The Effect of Chlorogenic Acid on Endoplasmic Reticulum Stress and Steroidogenesis in the Testes of Diabetic Rats: Study of mRNA Expressions of GRP78, XBP1s, 3β-HSD, and 17β-HSD

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Abstract. Diabetes mellitus (DM) is a chronic endocrine metabolic disorder that can increase the incidence of infertility. Excessive production of reactive oxygen species (ROS) can trigger oxidative stress reactions and reduce antioxidant content. The increase of ROS can exert an impact on the endoplasmic reticulum (ER) stress conditions and inhibit the steroidogenesis of the testes. The objectives of the current study were to determine the effect of chlorogenic acid (CGA) as an antioxidant in reducing ER stress conditions (mRNA expressions of GRP78, XBP1s), and to increase steroidogenesis (mRNA expressions of 3β-HSD and 17β-HSD) in the testis of DM rats. A total of 24 rats were randomly divided into six groups, namely: control group, DM1.5 months, DM2 months, and DM group were treated with CGA with three different doses (12.5 mg/kgBW, 25 mg/kgBW, and 50 mg/kgBW), respectively. Testicular tissue was isolated for examination of GRP78, XBP1s, 3β-HSD, and 17β-HSD mRNA expressions using RT-PCR. In the CGA1 group, GRP78 mRNA expression level was lower than in the DM2 group and was statistically different (p=0.021). XBP1s mRNA expression in the CGA1 group was lower and significantly different when compared to the DM2 group (p=0.018). The mRNA expression of 3β -HSD in the CGA1 group was higher than that in the DM1.5 and DM2 groups, which were statistically significant with p=0.000 and p=0,008. The comparison of 17β -HSD mRNA expression in the CGA1 group was higher than the DM1,5 and DM2 groups with p=0.013 and p=0.012. Administration of CGA at a dose of 12.5 mg/kgBW can reduce ER stress conditions and increase testicular steroidogenesis in DM rat models. These results were seen based on low GRP78 and XBP1s mRNA expressions, and high 3β-HSD and 17β-HSD mRNA expressions after CGA administration.

1 Introduction

Diabetes mellitus (DM) is a disease that has become the center of attention in the modern era because it causes a variety of physiological disorders [1]. One of the physiological disorders that can occur is chronic endocrine metabolic disorders [2]. DM triggers the occurrence of prolonged hyperglycemia which has the potential to produce reactive oxygen species (ROS) [1]. Excessive ROS production can trigger oxidative stress and reduce antioxidant content so it can cause infertility or subfertility in subjects with DM [3, 4]. ROS are by-products of metabolic pathways that cause cell damage by triggering endoplasmic reticulum (ER) stress, disrupting normal mitochondrial function, and increasing inflammation through activation of necrosis factor kappa beta (NF-ϰB) [1, 5].

ER stress can be characterized by the unfolded protein response (UPR) due to an imbalance between the entry of protein load and protein folding capacity. It has been reported by Morishima et al. and Rashid and Sil that hyperglycemic conditions lead to the accumulation of UPR in testis cells [2, 6]. Prolonged UPR can interfere with the normal function of the ER which is regulated by glucose-regulated protein 78 (GRP78) and X-box binding protein (XBP1). GRP78 and XBP1 act as transcription factors for genes encoding molecules and proteins involved in endoplasmic reticulum-associated degradation (ERAD) [4, 7].

Previous studies showed an increase in GRP78 and also XBP1 in the testes of DM rats through streptozotocin (STZ) induction [1]. The high expression of GRP78 and XBP1 can trigger apoptosis in testis cells. According to research by Shati and Keyhanmanesh et al., the presence of apoptotic events is characterized by high p53 expression in the testis cells of diabetic rats [8, 9]. This causes disruption of steroidogenesis in the synthesis of reproductive hormones [1, 10]. The synthesis of testosterone depends on the enzymes 3β-HSD and 17β-HSDwhich play a role in the Leydig cells to break down

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low-density lipoprotein (LDL) cholesterol into testosterone. Apoptotic conditions can result in decreased testosterone that indicates hypogonadism and leads to infertility [11]. Accordingly, bioactive compounds are needed that can overcome problems ranging from ER stress to a decrease in enzymes related to testosterone steroidogenesis which can disrupt the physiology of the male reproductive system.

Chlorogenic acid (CGA) is one of the bioactive components of phenolic acids contained in foods such as coffee. According to previous studies, CGA act as a therapy because it can be used as an antioxidant and anti-inflammatory [12, 13]. These antioxidant and anti-inflammatory properties of CGA can reduce insulin resistance, reduce hyperglycemic levels in glucose metabolism, and reduce the plasma lipid profile of obese rats [12, 14]. Administration of CGA was reported to cause a decrease in the expression of caspase-3 and Bcl which are markers of proapoptosis. The decrease in the expression of caspase-3 and Bcl was found in rat testis cells [9, 12, 15]. Therefore, it is hoped that the administration of CGA in hyperglycemic rats can reduce the occurrence of ER stress, apoptosis and improve the level of testosterone production, which is characterized by increased enzymes that regulate testosterone steroidogenesis pathways.

2 Materials and Methods

2.1 Materials

24 male Wistar rats (Rattus norvegicus) were selected at 8 weeks of age, and 150—250 grams body weight. Rats were maintained according to the standard laboratory conditions with the provision of diet and water ad libitum and 12-hour light and dark cycle cages. This study had been approved by the Medical and Health Research Ethics of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, with the reference number KE/FK/1086/EC/2020. Rats were divided into 6 groups: control (non-DM); DM1 (1.5 months DM), DM2 (2 months DM), CGA1 (1.5 months DM+12.5 mg/kgBW CGA); CGA2 (1.5 months DM+25 mg/kgBW CGA); and CGA3 (1.5 months DM+50 mg/kgBW CGA).

2.2 Animal Models

The rats were injected with STZ at a dose of 60 mg/kgBW dissolved in 0.1M citric acid pH 4.5 intraperitoneally. Rats were grouped as DM group, if the results of the examination of blood glucose levels through the tail vein were >250 mg/dL.

2.3 Chlorogenic Acid

CGA (Sigma Aldrich, C3878) was given at a dose of 12.5 mg/kgBW, 25 mg/kgBW, 50 mg/kgBW and administered to rats that had diabetes for 1.5 months, for 14 consecutive days intraperitoneally (i.p.) according to their group.

2.4 Euthanizing and Testes Harvesting

Rats were anesthetized with an intraperitoneal injection of ketamine (100 mg/kgBW) by the AVMA Guidelines for Euthanasia for Animals: 2013 Edition. Testes were harvested by surgically lowering the abdomen. The testes were placed into RNA preservation and then stored at -20oC until the RNA isolation process, cDNA preparation, and polymerase chain reaction (PCR) examination were carried out.

2.5 Reverse Transcriptase PCR (RT-PCR)

RNA was extracted using Trizol solution, followed by the RNA concentration quantification using nanodrop, and random primers were used in synthesizing the cDNA and then conducting RT-PCR using Master Mix (Go Taq green) for assessing the expression of the following genes: (1) Glucose-related protein-78/GRP78 (forward 5'-GGAGGATGTGGGCACGGTGGTC-3', reverse: 5'-GTCATTCCA AGTGCGTCCGATGAG-3'); (2) X-box binding protein-1 spliced/XBP1s (forward: 5'-CTGAGTCCGCAGCAGG-3', reverse: 5'-CTTGTCCAGAATGCCCAAAAGG-3'); (3) 3β-hydroxysteroid dehydrogenase/3β-HSD (forward 5'-TGTGCCAGCCTTCATCTAC-3', reverse 5'-CTTCTCGGCCATCCTTTT-3'); (4) 17β-hydroxysteroid dehydrogenase/17β-HSD (forward 5'-GACCGCCGATGAGTTTGT-3', reverse 5'-TTTGGGTGGTGCTGCTGT-3'); (5) βactin (forward 5'-GCAGATGTGGATCAGCAAGC-3', reverse 5'-GGTGTAAAACGCAGCTCAGTAA-3'). The gene expressions were quantified using densitometry analysis (ImageJ software), and the βactin gene was used to normalize the gene expressions (house-keeping gene).

2.6 Statistical Analysis

Statistical analysis was done using SPSS 22 for Windows (IBM, Chicago). The distribution of the data was priorly determined using the Shapiro–Wilk test. Determining the mean difference for each variable used one way ANOVA test and Kruskal Wallis test with means and standard deviation (SD). For multiple comparisons, the data were then examined using the post hoc Tukey and Mann Whitney. The level of statistical significance was $p < 0.05$.

3 Results and Discussion

3.1 Blood glucose level

The blood glucose level in the DM groups was higher than the control group and significantly different ($p=0.021$). Following treatment with CGA for 2 weeks, blood glucose levels were significantly lower ($p<0.05$) in the CGA1 and CGA2 groups compared to the DM groups (Table 1).

Table 1. Baseline characteristics of all experimental groups

	Groups					
	C	**DM1**	**DM2**	**CGA1**	**CGA2**	**CGA3**
Blood glucose level (mg/dL)	84.25 ± 26.12	518.5 ± 107.43*	637.5 ± 40.52*	136.75 ± 4.57#+	238 ± 148.9	525 ± 13.93
Testis weight (g)	1.24 ± 0.1	0.98 ± 0.14*	0.89 ± 0.14*	1.3 ± 0.14#+	1.05 ± 0.14	1.38 ± 0.07 #+
Testis weight/ tibia length (g/cm)	0.35 ± 0,02	0.3 ± 0,05	0.25± 0.03*	0.35 ± 0.02+	0.25± 0.03	0.33 ± 0.04+

Values are mean ± SD, n=4, *p<0.05 vs C; #p<0.05 vs DM1; +p<0.05 vs DM2

3.2 Testis weight and testis weight/tibia length

Testis weight decreased significantly (p=0.021) in the DM groups compared to the control group. Following intervention with CGA, there was a significant increase in CGA1 and CGA3 groups compared to DM groups (p<0.05). However, testis weight did not significantly differ between the CGA2 group compared to DM groups (p>0.05). Testis weight/tibia length significantly decreased (p=0.004) in the DM2 group compared to the control group. After CGA, there was a significant increase in the CGA1 and CGA3 groups compared to the DM2 group (p<0.05) (Table 1).

3.3 mRNA transcript levels of GRP78 and XBP1s in the testes

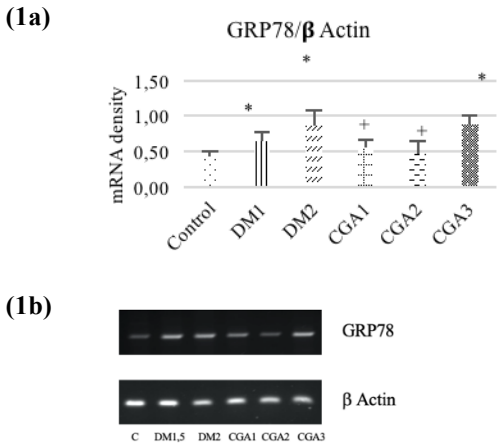


Fig 1. (a) Effect of CGA on mRNA transcript level of GRP78. Values are mean ± SD, n=4; **(b)** The amplified PCR product was examined using gel electrophoresis. *= p<0,05 vs C; += p<0,05 vs DM2

The mRNA transcript levels of GRP78 in the testes were significantly higher (p<0.05) in the DM groups, compared to the control group. Following intervention with CGA, GRP78 mRNA transcript levels were significantly lower (p<0.05) in the CGA1 and CGA2 groups, compared to the DM2 group. However, the mRNA transcript levels of GRP78 in CGA3 were significantly higher (p<0.05) than the control group (Fig 1a—b).

XBP1s transcript levels were not significantly higher (p>0.05) in the DM1 group, compared to the control group. In the DM2 group, XBP1s expressions were significantly higher (p<0.05) than the control group. After 2 weeks of CGA intervention, the mRNA transcript level of XBP1s was significantly lower (p<0.05) in CGA1 group, compared to the DM2 group. However, CGA2 and CGA3 were not significantly lower (p>0.05) than DM groups (Fig. 2a—b).

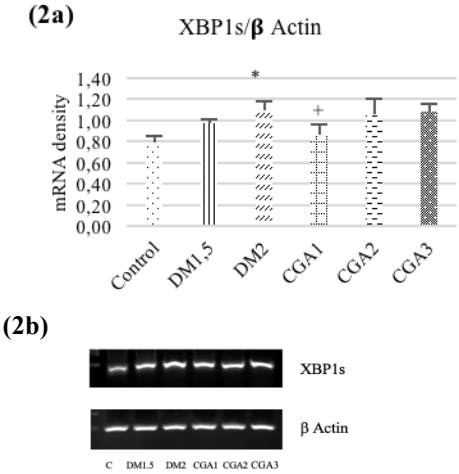


Fig 2. (a) Effect of CGA on mRNA transcript level of XBP1s. Values are mean ± SD, *n*=4; **(b)** The amplified PCR product was examined using gel electrophoresis. *= p<0,05 vs C; +=p<0,05 vs DM2

3.4 mRNA transcript levels of 3β-HSD and 17β-HSD in the testis

The mRNA transcript level of 3β-HSD did not significantly differ between DM and control groups,

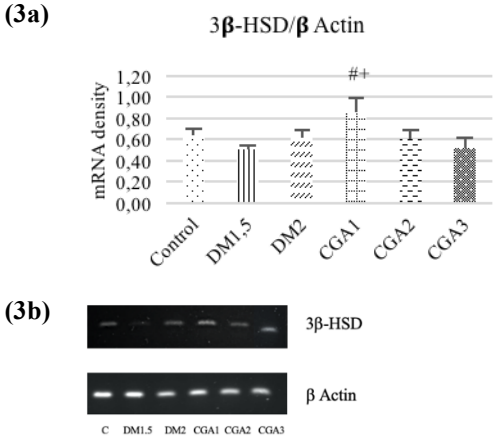
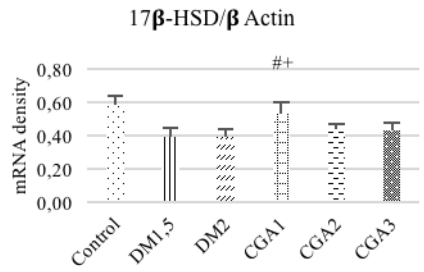


Fig 3. (a) Effect of CGA on mRNA transcript level of 3β-HSD. Values are mean ± SD, n=4; **(b)** The amplified PCR product was examined using gel electrophoresis. #= p<0.05 vs DM1; += p<0.05 vs DM2

although it was lower in the DM groups. Intervention with CGA1 significantly increased ($p<0.05$) the transcript level of 3β-HSD relative to DM groups. However, there was no significant difference ($p>0,05$) for transcript level 3β-HSD between CGA2 and DM groups, nor the CGA3 and DM groups (Fig 3a—b).

(4a)



(4b)

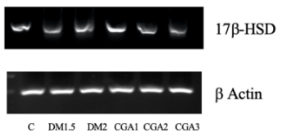


Fig 4. (a) Effect of CGA on mRNA transcript level of 17β-HSD. Values are mean ± SD, n=4; **(b)** The amplified PCR product was examined using gel electrophoresis. *= $p<0.05$ vs C; #=$p<0.05$ vs DM1; += $p<0.05$ vs DM2

17β-HSD transcript level in the testis was significantly lower ($p<0.05$) in the DM groups, compared to the control group. CGA1 treatment significantly increased ($p<0.05$) the mRNA transcript levels of 17β-HSD compared to DM groups. In the CGA2 and CGA3 groups, 17β-HSD mRNA transcript levels were not significantly different compared to the DM groups (Fig 4a—b).

STZ is a nitrosourea glucosamine compound that will bind to glucose transporter 2 (GLUT2) because it has a structure similar to glucose. This causes degeneration of the pancreatic islets of Langerhans, leading to a hyperglycemic state. Based on the examination of blood glucose in the study, blood glucose levels in the DM groups were higher than in the control group and the CGA treatment groups. A hyperglycemic condition has been shown to play a role in the emergence of oxidative stress. Oxidative stress can affect male infertility, such as vasculopathy, endothelial dysfunction, neuropathy in erectile tissue, and a decrease in the weight of the reproductive organs or testes [16].

Oxidative stress also affects apoptosis through the ER stress pathway which is characterized by the release of the GRP78 molecule through the activation of PERK and XBP1s with the activation of the IRE1-XBP1 pathway [1]. The results of this study showed that there was an increase in the expression of GRP78 and XBP1s mRNA in the DM groups. This occurs because the condition of testicular dysfunction due to hyperglycemia, hyperthermia, and varicocele in the testes will cause an increase in ROS and oxidative stress that can disrupt ER homeostasis.

In the DM group, there was a decrease in the mRNA expression of the 3β-HSD and 17β-HSD. We suspect that the decrease in the enzyme that catalyzes testosterone synthesis is due to apoptosis of testicular cells due to activation of apoptotic signaling pathways, and cell apoptosis mediated by mitochondria and ER stress. The results of this study are following previous studies conducted by Liu et al., (2017) which showed a decrease in the expression of 3β-HSD and 17β-HSDmRNA in the DM group [17].

The administration of CGA can cause an increase in testicular weight, inhibit ER stress conditions, and improve testosterone steroidogenesis in terms of decreasing blood glucose and transcriptomics. In the CGA1 group with a dose of 12.5 mg/kgBW, the results of testicular weight and testicular weight per tibia length were higher than the other treatment groups. This is supported by lower glucose levels. The expression of GRP78 and XBP1s mRNA in the CGA1 group also showed lower yields because CGA has the ability as an antioxidant so it can reduce the expression of these two RE stress markers. The expression of 3β-HSD and 17β-HSDmRNA was also found to be higher in the CGA1 group which acts as a marker of genes involved in steroidogenesis so that testosterone steroidogenesis continues to run well.

The CGA 3 group with a dose of 50 mg/kgBW showed higher blood glucose levels than the other CGA treatment groups. High glucose levels slightly decrease testicular weight and testicular weight per tibial length. In the GRP78 and XBP1s mRNA expression in the CGA3 group, there was an increase in the expression of the ER stress marker which was higher than in the CGA1 and CGA2 groups. In addition, the group also found lower expression of 3β-HSD and 17β-HSDmRNA. Bagdas et al., (2015) stated that the administration of polyphenolic compounds including CGA in high doses and for a long period can induce genotoxicity and increase ROS because these compounds have pro-oxidant activity under certain conditions [18].

4 Conclusion

Here, we demonstrated the administration of CGA at a dose of 12.5 mg/kgBW could reduce ER stress conditions and increase steroidogenesis in the testes of diabetic rats. These results were seen based on low GRP78 and XBP1s mRNA expressions, and high 3β-HSD and 17β-HSD expressions after CGA administration.

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