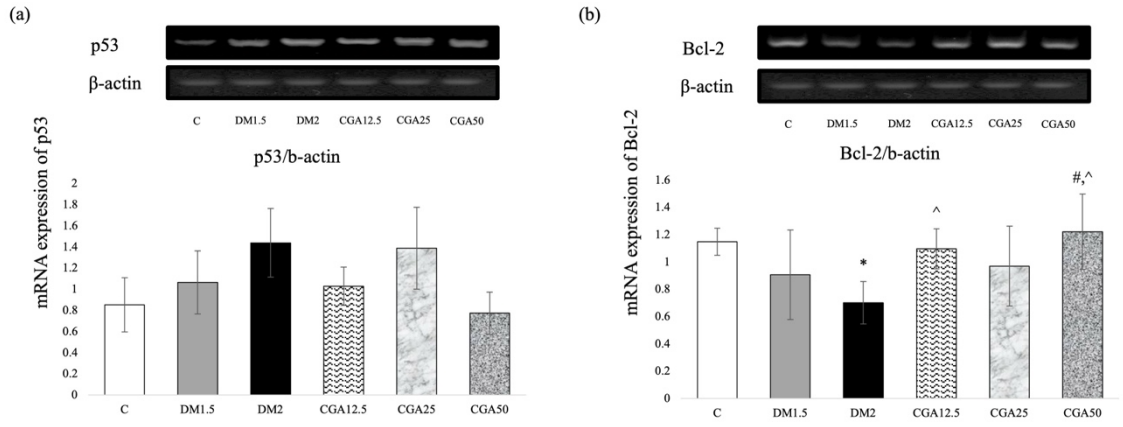

Figure 2. CGA effects in the frontal lobe of DM rats of (a) pro-apoptosis p53 and (b) anti-apoptosis Bcl-2 mRNA expressions. Data were represented as mean ± SD. (*$p < 0.05$ vs C; #$p < 0.05$ vs DM1,5; ^$p < 0.05$ vs DM2)

3.4. CGA increased antioxidant superoxide dismutase enzymes

The mRNA expression of SOD1/β-actin and SOD2/β-actin means ± SD among the six groups were shown in Figure 3 [18]. We did not observe any difference in SOD2 mRNA expression in the DM1.5 and DM2 groups compared to the control group. However, there was a significantly lowered SOD1 mRNA expression in the DM1.5 (p = 0.002) and DM2 (p = 0.037) groups compared to the C group. CGA treatment significantly increased SOD1 mRNA expression in the CGA12.5 (p = 0.024) and CGA25 (p = 0.016) groups compared to the DM1.5 group. Furthermore, the mRNA expression of SOD1 in the CGA50 group was lower than that in group C, which was statistically significant (p = 0.038). In addition to that, administration of CGA significantly elevated SOD2 mRNA expression in the CGA12.5 (p = 0.023), CGA25 (p = 0.008) and CGA50 (p = 0.003) groups compared to the DM1.5 group as well as the CGA12.5 (p = 0.013), CGA25 (p = 0.004) and CGA50 (p = 0.001) groups compared to the DM2 group. Additionally, the

CGA50 group also showed significantly higher SOD2 mRNA expression compared to the C group ($p = 0.039$). There was no difference between the three doses of chlorogenic acid administration on mRNA expression of SOD1 and SOD2.

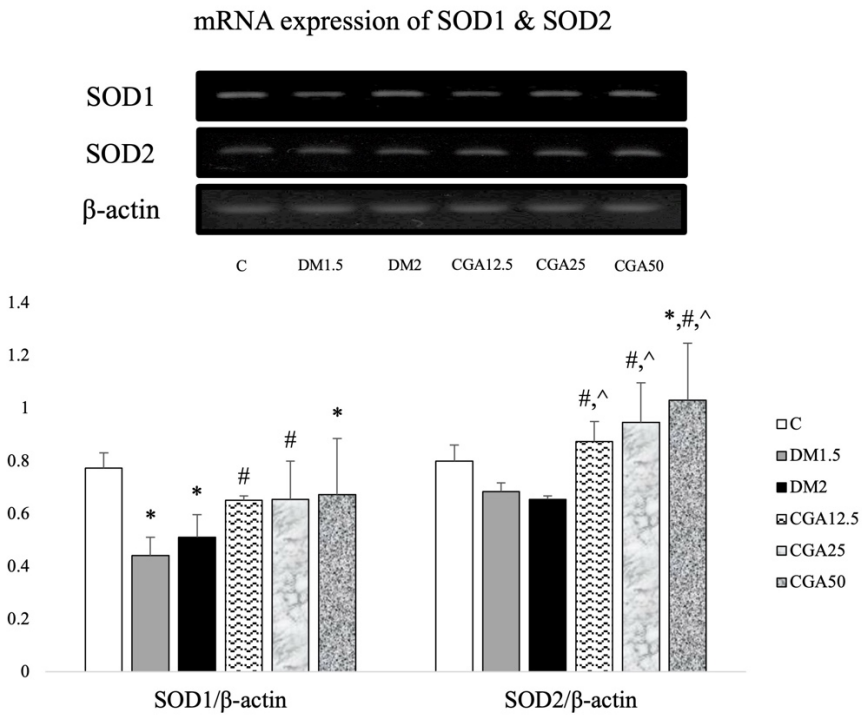

Figure 3. CGA effects in the frontal lobe of DM rats of SOD1 & SOD2 mRNA expressions. Data were represented as mean ± SD. (*$p < 0.05$ vs C; #$p < 0.05$ vs DM1.5; ^$p < 0.05$ vs DM2)

3.5. Amelioration of retention memory dysfunction statistically correlated with elevation of antioxidant SOD1 enzyme

The probe test path length was statistically correlated with the SOD1/β-actin mRNA expression (r = 0.414, $p = 0.029$, [n= 28]). However, a weak correlation was detected between the probe test path length and both SOD2/β-actin mRNA expression and blood glucose (r = 0.179, $p = 0.381$, [n= 26] and (r = -0.205, $p = 0.276$, [n= 30], respectively) Table 2 [18].

Table 2. Statistical analysis of correlation between probe test path length, blood glucose, and antioxidant superoxide enzyme

		Blood Glucose	SOD1/β-actin	SOD2/β-actin
Path Length	Correlation coefficient	-0.205	0.414	0.179
	Sig. (2-tailed)	0.276	0.029	0.381
	N	30	28	26

4. Discussion

This study examined the effect of various doses of CGA on the expression of oxidative stress and apoptosis genes in the frontal lobe of DM rats. Before termination, the blood glucose of each rat was measured. Blood glucose showed that the rats given STZ were > 250 mg/dl and/or were statistically significantly higher than the control, indicating that the experimental animal model had hyperglycemia and could be used as a further study of the DM model [17]. The administration of CGA at doses of 12.5 and 25 mg/kgBW in DM rats showed lower blood glucose level than those in the DM2 group. According to Miao and Xiang [19], CGA can delay glucose absorption in the intestine by inhibiting glucose-6-phosphate transferase I enzyme and lowering apical glucose transport driven by natrium gradient [20]. *In vitro* and *in vivo* studies also revealed that CGA lowered glucose export from the liver by inhibiting glucose-6-phosphatase [20-21].

This study revealed that CGA ameliorated memory function performance in diabetic rat. Diabetic rat demonstrated memory dysfunction with egocentric pattern and path length reduction of probe test as shown in Figure 1b&c [18], which represented as dysfunction of retention memory. The frontal lobe plays role in retention memory function [23] and

is one part of the brain that is sensitive to glycemic control [4]. Memory function can be determined by the preference of rodents in the platform area when the platform is not available, also known as the probe test [6]. If the animal can remember the location of the platform during the recognition test, it will swim a longer path length in the target quadrant [24]. This study is consistent with previous studies that animal models of type 1 DM involving the prefrontal cortex [6,24] and hippocampus [2] experienced impaired spatial learning and lost memory function.

Chlorogenic acid can restore spatial memory deficit conditions [14,25]. In recent study, CGA administration showed that DM rats tended to have good memory function, as reflected by the path length of the CGA12.5 and CGA50 groups were longer than DM2 group but had no difference in compared to C group. However, a previous study reported that CGA-treated groups (12.5, 25, and 50 mg/kgBW) had longer time in the target quadrant of the MWM probe test compared to the DM rats group [27]. The CGA12.5 group was also performed better performance on escape latency test compared to DM rats [28]. Other studies reported that CGA can prevent diabetes-induced learning and memory impairment [29]. Our study revealed that hypoglycemic condition and memory improvement based on MWM test (total path length) may be correlated each other, however it is beyond our study to prove whether CGA effect in memory improvement correlates to hypoglycemic effect. Future research with anti-hyperglycemic substance may be needed for elucidating these results.

The crucial pathogenic factor associated with diabetes-associated cognitive decline (DACD) is apoptosis signaling [2]. In recent study, there was no significant difference between groups in p53 mRNA expression in the frontal lobe of DM rats. However, the Bcl-2 mRNA expression of DM rats showed a significantly lower result compared to

control rats [18]. Previous study reported that higher mRNA expression of p53 in DM rats' hippocampus, which was localized by positive p53 immunoreactivity on hippocampus' pyramidal cells [28]. This study is consistent with previous study that after exposure to stress, neuronal culture cells underwent apoptosis, indicated by a shrinking nucleus and decreased expression of Bcl-2 [30]. DNA damage response due to diabetes activates the p53 protein that leads to the induction of cell cycle arrest, DNA repair, cell senescence, autophagy, and apoptosis [31]. Activated p53 induces apoptosis both via extrinsic and intrinsic pathways [32]. In extrinsic pathway, p53 induces expressions of death receptor on cell surface, thus cell undergoes apoptosis [33]. In the intrinsic pathway, apoptosis is mediated by mitochondria where activated p53 causes Bax, NOXA, PUMA, cytochrome C and other mitochondrial apoptosis factors to release to the cytosol and form apoptosome which initiates caspase-9 and activates apoptosis executor [8,31]. The B-cell lymphoma 2 (Bcl-2) family is a key regulator of apoptosis because it bridges extrinsic and intrinsic pathways [35]. The Bcl-xL and Bcl-2 proteins play a role in anti-apoptosis, inhibiting Bax and BAK proteins [35].

After chlorogenic acid administration, this study demonstrated a tendency to reduced p53 mRNA expression in the CGA12.5 and CGA50 groups than in the DM2 group [18]. Chlorogenic acid administration returned the condition as shown by lower pro-apoptosis p53 and Bax mRNA expression [28]. The CGA dose of 12.5 and 50 mg/kgBW in DM rats showed significantly higher anti-apoptotic Bcl-2 mRNA expression compared to the 2-month DM group [18]. This recent study supports previous studies that stated that chlorogenic acid has a neuroprotective effect by increasing anti-apoptotic proteins Bcl-2 and Bcl-xL and decreasing caspase-3 cleavage in H2O2-induced neuronal cells [36]. *In*

vivo studies have also shown that chlorogenic acid can protect hippocampal pyramidal cells by increasing the mRNA expression of Bcl-2 [16].

Many evidence state that apoptosis in many diseases might be evoked by enhanced oxidative stress and inflammatory reactions [2]. In recent study, impaired memory function was followed by decreased mRNA expression of SOD1 in the frontal lobe of DM rats in compared to the control [18]. Oxidative stress has been proven to play a role in cognitive decline related to hyperglycemia [37]. Streptozotocin-induced diabetes animal models are widely used to study oxidative stress as a pathogenesis of memory impairment due to diabetes [24,36]. Many studies have reported that the concentration of ROS is significantly increased in the brain of DM rats [39]. However, under hyperglycemic conditions, the antioxidant SOD enzyme is unable to eliminate ROS [10]. Previous study also stated that STZ-induced mice showed a decreased activity, mRNA and protein expression of antioxidant SOD1 and catalase enzymes [40]. Oxidative stress in cells can affect the translocation of transcription factors to the nucleus [41]. Therefore, a decrease in the antioxidant enzyme in diabetes could occur due to oxidation of transcription factors that initiated the transcription process [40].

High blood glucose interferes electron transport in mitochondria, which will result in increased oxygen oxidation by co-enzyme Q and form superoxide [42]. Manganese superoxide dismutase (Mn-SOD/SOD2) is an antioxidant enzyme that is responsible for mitochondrial detoxification against oxygen radicals by catalysing the dismutation of superoxide free radicals [43]. Hyperglycaemic conditions can cause ROS overproduction which further reduces the antioxidant capacity of cells [42]. In this study, there was no difference of SOD2 mRNA expression in the frontal lobe between the DM and control rats. There is a dynamic regulation of antioxidant enzymes to maintain the redox balance

[43]. It is caused by ROS, as second messengers, will trigger a lot of activity against radicals that can harm cells at basal conditions [44]. An *in vitro* study reported that the decrease in SOD1 activity was due to increased SOD2 expression [43]. Other study reported decreased total SOD and SOD1 activity but increased SOD2 activity in the heart of DM mice [45]. Furthermore, in the early diabetes, tissue increases the activity of antioxidant enzymes to defend against hyperglycaemia-induced ROS production [45].

Chlorogenic acid can increase the expression of antioxidant enzymes by donating its hydrogen atoms to free radicals [16]. In this study, administration of CGA doses of 12.5 and 25 mg/kgBW showed significantly higher SOD1 mRNA expression in compared to DM rats without CGA. In addition, the administration of CGA showed significantly higher SOD2 mRNA expression than the DM rats. In this present study, the longer path length in the target area by the DM rats with CGA also statistically correlated with higher antioxidant SOD1 mRNA expression, but weakly correlated with lower blood glucose (Table 2) [18]. In the previous study stated that CGA, as an antioxidant, acts by chelating cations [46]. CGA chelated the copper ion of SOD1 and the manganese ion of SOD2 and deposited them on the liver of obese Zucker rats [46]. Phenol compounds in CGA can also stimulate nuclear factor-erythroid 2-related factor 2 (Nrf2), a transcription factor that regulates genes encoding various antioxidant enzymes, to bind to antioxidant response elements (ARE) [19].

The DM1.5 group was made to observe any progression of complications due to DM in the frontal lobe of rats. Memory function in 1.5 months after STZ administration had not shown any disturbance compared to control. There was also no significant difference in the expression of the observed genes mRNA at 1.5 months after STZ administration compared to the control. The pathogenesis from hyperglycemia in diabetic

encephalopathy, which is characterized by changes in cognitive function and nervous system structure, is a long process accompanied by other metabolic abnormalities [47]. Five weeks after STZ administration showed no clear cognitive deficit but revealed an increased rate of apoptosis in the hippocampus in rats, which was confirmed by the TUNEL assay [47]. Administration of STZ for diabetic complications study could be repeated at the seventh week [17]. Then, it could be used for several more weeks or months [17].

This study used several doses of intraperitoneal CGA to determine the relationship between dose and effect on the frontal lobe of DM rats. However, no differences were found between the three doses in memory function and the examined genes of oxidative stress and apoptotic. This study also demonstrated inconsistency in the CGA administration at a dose of 25 and 50 mg/kgBW. Chlorogenic acid is not well absorbed by the gastrointestinal tract when given orally and its concentrations are more stable when administered intraperitoneally [48]. Previous studies reported that chlorogenic acid at a dose of 30 mg/kgBW had a positive effect on brain damage due to focal [26] and global [16] cerebral ischemia. High doses of CGA have been reported to induce 4/6 rat death, while low dose CGA was considered non-toxic [49].

Another possible mechanism that may affect brain injury and cognitive dysfunction associated with hyperglycemia is vasculopathy or angiopathy [7]. The animal model of diabetes stroke showed endothelial cell dysfunction and increased vascular permeability [50]. Interestingly, CGA could prevent spatial memory deterioration and affect the vascular response by decreasing the expression of vasoconstrictor ET-1 and increasing the expression of endothelial cell CD31 in global brain ischemic rats [16]. Further

research related to the effect of chlorogenic acid on the vascular response in memory dysfunction due to diabetes mellitus will be worth investigating.

5. Conclusion

Chlorogenic acid (CGA) treatment in the frontal lobe of DM rats might improve memory function by increasing its antioxidant and anti-apoptosis properties. Further basic-to-clinical research are considered because CGA has antioxidant and anti-apoptosis effect that will likely result in clinical benefit as a supplementary therapy in treating or preventing memory dysfunction related to diabetic condition.

Acknowledgment

This work was funded by Academic Excellence UGM and Dana Masyarakat of Prof. Dwi Cahyani Ratna Sari, MD. Some of the data were used for completing master program of Ramadhea Laila Afifa An-Nur Willya Saputri. The authors also thank Mr. Mulyana for maintaining the animal and laboratory assistant.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this study.

References

1. Khan MAB, Hashim MJ, King JK, Govender RD, Mustafa H et al. Epidemiology of type 2 diabetes - global burden of disease and forecasted trends. Journal of Epidemiology and Global Health 2020; 10 (1): 107–111. https://doi.org/10.2991/JEGH.K.191028.001

2. Xianchu L, Kang L, Beiwan D, Huan P, Ming L. Apocynin ameliorates cognitive deficits in streptozotocin–induced diabetic rats. Bratislava Medical Journal 2021; 122 (1): 78–84. https://doi.org/10.4149/BLL_2021_010

3. Cox D, Kovatchev B, Gonder-Frederick L, Summers K, Mccall A et al. Relationships between hyperglycemia and cognitive performance among adults

with type 1 and type 2 diabetes. Diabetes care 2005; 28 (1): 71–77. https://doi.org/10.2337/diacare.28.1.71

4. Perantie DC, Wu J, Koller JM, Lim A, Warren SL et al. Regional brain volume differences associated with hyperglycemia and severe hypoglycemia in youth with type 1 diabetes. Diabetes Care 2007; 30 (9): 2331–2337. https://doi.org/10.2337/dc07-0351

5. Yonguc GN, Dodurga Y, Adiguzel E, Gundogdu G, Kucukatay V et al. Grape seed extract has superior beneficial effects than vitamin E on oxidative stress and apoptosis in the hippocampus of streptozotocin induced diabetic rats. Gene 2015; 555 (2): 119–126. https://doi.org/10.1016/j.gene.2014.10.052

6. Vorhees C V, Williams MT. Morris water maze: Procedures for assessing spatial and related forms of learning and memory. Nature Protocols 2006; 1 (2): 848–858. https://doi.org/10.1038/nprot.2006.116

7. Hamed SA. Brain injury with diabetes mellitus: evidence, mechanisms and treatment implications. Expert Review of Clinical Pharmacology 2017; 10 (4): 409–428. https://doi.org/10.1080/17512433.2017.1293521

8. Sanna RS, Muthangi S, Chandrasekhar CS, Devi SA. Grape seed proanthocyanidin extract and insulin prevents cognitive decline in type 1 diabetic rat by impacting Bcl-2 and Bax in the prefrontal cortex. Metabolic Brain Disease 2019; 34 (1): 103–117. https://doi.org/10.1007/s11011-018-0320-5

9. Sifuentes-Franco S, Padilla-Tejeda DE, Carrillo-Ibarra S, Miranda-Díaz AG. Oxidative stress, apoptosis, and mitochondrial function in diabetic nephropathy. International Journal of Endocrinology 2018; 2018 (1). https://doi.org/10.1155/2018/1875870

10. Setyaningsih WAW, Arfian N, Fitriawan AS, Yuniartha R, Sari DCR. Ethanolic extract of centella asiatica treatment in the early stage of hyperglycemia condition inhibits glomerular injury and vascular remodeling in diabetic rat model. Evidence-based Complementary and Alternative Medicine 2021; 2021 (6). https://doi.org/10.1155/2021/6671130

11. Miyashita T, Reed JC. Tumor Suppressor p53 Is a Direct Transcriptional Activator of the Human bax Gene. Cell 1995; 80 : 293–299.

12. Erster S, Moll UM. Stress-induced p53 runs a direct mitochondrial death program: Its role in physiologic and pathophysiologic stress responses in vivo. Cell Cycle 2004; 3 (12): 1492–1495. https://doi.org/10.4161/cc.3.12.1318

13. Li GY, Xie P, Li HY, Hao L, Xiong Q et al. Involment of p53, Bax, and Bcl-2 pathway in microcystins-induced apoptosis in rat testis. Environmental Toxicology 2011; 26 (2): 111–117. https://doi.org/10.1002/tox.20532

14. Naveed M, Hejazi V, Abbas M, Kamboh AA, Khan GJ et al. Chlorogenic acid (CGA): A pharmacological review and call for further research. Biomedicine & Pharmacotherapy 2018; 97 : 67–74. https://doi.org/https://doi.org/10.1016/j.biopha.2017.10.064

15. Ye H-Y, Li Z-Y, Zheng Y, Chen Y, Zhou Z-H et al. The attenuation of chlorogenic acid on oxidative stress for renal injury in streptozotocin-induced diabetic nephropathy rats. Archives of Pharmacal Research 2016; 39 (7): 989–997. https://doi.org/10.1007/s12272-016-0771-3

16. Hermawati E, Arfian N, Mustofa M, Partadiredja G. Chlorogenic acid ameliorates memory loss and hippocampal cell death after transient global ischemia. European Journal of Neuroscience 2020; 51 (2): 651–669. https://doi.org/10.1111/ejn.14556

17. Furman BL. Streptozotocin-Induced Diabetic Models in Mice and Rats. Current Protocols 2021; 1 (4). https://doi.org/10.1002/cpz1.78

18. Saputri RLAAW, Yunus J, Arfian N. The effect of chlorogenic acid on oxidative stress and apoptosis in the frontal lobe of DM rats. Master, Universitas Gadjah Mada, Yogyakarta, Indonesia, 2022.

19. Miao M, Xiang L. Pharmacological action and potential targets of chlorogenic acid. Advances in Pharmacology 2020; 87 : 71–88. https://doi.org/10.1016/bs.apha.2019.12.002

20. McCarty MF. A chlorogenic acid-induced increase in GLP-1 production may mediate the impact of heavy coffee consumption on diabetes risk. Medical Hypotheses 2005; 64 (4): 848–853. https://doi.org/https://doi.org/10.1016/j.mehy.2004.03.037

21. Arion WJ, Canfield WK, Ramos FC, et al. Chlorogenic acid and hydroxynitrobenzaldehyde: new inhibitors of hepatic glucose 6-phosphatase. Archives of Biochemistry and Biophysics 1997; 339 (2): 315–322. https://doi.org/https://doi.org/10.1006/abbi.1996.9874

22. Herling AW, Burger H-J, Schubert G, Hemmerle H, Schaefer H-L et al. Alterations of carbohydrate and lipid intermediary metabolism during inhibition of glucose-6-phosphatase in rats. European Journal of Pharmacology 1999; 386 (1): 75–82. https://doi.org/https://doi.org/10.1016/S0014-2999(99)00748-7

23. Patestas MA, Gartner LP. *A Textbook of Neuroanatomy*. 1st ed. United Kingdom: Blackwell Publishing; 2006.

24. Jafari AI, Sankian M, Ahmadpour S, Varasteh ARZ, Haghir H. Evaluation of Bcl-2 family gene expression and Caspase-3 activity in hippocampus STZ-induced diabetic rats. Experimental diabetes research 2008; 2008 : 638467. https://doi.org/10.1155/2008/638467

25. Ahmadi M, Rajaei Z, Hadjzadeh MA, Nemati H, Hosseini M. Crocin improves spatial learning and memory deficits in the Morris water maze via attenuating cortical oxidative damage in diabetic rats. Neuroscience Letters 2017; 642 (6): 1–6. https://doi.org/10.1016/j.neulet.2017.01.049

26. Lee TK, Kang IJ, Kim B, Sim HJ, Kim DW et al. Experimental pretreatment with chlorogenic acid prevents transient ischemia-induced cognitive decline and neuronal damage in the hippocampus through anti-oxidative and anti-inflammatory effects. Molecules 2020; 25 (16). https://doi.org/10.3390/molecules25163578

27. Munawaroh F, Sari DCR, Arfian N. Pengaruh pemberian chlorogenic acid (CGA) terhadap fungsi memori dan inflamasi lobus frontal pada tikus model diabetes mellitus. Master, Universitas Gadjah Mada, Yogyakarta, Indonesia, 2022 (in Bahasa Indonesia).

28. Susilowati A, Sari DCR, Arfian N. Pengaruh asam klorogenat (chlorogenic acid) terhadap perbaikan disfungsi memori, ekspresi mRNA p53, Bax, dan Bcl-2 pada tikus model diabetes mellitus. Master, Universitas Gadjah Mada, Yogyakarta, Indonesia, 2021 (in Bahasa Indonesia).

29. Stefanello N, Schmatz R, Pereira LB, Rubin MA, Da Rocha JBT et al. Effects of chlorogenic acid, caffeine, and coffee on behavioral and biochemical parameters of diabetic rats. Molecular and Cellular Biochemistry 2014; 388 (1): 277–286. https://doi.org/10.1007/s11010-013-1919-9

30. Bossenmeyer-Pourié C, Lièvre V, Grojean S, Koziel V, Pillot T et al. Sequential Expression Patterns of Apoptosis- and Cell Cycle-Related Proteins in Neuronal Response to Severe or Mild Transient Hypoxia. Neuroscience 2002; 114 (4): 869–882.www.neuroscience-ibro.comAccessed

31. Ou H-L, Björn S. DNA Damage Responses and p53 in the Aging Process. Blood 2018; 131 (5): 488–495.http://ashpublications.org/blood/article-pdf/131/5/488/1465962/blood746396.pdfAccessed

32. Ryan KM, Phillips AC, Vousden KH. Regulation and function of the p53 tumor suppressor protein. Current Opinion in Cell Biology 2001; 13 : 332–337.

33. D'Arcy MS. Cell death: a review of the major forms of apoptosis, necrosis and autophagy. Cell Biology International 2019; 43 (6): 582–592. https://doi.org/https://doi.org/10.1002/cbin.11137

34. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. Nature Reviews Cancer 2002; 2 (4): 277–288. https://doi.org/10.1038/nrc776

35. Wanner E, Thoppil H, Riabowol K. Senescence and Apoptosis: Architects of Mammalian Development. Frontiers in Cell and Developmental Biology 2021; 8 . https://doi.org/10.3389/fcell.2020.620089

36. Kim J, Lee S, Shim J, Kim HW, Kim J et al. Caffeinated coffee, decaffeinated coffee, and the phenolic phytochemical chlorogenic acid up-regulate NQO1 expression and prevent H 2O 2-induced apoptosis in primary cortical neurons. Neurochemistry International 2012; 60 (5): 466–474. https://doi.org/10.1016/j.neuint.2012.02.004

37. Lejri I, Agapouda A, Grimm A, Eckert A. Mitochondria and oxidative stress-targeting substances in cognitive decline-related disorders: from molecular mechanisms to clinical evidence. Oxidative Medicine and Cellular Longevity 2019: 1–26. https://doi.org/10.1155/2019/9695412

38. Yi SS, Hwang IK, Kim DW, et al. The chronological characteristics of SOD1 activity and inflammatory response in the hippocampi of STZ-induced type 1 diabetic rats. Neurochemical Research 2011; 36 (1): 117–128. https://doi.org/10.1007/s11064-010-0280-6

39. Matough FA, Budin SB, Hamid ZA, Alwahaibi N, Mohamed J. The role of oxidative stress and antioxidants in diabetic complications. Sultan Qaboos University medical journal 2012; 12 (1): 5–18. https://doi.org/10.12816/0003082

40. Sadi G, Yilmaz O, Güray T. Effect of vitamin C and lipoic acid on streptozotocin-induced diabetes gene expression: mRNA and protein expressions of Cu-Zn SOD and catalase. Molecular and Cellular Biochemistry 2008; 309 (1–2): 109–116. https://doi.org/10.1007/s11010-007-9648-6

41. Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. The FASEB Journal 1996; 10 (7): 709–720. https://doi.org/10.1096/fasebj.10.7.8635688

42. Brownlee M. The Pathobiology of Diabetic Complications A Unifying Mechanism. Banting Lecture 2004.http://diabetesjournals.org/diabetes/article-pdf/54/6/1615/381945/zdb00605001615.pdfAccessed

43. Vincent AM, Russell JW, Sullivan KA, Backus C, Hayes JM et al. SOD2 protects neurons from injury in cell culture and animal models of diabetic neuropathy. Experimental Neurology 2007; 208 (2): 216–227. https://doi.org/10.1016/j.expneurol.2007.07.017

44. Tang XQ, Feng JQ, Chen J, Chen PX, Zhi JL et al. Protection of oxidative preconditioning against apoptosis induced by H2O2 in PC12 cells: mechanisms via MMP, ROS, and Bcl-2. Brain Research 2005; 1057 (1–2): 57–64. https://doi.org/10.1016/j.brainres.2005.07.072

45. Lei S, Liu Y, Liu H, Yu H, Wang H et al. Effects of N-acetylcysteine on nicotinamide dinucleotide phosphate oxidase activation and antioxidant status in heart, lung, liver and kidney in streptozotocin-induced diabetic rats. Yonsei Medical Journal 2012; 53 (2): 294–303. https://doi.org/10.3349/ymj.2012.53.2.294

46. Rodriguez De Sotillo D v, Hadley M. Chlorogenic acid modifies plasma and liver concentrations of: cholesterol, triacylglycerol, and minerals in (fa/fa) Zucker rats. Journal of Nutritional Biochemistry 2002; 13 : 717–726.

47. Dong M, Ren M, Li C, Zhang X, Yang C et al. Analysis of metabolic alterations related to pathogenic process of diabetic encephalopathy rats. Frontiers in Cellular Neuroscience 2019; 12 (1). https://doi.org/10.3389/fncel.2018.00527

48. Bagdas D, Etoz BC, Ozturkoglu SI, Cinkilic N, Ozyigit MO. Effects of systemic chlorogenic acid on random-pattern dorsal skin flap survival in diabetic rats. Biological and Pharmaceutical Bulletin 2014; 37 (3): 361–370. https://doi.org/10.1248/bpb.b13-00635

49. Zeiger E, Tice R. Chlorogenic Acid [327-97-9] and Caffeic Acid [331-39-5]. Review of Toxicological Literature 1998.

50. Li W, Qu Z, Prakash R, Chung C, Ma H et al. Comparative analysis of the neurovascular injury and functional outcomes in experimental stroke models in diabetic Goto-Kakizaki rats. Brain Research 2013; 1541 : 106–114. https://doi.org/10.1016/j.brainres.2013.10.021

Table 1. Primer sequences for reverse-transcriptase PCR

Gene	Primer sequence
SOD1	Forward: 5'-GCGGTGAACCAGTTGTGGTG-3' Reverse: 3'-AGCCACATTGCCCAGGTCTC-5'
SOD2	Forward: 5'-ATGTTGTGTCGGGCGGCGTGCAGC-3' Reverse: 3'-GCGCCTCGTGGTACTTCTCCTCGGTG-5'
p53	Forward: 5'-CCCCTGAAGACTGGATAACTGT-3' Reverse: 3'-ATTAGGTGACCCTGTCGCTG-5'
Bcl-2	Forward: 5'-GCGTCAACAGGGAGATGTCA-3' Reverse: 3'-TTCCACAAAGGCATCCCAGC-5'
β-actin	Forward: 5'-TTCCACAAAGGCATCCCAGC-3' Reverse: 3'-TTCCACAAAGGCATCCCAGC-5'

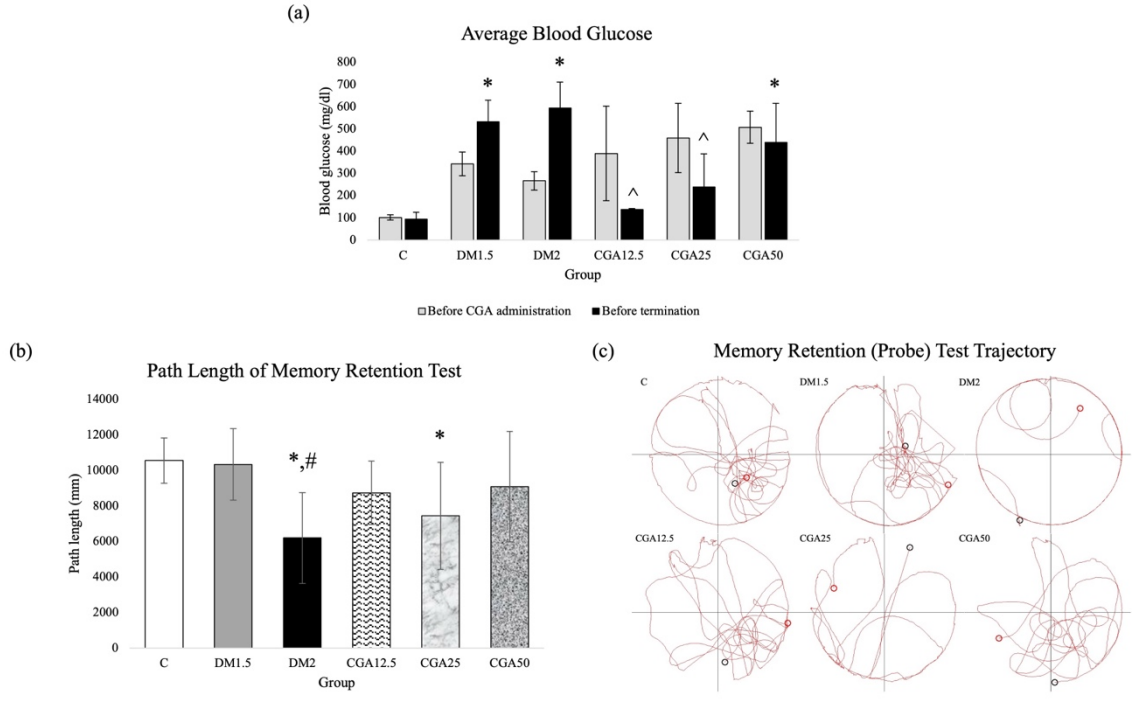

Figure 4. (a) Average blood glucose level before CGA administration vs. before termination, (b) CGA effects on path length of memory retention test and (c) theirs

probe test trajectory. Data were represented as mean ± SD. (*$p < 0.05$ vs C; #$p < 0.05$ vs DM1.5; ^$p < 0.05$ vs DM2)

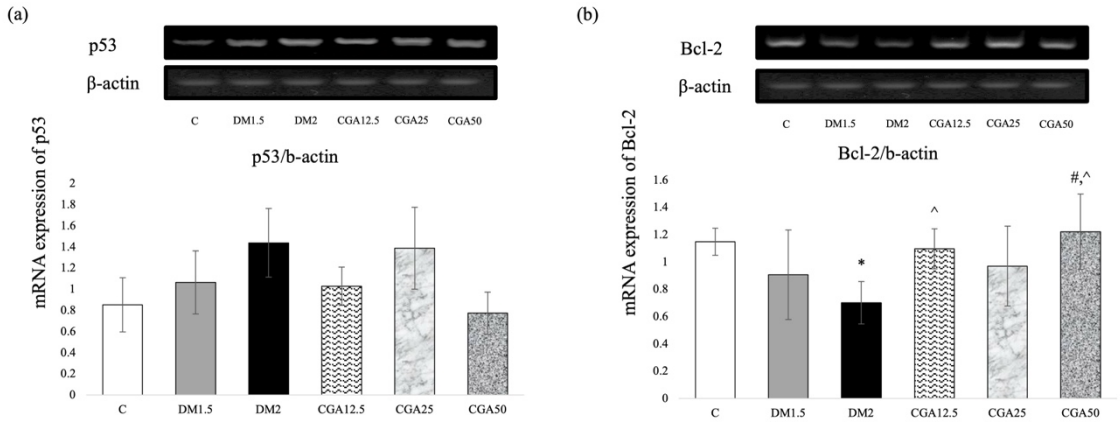

Figure 5. CGA effects in the frontal lobe of DM rats of (a) pro-apoptosis p53 and (b) anti-apoptosis Bcl-2 mRNA expressions. Data were represented as mean ± SD. (*$p < 0.05$ vs C; #$p < 0.05$ vs DM1,5; ^$p < 0.05$ vs DM2)

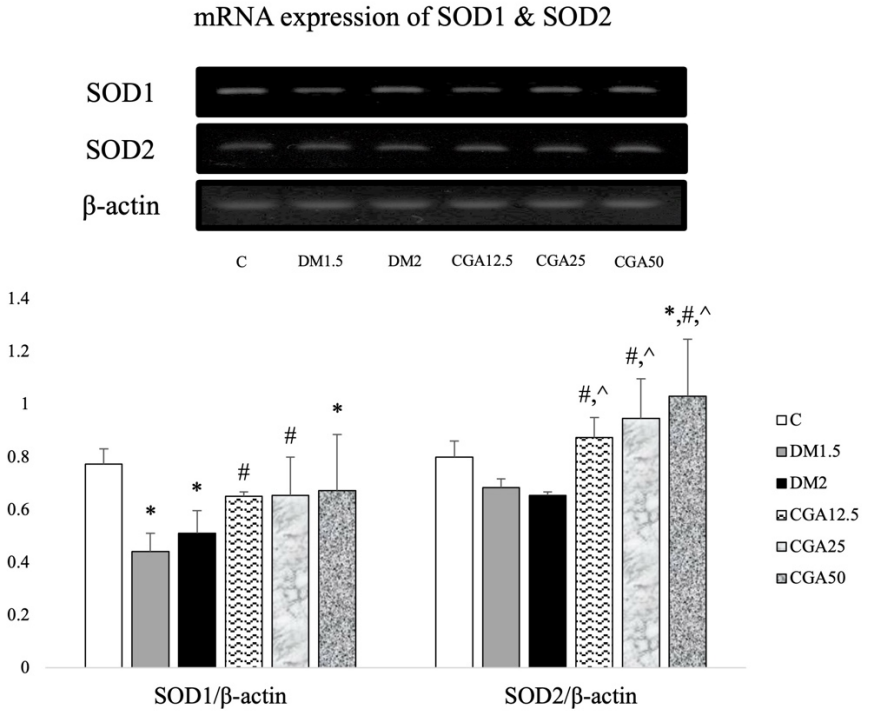

Figure 6. CGA effects in the frontal lobe of DM rats of SOD1 & SOD2 mRNA expressions. Data were represented as mean ± SD. (*$p < 0.05$ vs C; #$p < 0.05$ vs DM1.5; ^$p < 0.05$ vs DM2)

Table 2. Statistical analysis of correlation between probe test path length, blood glucose, and antioxidant superoxide enzyme

		Blood Glucose	SOD1/β-actin	SOD2/β-actin
Path Length	Correlation coefficient	-0.205	0.414	0.179
	Sig. (2-tailed)	0.276	0.029	0.381
	N	30	28	26