**ORIGINAL ARTICLE**

**Chlorogenic acid may improve memory function and decrease inflammation of frontal lobe in diabetic rat**

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**ABSTRACT**

Introduction: Diabetes Mellitus (DM) is a chronic disease with many complications, one of which is diabetic encephalopathy which is characterised by memory dysfunction. Hyperglycaemia that occurs in DM will activate inflammatory pathways in neurons, including NF-κB pathway. Activation of this pathway produces proinflammatory agents such as MCP-1 and IL-6, which activate glial cells. Activation of glial cells is characterised by Glial Fibrillary Acid Protein (GFAP). Chlorogenic acid (CGA) has been reported to have anti-inflammatory effects and can improve memory function. This research aimed to determine the effect of CGA as anti-inflammation, its effect on memory function, mRNA expression of NF-κB, MCP-1, IL-6, and GFAP of frontal lobe.

Materials and Methods: A total of 24 male rats were randomly divided into six groups: control, DM 1.5 month (DM1.5), DM 2 months (DM2) and the group with three different doses of CGA 12.5 (CGA1), 25 (CGA2), and 50 (CGA3) mg/KgBW. Frontal lobe tissue is taken for analysis of mRNA expression for NF-κB, MCP-1, IL-6, and GFAP using Reverse Transcriptase PCR (RT-PCR). Samples were also taken for histopathology preparation and stained by immunohistochemistry method using anti-GFAP antibodies to observe glial cell activation in frontal lobe tissue.

Results: The group that was given CGA at all doses have statistically significant better memory function, i.e. DM2 versus CGA1 (p = 0.036), CGA2 (p = 0.040), and CGA3 (p = 0.021). The result of mRNA expression in NF-κB was lower in the group given CGA, i.e. DM2 compared to CGA2 (p = 0.007). mRNA expression of MCP-1 was significantly lower in all CGA treatment groups compared to the non-CGA group (p = 0.000). IL-6 mRNA expression was lower than the group not given CGA, DM compared to CGA2 (p = 0.028). GFAP mRNA expression was lower than the group given CGA in DM, DM2 group compared to CGA1 (p = 0.04) and CGA3 (p = 0.004).

Conclusion: Administration of CGA can improve memory function at all doses given, and can reduce brain inflammatory activity, especially in the CGA2 group.

**KEYWORDS:**
Chlorogenic acid, Diabetes Mellitus, Memory, Inflammation, frontal lobe

**INTRODUCTION**

Diabetes Mellitus (DM) is a metabolic disorder caused by the inability of insulin production by the pancreas or resistance by end organ tissues and results in high blood glucose levels (hyperglycaemia) and can be followed by a progressive decrease in pancreatic beta cell function.¹ DM is a chronic disease that can develop into many complications in various organs. One of the most dangerous complications of chronic hyperglycaemia is diabetic encephalopathy which is characterised by decreased cognitive function and motor dysfunction.²

Hyperglycaemia that occurs in DM will activate inflammatory pathways in neurons, then neurons will release inflammatory agents and activate glial cells. Inflammation in neurons involves several pathways, one of which is the PKC pathway. The activated PKC pathway will activate NF-κB in the nucleus then will express proinflammatory agents such as MCP-1 and IL-6.³ Metabolic dysfunction and oxidative stress will also cause changes that occur rapidly and activate glial cells. The main indicator of this response is Glial Fibrillary Acid Protein (GFAP). Abnormal GFAP expression is mostly concentrated in the cortex and hippocampus.⁴ Inflammation that occurs due to hyperglycaemia will then cause damage to the cerebrum called diabetic encephalopathy. Diabetic encephalopathy shows symptoms of decreased cognitive function, including memory.⁵ The brain regions most susceptible to damage due to DM are the hippocampus and frontal cortex.⁶

One of the functions of the frontal cortex is to play a role in working memory. Lesions in the frontal lobe will result in memory dysfunction including spatial memory and working memory.⁷ Several studies, one of which is by inhibiting GSK-3β, have shown that this inhibition prevents cognitive dysfunction in DM rats using the Morris water maze assay, and shows lower expression of proinflammatory agents in the brain.⁸ Administration of antioxidants in the form of crocin compounds also has a beneficial effect in improving memory dysfunction, and reducing hyperglycaemia and oxidative stress in DM rats by streptozotacin induction.⁹ Administration of chlorogenic acid (CGA) in rats with cognitive dysfunction induced with intracerebroventricular streptozotocin demonstrates prevention of cognitive dysfunction.¹⁰ CGA is also known to inhibit memory deficits and hippocampal cell death in mice with a transient global ischemia model.¹¹

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CGA is one of the polyphenol compounds in coffee that has the highest antioxidant content, which is ± 200 550 mg/cup of coffee with 26% activity compared to beta carotene (0.1%), alpha tocopherol (0.5%) and vitamin C (8.5%). CGA is the most widely consumed polyphenol compound and reported to have antioxidant and anti-inflammatory effects. CGA has been reported to inhibit mRNA expression and levels of the cytokine interleukin-8 (IL-8). The study also reported the neuroprotective effect of CGA on scopolamine-induced memory and learning impairment in mice and found that decaffeinated CGA-rich instant coffee was able to protect against learning and memory disorders through cholinergic and antioxidant mechanisms. Several pre-clinical studies conducted to determine the effects of CGA on cognitive function in rodents have also shown that CGA has the potential to improve spatial learning and memory, reduce memory impairment, reduce anxiety, improve motor function and protect against ischemia-induced neuronal damage.

Inflammation of neurons that occurs due to hyperglycaemia in DM is a serious problem and can interfere with cognitive function and memory and one of the vulnerable parts of the brain is the hippocampus and frontal cortex. There is no study that clarifies the effect of CGA on memory dysfunction and frontal lobe inflammation caused by DM. Therefore, further research is needed regarding the effect of CGA on memory dysfunction and frontal cortex inflammation by studying the frontal lobe mRNA expression of GFAP, MCP-1, NF-xB and IL-6 in diabetic rats.

MATERIALS AND METHODS

Design

This research is a quasi-experimental study with a post-test only controlled group design used 2-month-old male Rattus norvegicus Wistar strain with a body weight (BW) of 150-200 grams obtained from the University of Muhammadiyah Yogyakarta. Rats (n=24) are divided into six groups: control; DM1.5; DM2; CGA1; and CGA3 groups. This research has been approved by the ethics committee of the Faculty of Medicine, Universitas Gadjah Mada with number KE/FR/1117/EC/2020.

Diabetic Induction

DM model was prepared by injection of streptozotocin (60 mg/kg) dissolved in 0.1 M citric acid pH 4.5). DM is defined by measuring glucose levels that are more than 250 mg/dL by checking blood sugar from the tail vein.

CGA Administration

CGA was dissolved using PBS was administered by intraperitoneal (IP) injection with a total volume of 1 ml/kgBW. Three variation of CGA dosage: 12.5 mg/kgBW (CGA1), 25 mg/kgBW (CGA2) and 50 mg/kgBW (CGA3). CGA was administered to CGA1, CGA2, CGA3 groups for 14 consecutive days.

Probe Test

All six groups were assessed with probe test before termination using Morris water maze to assess memory dysfunction. Rats were being trained to search probe in 5 days before undergo probe test.

RESULTS

Blood Glucose Level

Blood glucose levels are divided into two types in each group: GDS1 taken before CGA administration (0.9% NaCl administration in the DM group) and data after CGA administration (Figure 1). Previously all data were tested for normality using the Saphiro-Wilk test with blood glucose administration (Figure 1). Previously all data were tested for normality using the Saphiro-Wilk test with blood glucose distribution results (p ≥ 0.05) so a paired t-test statistical test was used. Meanwhile, the CGA2 group showed an abnormal distribution (p = 0.007) so the Wilcoxon statistical test was used.

Probe Test

The results of the probe test for the six groups are presented in Table I. The control group had the longest time in the correct quadrant (Q4), while the time in the DM1.5 and DM2 groups gradually decreased. In the three groups that were given CGA, the time in quadrant 4 was longer than in the DM2 group.

Termination

Termination was carried out on day 60. In accordance with the AVMA guidelines for euthanasia for animals: 2013 Edition, the termination of the experimental animals was carried out using ketamine at a dose of 100 mg/kgBW which was injected intraperitoneally. Frontal lobe tissue was taken and immediately stored in a 1.5 ml tube filled with RNA preservation solution at -20°C in the anatomy laboratory.

Reverse Transcriptase PCR (RT-PCR)

Frontal lobe tissue used for RNA extraction and RNA will then be used for the production of cDNA. cDNA was used for RT-PCR and followed by electrophoresis procedure. The mRNA expression test for NF-xB (ICACTCTCTTTTGGAGGT; rTGATATAAGGCGTTAC), MCP-1 (F C A G T C T T G T C A G C T T C T) rAGATATCCATGGAGGAAATAG, IL-6 (F TTG GAT G T C T T G T C T T A G C C; rTCTACCCCCAACTCCTCAATGGCT), and GFAP (F CGA A C G A G T C T T T G G A G A G G; rTAGAGGAAATGGTGATGCGG) was performed by densitometric analysis after electrophoresis and β-actin used as a house keeping gene.

Immunohistochemical (IHC) Staining

IHC staining was performed at the same time for each tissue group using Abcam Rabbit Anti-GFAP antibody and BIOTnA mouse/rabbit probe HRP labelling kit with DAB brown. Observation of the results of IHC staining was carried out under a light microscope in the entire field of view of the frontal lobe cortex tissue, with a magnification of 400 times. The assessment was carried out by observing the brown colour of the coronal section of the frontal lobe histology.

Data Analysis

The data obtained were tested with the Shapiro Wilk test to determine data distribution. One way ANOVA test and post hoc Least Significant Difference (LSD) test used for normal data distribution and Kruskal Wallis test and Mann Whitney post-hoc test for non-normal data distribution. The value of p < 0.05 was used as the significance criteria.

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Table I: Mean, SD, and SEM of probe test result (in Q4)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SD (seconds)</th>
<th>SEM (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>64.466 ± 6.431</td>
<td>2.876</td>
</tr>
<tr>
<td>DM1.5</td>
<td>52.306 ± 13.152</td>
<td>5.882</td>
</tr>
<tr>
<td>DM2</td>
<td>33.05 ± 6.159</td>
<td>2.754</td>
</tr>
<tr>
<td>CGA1</td>
<td>48.106 ± 10.264</td>
<td>4.590</td>
</tr>
<tr>
<td>CGA2</td>
<td>47.782 ± 5.795</td>
<td>2.591</td>
</tr>
<tr>
<td>CGA3</td>
<td>49.065 ± 12.530</td>
<td>5.115</td>
</tr>
</tbody>
</table>

Fig. 1: Blood glucose level

Fig. 2: Graph of the length of time the rat is in the right quadrant. There was a significant difference between the control group and DM2 (p = 0.000); control group with CGA1 (p = 0.022); control group with CGA2 (p = 0.024); and the control group with CGA3 (p = 0.026). There was also a significant difference in the DM2 group with CGA2 (0.040).

*: Significantly different to control; #: Significantly different to DM2
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**Fig. 3:** Rats' trajectory in probe test A: control, B: DM1.5, C: DM2, D: CGA1, E: CGA2, F: CGA3

**Fig. 4:** NFkB, IL-6, MCP-1 and GFAP mRNA expression. DM2 has significantly higher mRNA expression in to control in all inflammatory marker and glial activation marker. There was a significant difference between the control group and DM2 in all mRNA expressions of inflammatory agent genes and glial activation marker genes. CGA2 has significantly lower mRNA expression of inflammatory agent to DM2. There is inconsistent result of GFAP mRNA expression in CGA groups. *: Significantly different from the control; #: Significantly different from DM2
The results of the probe test from the six groups were tested for normality using the Shapiro-Wilk test with the results of all groups being normally distributed \((p \geq 0.05)\), then the data was tested for one-way ANOVA with post-hoc LSD. The test results showed a significant level of \(p = 0.005\) indicating a statistically significant difference in at least one group with each other.

After further testing with post-hoc LSD, the control group time was not significantly different from the DM1.5 group, but significantly different from the DM2 group; this indicates that the memory function in the DM2 group was lower than the control group. The three groups given CGA had a higher and statistically significant difference with time in the DM2 group, whereas between the three groups given CGA there was no statistical difference.

**Probe Test Trajectory**

In addition to looking at the length of time in the right quadrant (Q4-bottom right), the researchers also observed the trajectory that the rats took. In the control group, the rat focused more on looking at Q4, while in the DM1.5 and DM2 groups, the rat also circled a lot of other quadrants, and it was very visible in the DM2 group trajectory, the rat searched did not focus on the correct quadrant but almost the entire quadrant was surrounded by rat. In addition, in the DM2 group trajectory images, rat do not appear to have a search focus on Q4 (Figure 3).

**Frontal Lobe mRNA NFKB Expression**

The results of the NFKB densitometry data were first tested for normality with the Shapiro-Wilk test with the results that all groups were normally distributed \((p \geq 0.05)\). Then, a one-way ANOVA test was carried out with post-hoc LSD, showing that the DM1.5 group had higher NFKB mRNA expression than the control group. Meanwhile in the CGA group, the three groups, namely CGA1, CGA2, and CGA3 had lower NFKB mRNA expression than the DM1.5 and DM2 groups, and were statistically significant in the three groups. Meanwhile, between the three groups that were given CGA, there was no statistical difference in the expression of NFKB mRNA.

**Frontal Lobe mRNA MCP-1 Expression**

Data was tested by one-way ANOVA test with \(p=0.000\). After the post-hoc LSD test, the control group was statistically significant with the DM1.5 group \((p=0.000)\) and the DM2 group \((p=0.000)\), indicating that both DM models had higher MCP-1 mRNA expression than the control group. Meanwhile in the CGA group, the three groups, namely CGA1, CGA2, and CGA3 had lower MCP-1 mRNA expression than the DM1.5 and DM2 groups, and were statistically significant in the three groups. Meanwhile, between the three groups that were given CGA, there was no statistical difference in the expression of MCP-1 mRNA.

**Frontal Lobe mRNA IL-6 Expression**

One-way ANOVA test was carried out with the results of \(p=0.013\); Then from the post-hoc test there was a significant difference between the DM1.5 group; DM2; CGA1, and CGA3 with the control group, the results of the CGA 1 group had lower and statistically significant IL-6 mRNA expression compared to the DM2 group, and not significantly different from control.\(^9\)

**Frontal Lobe mRNA GFAP Expression**

One-way ANOVA test was performed with \(p=0.04\) and after the post-hoc LSD test, the control group was not significantly different from the DM1.5 group \((p=0.056)\) but the DM1.5 group showed higher GFAP expression but not statistically significant. Then the control group was significantly different from the DM2 group \((p=0.04)\), indicating that the DM2 group had a higher significant GFAP expression than the control group. In the three groups given CGA, all three had lower GFAP expression than the DM2 group, but it was only significant in the CGA1 and CGA 3 groups. Meanwhile, in the three groups given CGA, there was no statistically significant difference with each other.
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**IHC anti-GFAP Antibody**
On IHC-anti-GFAP staining of the frontal lobe, positive IHC is indicated by the presence of a brown colour. In the DM group, both DM1.5 and DM2, the brown colour which indicated the GFAP protein was more abundant and thicker than in the control group. In the group given CGA, the brown colour appeared less than in the DM group.19

**DISCUSSION**
In this study, we wanted to find out the effect of CGA administration on memory function (probe test), mRNA expression of pro inflammatory factors (NF-kB, MCP-1, and IL-6), as well as protein expression of glial activation markers as GFAP, in DM-induced rat using streptozotocin. We also observed the appearance of anti-GFAP IHC staining as well as the trajectories taken by rat in the probe test of Morris water maze. In the results of the blood glucose test, the average blood glucose was higher in both DM groups, and in the three CGA groups, this showed that the creation of DM models in rat was successful. In the group that obtained CGA at doses of 12.5 mg/kgBW (CGA1) and 25 mg/kgBW (CGA2) showed decrease in blood glucose levels after 2 weeks of CGA administration but did not differ significantly. In the CGA3 group, the average glucose increased but was also not significant, it is likely that this happened because CGA doses of 50 mg/kgBW or higher had genotoxic effects on the bone marrow and stress on vital organ.22 This is inconsistent with previous studies, a clinical trial that showed significant reductions in fasting glucose in humans with impaired glucose tolerance (IGT) after capsule administration containing CGA 400 mg three times a day for 12 weeks.22 In addition, in clinical trials of metabolic syndrome patients who were given green coffee extract 400 mg twice a day for 8 weeks also lowered the average fasting blood sugar.22 Whereas in another study in DM rats with streptozotocin induction as well, CGA at doses of 100 mg/kgBW and 150 mg/kgBW orally for 28 days rats significantly lowered the blood glucose levels of rats, in this study doses of 100 mg/kgBW rats had better blood glucose reduction results.22

The antidiabetic effect on CGA comes from various mechanisms, especially CGA has the effect of inhibiting glucose absorption in the small intestine by inhibiting glucose-6-phosphate translocase as well as inhibiting glucose-6-phosphatase in liver. In addition, CGA will inhibit the uptake of glucose from the intestines by inhibiting the α-glucosidase by reducing the synergistic transportation of glucose so that blood glucose will decrease.24 In this study, CGA was injected intraperitoneally so that it might reduce the effect of CGA as an anti-glycaemic, thus causing an insignificant decrease in blood glucose levels in all CGA groups.

In the probe test, researchers looked at how long the rat would look for platforms in the right quadrant for 120 seconds and observed the trajectory that the rat passed. Previously, for 5 days, rats were trained to swim in search of platforms. This test showed memory ability in rat, in this study it was found that DM-induced rat, for both 1.5 months and 2 months had worse memory than control group, but only the DM2 group differed significantly. This data indicates that there has been memory impairment due to DM in rat progressively and in DM2 months the rat’s memory is significant, this is also supported by a picture of the trajectory that rat passed with DM, rats appeared to rotate around the entire quadrant in both DM2 groups. The results of the probe test Morris water maze showed that the resulting DM model experienced the progressivity of memory worsening from the DM1.5 group to the DM2 group. Other studies on rat also showed that the group of rat that had higher glucose levels showed worse spatial memory test results on Morris water maze.25 In the entire CGA group, the results of rat travel time in Q4 were higher, indicating better memory than in the 2-month DM group, but not yet equivalent to the control group. Meanwhile, in the three groups given by CGA, there were no statistical differences between them. This suggests that administering CGA at all three doses can improve the effect of improving memory function caused by DM, although there is no dose-dependent sign of CGA administration yet. On the trajectory passed by the rats, it was also seen that the trajectory passed by the CGA group had more focus on Q4 compared to the DM group. This is in accordance with other studies on the effect of CGA in improving scopolamine-induced memory function decline, namely at doses of 6 mg/kgBW and 9 mg/kgBW showing improvements in probe test results.23 Administration of CGA also showed improvement in decreased memory impairment due to transient ischemia at doses of 2.5 mg/kgBW, 5 mg/kgBW and 10 mg/kgBW.22 This study is in accordance with the results of previous studies, with the administration of CGA 50 mg/kgBW can improve memory in rats which experienced a decrease in memory function by inducing intra-ventricular streptozotocin.24

Better memory function in groups with CGA administration may occur because CGA has anti-inflammatory effects. This is in accordance with studies using the administration of GSK-3β inhibitors (SB216763) which are one of the inhibitors of inflammatory processes in DM model, and also showed memory improvement and decreased levels of IL-6, TNF-α, and NF-kB protein expression.25 Increased inflammatory markers in neural tissue (neuroinflammatory) that occur in DM along with BBB damage are socialized with memory dysfunction.26 In accordance with the theory of CGA has an anti-inflammatory effect so that memory function is better in the group with CGA administration.

Inflammation in neurons involves several pathways, one of which is the CCP pathway. The activated PKC pathway will activate NF-kB on cytosol, NF-kB which is a transcription factor then goes to the nucleus thus initiating transcription from proinflammatory factors such as MCP-1 and IL-6-4. Inflammation in neurons will activate glia cells.27 Metabolic dysfunction and oxidative stress will also cause rapid changes and activate glia cells. The main indicator of this response is glial fibrillary acid protein (GFAP). Abnormal GFAP expression is mostly concentrated in the cortex and hippocampus.28 Other research results (Datusalia dan Sharma, 2014) also states that GSK-3β inhibitors can reduce inflammation and improve memory function in DM models, this is in accordance with the properties of CGA, one of which has molecular docking in AKT and can reduce the activity of GSK-3β.29
In this study, the group with CGA dose administration of 25 mg/kgBW had significantly lower expression of NF-κB mRNA of the frontal lobe than in the DM2 group. This shows that at these doses CGA can reduce inflammatory activity that occurs in the CCP pathway. ChCGA can bind to PH domain of AKT, a protein kinase, making AKT phosphorylated into p-AKT. The active AKT then causes Glycogen synthase kinase-3 (GSK-3) inactivation. GSK-3 is a protein kinase that is widely found in the brain, GSK-3β contributes to abnormal phosphorylation of microtubule-bound proteins associated with the incidence of Alzheimer's disease. GSK-3 has two isoforms namely GSK-3α and GSK-3β. GSK-3α is found mainly in the hippocampus, cortex, striatum, and cerebellum. While GSK-3β is found in all parts of the brain. GSK-3β regulates inflammation in neurons by means of NF-κB maintaining the integrity of TLR, NEMO, as well as inhibiting the accumulation of CREB. Decreased activity on GSK-3β because of CGA will further decrease the activity of NF-κB. This is in accordance with the results of this study, namely in DM, especially DM 2 months, mRNA expression of the frontal lobe from NF-κB increased compared to controls, while in the group given CGA decreased compared to the DM2 group, in this study, especially in the CGA2 group.

In the results of mRNA expression NF-κB and IL6 was lower in the group given CGA2 than DM2. mRNA expression of MCP-1 was significantly lower in all CGA treatment groups compared to the non-CGA groups (DM1.5; DM2); while in GFAP mRNA expression was lower in CGA1 and CGA3 groups than DM2. Treatment with chlorogenic acid (CGA) may improve memory function at all doses given, and can reduce brain inflammatory activity, especially in the CGA2 group.

Hyperglycaemia activates the CCP pathway where NF-κB experiences increased activity. NF-κB which is a transcription factor will then enter the nucleus and bind to the DNA. NF-κB bonding with DNA will induce transcription from proinflammatory agents such as IL-6, TNF-α, IL-1β. In accordance with the theory, the expression of mRNA IL-6 frontal lobes in the CGA2 group was lower and statistically more in the group with CGA administration than not, corresponding to the expression of NF-κB mRNA which also decreased in the CGA2 group.

In the results of mRNA expression NF-κB and IL-6 frontal lobe decreased significantly only in the CGA2 group. This can occur because there is a possibility that the dose of CGA1 is too small, while the dose of CGA3 or higher has a genotoxic effect on the bone marrow and stress on the organs of vital. This is also consistent with previous studies that looked at GFAP expression on the retina with dm models. The existence of a trend that does not correspond to this increase in doses may be due to the regulation of GFAP production itself occurring not only at the transcription level, but also the post-translational level. However, in the staining of the anti-GFAP IHC on the frontal lobe, it was seen that the DM group expressed many positive signs and decreased in all three groups given CGA.

Given this result of the study, we hope that the use of CGA in memory dysfunction can be considered. This study has various limitations, including: this study did not examine other factors such as apoptosis of neuron cells, this study did not count the number of cells and synapses that affect memory.

**CONCLUSION**

The group given CGA at all doses have statistically significant better memory function than DM2 group in probe test Morris water maze. The result of mRNA expression in NF-κB and IL6 was lower in the group given CGA2 than DM2. mRNA expression of MCP-1 was significantly lower in all CGA treatment groups compared to the non-CGA groups (DM1.5; DM2); while in GFAP mRNA expression was lower in CGA1 and CGA3 groups than DM2. Treatment with chlorogenic acid (CGA) may improve memory function at all doses given, and can reduce brain inflammatory activity, especially in the CGA2 group.

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