Chlorogenic Acid Ameliorates Liver Function in Association with Bax Downregulation, P53 Downregulation and Bcl-2 Upregulation in Diabetic Wistar Rat

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Abstract. Hyperglycemia in Diabetes mellitus (DM) induces oxidative stress and mitochondrial disruptions in the liver, thereby triggering apoptosis through Bcl-2 family signaling. Excessive apoptosis leads to liver dysfunction. Chlorogenic acid (CGA) was elucidated in this study in preventing liver dysfunction as a progression of DM. The diabetic model was conducted in Wistar rats, divided into six groups. Blood examinations were done to measure the levels of blood glucose, SGOT, and SGPT. The liver was harvested for analysis of SOD2, Bax, and Bcl-2 mRNA. A paraffin section was used for p53 immunostaining. Liver dysfunction occurred in the DM 2 months group indicated by higher levels of SGPT and SGOT, higher expression of Bax, and lower expression of Bcl-2 compared to the control group. Giving CGA 12.5 mg/kgBW ameliorated blood glucose and liver enzyme levels, which were associated with lower expression of Bax, lower signaling of p53, and higher expression of Bcl-2 compared to DM 2 months group. Administration of CGA did not affect SOD2 expression in diabetic rats. CGA may attenuate liver dysfunction in diabetic rats through downregulation of Bax and p53, and upregulation of Bcl-2 signaling.

1 Introduction

Diabetes mellitus (DM) is a serious long-term health problem that occurs in many countries, and can reduce life expectancy by as much as 5-10 years due to complications to various organs [1]. The DM disease is a group of metabolic diseases. DM is characterized by hyperglycemia due to defects in insulin secretion, insulin action, or both. Both type 1 and type 2 diabetes can result in a progressive decrease in cell mass and function which clinically manifests as hyperglycemia [2]. Prolonged hyperglycemia in DM causes complications in the form of glucotoxicity which causes toxic effects on cell function and continues as organ dysfunction [3].

Streptozotocin (STZ) injection to induce type 1 DM has been widely applied in experimental animal models. STZ induction affects the mitochondrial mechanism in the existence of cellular glucotoxicity through the electron transport chain [4]. The injection of STZ causes damage to the beta-cells of the pancreas. Lack of beta cells affects the insulin. Lack of insulin causes hyperglycemia because glucose cannot be carried into cells and continues to accumulate in the blood. Hyperglycemic conditions cause disturbances in the apoptotic process associated with oxidative stress [5].

Chronic hyperglycemia is a trigger for liver molecular changes due to metabolic toxic substances. The liver plays a role in maintaining blood glucose levels and blood cholesterol levels. Another function of the liver is as a detoxification site [6]. When there is repeated damage to hepatocytes over a long period of time, the multiplication of liver cells is followed by the development of a number of connective tissues that contain many collagen fibers [7]. Several molecular mechanisms mediated by chronic hyperglycemia include the polyol pathway, protein kinase C pathway, increased oxidative stress, and increased hexosamine pathway. Those mechanisms trigger long-term reactions in the form of oxidative stress, inflammation, apoptosis, and even tissue fibrosis [8].

Glucose causes oxidative stress through the induction of free radicals and reduces endogenous antioxidants as a defense for diabetic patients. Exposure to high glucose for a long time can increase the production of reactive oxygen species (ROS). Excessive ROS results in a poor metabolic cycle that can trigger irreversible tissue damage. Excessive ROS production is associated with changes in mitochondrial morphology [9]. Increased ROS due to hyperglycemia correlates with increased mitochondrial enzyme activities and cell proliferation [10].

The apoptotic response associated with oxidative stress involves apoptotic regulatory genes. Regulatory genes that are involved in the apoptotic process belong to
the BCL2 family, including the Bax and Bcl-2 genes. The high rate of apoptosis induction is caused by high expression of regulatory gene Bax and low expression of Bcl-2 which activates caspase 3 [11]. In addition to regulatory genes, oxidative stress also triggers the apoptotic protein p53 which affects glucose metabolism and plays a role in the formation of caspase 3 through the intrinsic pathway [12].

Chlorogenic acid (CGA) is a phenolic compound from green coffee that has been investigated as a hepatoprotector due to its anti-inflammatory and antioxidant properties. The anti-inflammatory activities occur through the inhibition of nuclear factor-kappa B (NFκB) activation [10]. CGA as an antioxidant resists free radicals, increases resistance to changes in low density lipoprotein (LDL) to lipid peroxide, prevents carcinogenesis in the large intestines, liver, tongue, by preventing DNA damage [13]. CGA is recognized as an antidiabetic to reduce intestinal glucose transport through regulation of the sodium gradient, suppress gluconeogenesis in the liver by inhibiting glucose-6-phosphatase activity, and increase glucose uptake in myotubes and adipocytes [5].

This study was conducted to see the effects of oxidative stress due to hyperglycemia on the liver of type 1 diabetic rats. Liver damage was seen on exposure to diabetes for 1.5 months and 2 months. CGA in three different doses was used to examine the hepatoprotective effect through endogenous antioxidants and antiapoptotic mechanisms.

2 Material and Methods

2.1 Subject

This study used male Rattus norvegicus Wistar strain rats aged 2 months with a body weight of 150-200 gram. The diabetic model was conducted in Wistar rats, divided into six groups: control, DM 1.5 months, DM 2 months, DM 1.5 months with CGA 12.5 mg/kgBW (CGA1), DM 1.5 months with CGA 25 mg/kgBW (CGA2), and DM 1.5 months with CGA 50 mg/kgBW (CGA3). This research has obtained ethical clearance from the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada with the reference number KE/FK/1261/EC/2020.

2.2 Animal Model of Diabetes mellitus

Induction of diabetes used single intraperitoneal injection of 60 mg/kgBW Streptozotocin (STZ). Diabetes induction was successful if it showed an increase in blood sugar level > 200 mg/dl.

2.3 Chlorogenic Acid (CGA)

Chlorogenic Acid (CGA) was pure solid preparation from Sigma-Aldrich® which is dissolved in PBS with 3 variations in dosages: 12.5 mg/kgBW, 25 mg/kgBW and 50 mg/kgBW. CGA was administered to DM rats intraperitoneally at the end of the 6th week, every day for 2 weeks [4].

2.4 Liver function

Liver function was measured by SGOT, SGPTP and blood glucose levels in rat blood serum taken through the retro orbital vein. Blood samples were examined by the enzymatic method in the Clinical Pathology Laboratory of Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada.

2.5 Euthanasia

Anesthesia was performed by intraperitoneal injection of ketamine 100 mg/kgBW. After being anesthetized, 1 ml of blood was taken from the retro orbital vein for examination of GDS, SGPT, and SGOT. After termination by cardiac puncture, the medial lobe liver tissue of the rats was taken. Some were stored in tubes filled with NBF solution for immunohistochemical staining. Some were stored in later RNA solution, in a refrigerator at -20°C for later examination of mRNA using the RT-PCR method.

2.6 Reverse transcriptase-PCR

RT-PCR was preceded by RNA extraction and cDNA making from liver tissue. The biomarker of endogenous antioxidants was SOD2. The biomarker of the apoptosis regulatory gene was the expression of Bax and Bcl-2 mRNA Expressions of mRNA SOD2, Bax, and Bcl-2 of rat hepatocytes were measured by RT-PCR examination. The results of the electrophoretic band density were measured using ImageJ® software. The mRNA expression density of each gene was compared with the housekeeping gene β actin. Primers used by the Macrogen® brand include:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Product</th>
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<tbody>
<tr>
<td>Bcl-2 F</td>
<td>GGTTGAAAAGCAGCTCAGTAA</td>
<td>150 bp</td>
</tr>
<tr>
<td>Bcl-2 R</td>
<td>GGCGCTCGTGTACTTC-TCCTCGGTG</td>
<td>150 bp</td>
</tr>
<tr>
<td>Rax F</td>
<td>GGTTTTCATCC-AGGATCGACA</td>
<td>150 bp</td>
</tr>
<tr>
<td>Rax R</td>
<td>ATCCACATCAGC-AATCATCTCTTG</td>
<td>150 bp</td>
</tr>
<tr>
<td>RBC-1 F</td>
<td>GCCGCAAC-AGGAGATGTC</td>
<td>150 bp</td>
</tr>
<tr>
<td>RBC-2 R</td>
<td>TTCCACAAAGGACATCCAGC</td>
<td>150 bp</td>
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2.7 Immunohistochemical stain

The p53 expression as biomarker of the apoptosis in rat hepatocytes was obtained from direct immunohistochemical staining using a kit from BIOTnA Biotech® with rabbit polyclonal antibody anti-p53. The expression of p53 was examined under a light microscope with 400x magnification. Positive staining was an image of the hepatocyte nucleus which was painted brown. [14]. The positive staining results were calculated and compared with the entire nucleus in the field of view and presented as a percentage of the average nucleus [15].

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Figure 1. Levels of SGPT and SGOT after experimental. SGPT and SGOT levels were significantly higher in the DM2 group compared with control. In the administration of CGA, SGPT and SGOT levels were significantly lower in CGA 1 and CGA 2 groups compared with DM2 group. K = control, DM 1.5 = DM 1.5 months rats, DM 2 = DM 2 months rats, CGA1 = DM1.5 + CGA12.5 mg/kgBW, CGA2 = DM1.5 + CGA25mg/kgBW, CGA3 = DM1.5 + CGA50mg/kgBW. * = p<0.05 vs K; # = p<0.05 vs DM2.

3 Results and Discussion

3.1 Blood glucose levels

Table 1. Blood Glucose in Control and Experimental Groups of rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/dl)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>84.25 ± 13.06</td>
</tr>
<tr>
<td>DM 1.5 months</td>
<td>467.75 ± 101.4³</td>
</tr>
<tr>
<td>DM 2 months</td>
<td>594.5 ± 57.75 unrest</td>
</tr>
<tr>
<td>CGA + CGA 12.5 mg/kgBW</td>
<td>136.75 ± 2.29²</td>
</tr>
<tr>
<td>CGA + CGA 25 mg/kgBW</td>
<td>238 ± 74.45³</td>
</tr>
<tr>
<td>CGA + CGA 50 mg/kgBW</td>
<td>525 ± 6.97</td>
</tr>
</tbody>
</table>

*p<0.05 versus control
#p<0.05 versus DM 2 months

The average blood glucose profile of each group at the end of the study can be seen in Table 1. Administration of STZ injection succeeded in causing hyperglycemia in both the 1.5 months DM and 2 months DM groups which was characterized by glucose levels of >200 mg/dl (Table 1). DM 2 months group was conducted to see the progressivity of DM without treatment. Administration of CGA doses of 12.5 mg/kgBW and 25 mg/kgBW in DM 1.5 months group caused glucose levels to be lower than in the DM 2 months group. The effect of lowering glucose after administration of CGA occurred in association with suppression of gluconeogenesis in the liver through inhibition of glucose-6-phosphatase activity. CGA increases the stimulation of glucose transport by muscle GLUT 4 via the AMPK pathway [5].

3.2 SGPT and SGOT Levels

In Wistar rats, the normal value of SGPT is 10-40 IU/L, while for SGOT it is 50-150 IU/L [23]. These enzymes play a role in the process of gluconeogenesis by catalyzing the transfer of aspartic acid or alanine to ketoglutaric acid to form oxaloacetic acid and pyruvic acid. SGOT is present in the cytosol and mitochondria but is nonspecific in the liver. Elevated SGOT may be a sign of extrahepatic disorders. SGPT is a cytosolic enzyme that is found in high concentrations in the liver. Both of these enzymes are released into the circulation in hepatocellular damage or unwanted cell death [20].

SGPT levels in DM 1.5 and DM 2 groups were significantly higher than in the K group. Administration of CGA at a dose of 12.5 mg/kgBW and CGA 25 mg/kgBW affected lower SGPT levels in the liver of DM rats. SGOT levels in the DM1.5 group did not show significantly higher levels than in the K group. The SGOT level was significantly higher in the DM2 group than the K group. Administration of CGA at doses of 12.5 mg/kgBW, 25 mg/kgBW, and 50 mg/kgBW affected lower SGOT levels in the liver of DM rats (Figure 1).

The attenuation of liver enzyme levels was Administration of STZ injection succeeded in causing hyperglycemia in both the 1.5 month and 2 month DM group which was characterized by glucose levels of more than 200 mg/dl (Table 1). related to the anti-inflammatory effect of CGA through inhibition of nuclear factor-kappa B (NFkB) thus preventing the release of pro-inflammatory cytokines. In addition, lowering blood glucose levels due to CGA administration leads to reduced oxidant stress and the formation of ROS. The reduction of ROS leads to improved liver function [16].

3.3 Expression of SOD 2

SOD2 expression in DM 1.5 months and DM 2 months groups was significantly higher than the K group. After administration of CGA, the expression of SOD2 in the CGA3 group was higher than in the DM 2 months group, but not significantly. Based on the results of statistical tests, the administration of CGA had no significant effect on the expression of SOD2 in the liver of DM rats (Figure 2a).
Figure 2. (a) The electrophoresis band and expression of SOD2. The SOD 2 was significantly higher in the DM 1.5 group, DM2 group, and CGA3 group compared with the control. (b) The electrophoresis band and expression of Bax. The DM2 group showed higher Bax expression than control. In the administration of CGA, CGA 1 and CGA 3 groups showed lower expression of Bax compared with DM2 group. (c) The electrophoresis band and expression of Bcl-2. The DM1.5 and DM2 groups showed significantly lower Bcl-2 expression compared with control. In the administration of CGA, CGA1 group showed higher Bcl-2 expression compared with DM2 group. K = control, DM 1.5 = DM 1.5 months rats, DM 2 = DM 2 months rats, CGA1 = DM 1.5 months + CGA12.5mg/kgBW, CGA2 = DM 1.5 months + CGA25mg/kgBW, CGA3 = DM 1.5 months + CGA50mg/kgBW. *=p<0.05 vs K; #=p<0.05 vs DM2, ##=p<0.05 vs DM1.5.

Previous studies have shown that SOD2 decreased in DM patients, causing superoxide accumulation that causes tissue damage and vascular complications [17]. In this study, the expression of the endogenous antioxidant SOD2 in DM rats for 2 months was higher than in normal rats. It was different from previous study due to genetic polymorphism in the SOD2 gene [17].

CGA functions as a non-enzymatic antioxidant that works by donating hydrogen atoms to reduce free radicals and prevent oxidation reactions. The role of CGA as an antioxidant works if there is oxidation in the system involving metals such as Cu and Fe to form ROS [18]. The antioxidant effect of CGA is obtained through its anti-diabetic properties. Decreased blood glucose indirectly inhibited oxidative stress and reduced ROS accumulation in liver cells [10].

3.4 Expression of Bax and Bcl-2

The induction of liver cell apoptosis in this study was indicated by the high expression of the pro-apoptotic regulatory gene Bax in the DM 2 months group, and the low expression of the anti-apoptotic regulatory gene Bcl-2 in the DM 1.5 months and DM 2 months groups (Figure 2b, 2c). Treatment of CGA in DM 1.5 months group at a dose of 12.5 mg/kgBW showed lower pro-apoptotic gene Bax and higher anti-apoptotic Bcl-2 significantly compared to the DM 2 months group.

Increasing in apoptosis induction was caused by upregulation in the pro-inflammatory cytokines IFN-γ and TNF-α which affected in an increase of ROS production and p53 induction, thereby triggering apoptosis [19]. The upregulation of pro-apoptotic regulatory genes in DM for 2 months was in line with impaired liver function in the form of high SGOT and SGPT. This study supports previous studies suggesting that hepatocellular damages and unwanted liver cell death trigger the release of SGOT and SGPT enzymes into the bloodstream [20].

3.5 Expression of p53 protein

Expression of p53 as protein on immunohistochemical staining resulted in a brown image of the hepatocyte nucleus. The expression of a brown-colored nucleus was shown in the DM 1.5 months and DM 2 months groups. In the CGA1 and CGA2 groups, less brown staining was seen than in the DM2 group (Figure 3a). Liver cells undergoing apoptosis have eosinophilic cytoplasm. In addition, the apoptotic liver cells showed an irregular appearance, shrinking, and the nucleus became fragmented [7].

Analyzing results on the percentage of p53 expression showed the DM 1.5 months (14.72 %) and DM 2 months (14.89%) groups had a higher expression than control, but it was not significant. Administration of CGA doses of 12.5 mg/kgBW and 25 mg/kgBW significantly affected p53 expression in the liver cells of DM rats (Figure 3b).

The immunohistochemical staining of expression of p53 in the liver of DM rats as a marker of apoptosis induction according to this study did not give a significant difference compared to normal liver. This was indicated by the positive stain of p53 expression only reaching 14% in the DM group. This study supported the research of Alves, in 2004 which found that p53 expression in liver cell staining tends to be unstable and has a short half-life. In the case of liver cancer, there may be a mutation of p53 that causes its overexpression and is stable so that it can be stained with immunohistochemical staining [21].
Figure 3. (a). The immunohistochemical microscopy of p53 in liver tissue. The brown-colored nucleus (black arrow) is the expression of p53 as a marker of liver cell apoptosis. The DM1.5 and DM2 groups showed more brown color than the K group, but statistically not significant. The CGA1 and CGA2 groups showed less brown color than the DM2 group. Image using 400x magnification microscope, 50 μm scale. (b). Percentage of p53 expression in liver cells at the end of the study. In CGA administration, p53 expression was significantly lower in CGA1 and CGA2 groups compared with the DM2 group. K = control, DM 1.5 = DM 1.5 months rats, DM 2 = DM 2 months rats, CGA1 = DM 1.5 months + CGA12.5mg/kgBW, CGA2 = DM 1.5 months + CGA25mg/kgBW, CGA3 = DM 1.5 months + CGA50mg/kgBW. *p<0.05 vs K; #p<0.05 vs DM2; +p<0.05 vs CGA1; $p<0.05 vs CGA2.
In this study, the liver apoptosis induction of DM rats was significantly shown by the high expression of the Bax gene and the low expression of Bcl-2 (Figure 2). Administration of CGA affected liver cell repair through downregulation of p53 protein and the Bax pro-apoptotic gene, as well as upregulation of the antiapoptotic gene Bcl-2. Ameliorating of liver function happens through two mechanisms. CGA has a role as an antidiabetic that helps lower blood glucose levels through activation of muscle glucose transport [5]. Decreasing blood glucose reduced oxidative stress in mitochondria, prevented activation of p53, and reduced the induction of apoptosis. CGA also worked as a non-enzymatic antioxidant that reduces ROS formation, prevents mitochondrial membrane damage, and reduces the induction of apoptosis [22]. The hepatoprotective function of CGA occurs through antidiabetic, antioxidant, and anti-apoptotic mechanisms.

This study showed the effect of chronic hyperglycemia on liver disorders. Exposure to hyperglycemia for 1.5 months has not shown significant differences in SGOT levels and Bax gene expression compared to controls. Significant differences in SGOT and SGPT levels, as well as Bax and Bcl-2 expressions, were shown by exposure to hyperglycemia for 2 months.

4 CONCLUSIONS

This study showed liver disorders in DM for 2 months through high levels of SGOT and SGPT enzymes, high expression of the pro-apoptotic regulatory gene Bax, low expression of the anti-apoptotic regulatory gene Bcl-2, and high antioxidant SOD2. Giving CGA 12.5 mg/kgBW had a significant effect on ameliorating liver function of DM rats as shown by low levels of SGOT and SGPT enzymes, low expression of Bax and p53, and high expression of Bcl-2. Giving CGA had no effect on SOD2 levels in DM rats. This study supports similar studies with additional data on SGOT, SGPT, expression of SOD2, Bax, and Bcl-2 genes, and immunohistochemical features of p53.

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