Chlorogenic acid ameliorates muscle wasting by upregulating mRNA expressions of calcineurin and PGC-1α in diabetic rat model

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ABSTRACT

Introduction: Muscle health in diabetes mellitus (DM) is often neglected, which leads to muscle wasting. Increased reactive oxygen species in DM could decrease antioxidant enzymes such as superoxide dismutase-1 (SOD-1) and -2 (SOD-2) and inhibit calcineurin (CN) and PGC-1α signalling pathways. Chlorogenic acid (CGA) is known as a potent antioxidant and activators of CN and PGC-1α. This study aimed to determine the effect of CGA on mRNA expressions of SOD-1, SOD-2, CN and PGC-1α in inhibiting the progression of DM to muscle wasting.

Materials and Methods: This study was conducted at Department of Anatomy, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada starting on July 20th, 2020. A total of 24 male Wistar rats were randomly divided into six groups (four rats per group), i.e., control, DM 1.5 months (DM1.5), and DM 2 months (DM2); and DM groups treated with CGA in three different doses, namely CGA1 (12.5 mg/kg BW), CGA2 (25 mg/kg BW), and CGA3 (50 mg/kg BW). Control group was only injected with normal saline, while diabetic model was induced by intraperitoneal injection of streptozotocin. Blood glucose levels were measured twice (one week after diabetic induction and before termination). The soleus muscle tissue was harvested to analyse the mRNA expressions of SOD-1, SOD-2, CN and PGC-1α using RT-PCR. In addition, the tissue samples were stained with immunohistochemistry for CN and haematoxylin-eosin (HE) for morphologic analysis under light microscopy.

Results: The mRNA expressions of SOD-1 and SOD-2 in the CGA1 group were relatively higher compared to the DM2 groups. The mRNA expression of CN in the CGA1 group was significantly higher compared to the DM2 group (p = 0.008). The mRNA expression of PGC-1α in the CGA1 group was significantly higher compared to the DM2 group (p = 0.025). Immunohistochemical staining showed that CN-immunopositive expression in the CGA1 group was more evident compared to the other groups. Haematoxylin-eosin staining showed that muscle tissue morphology in the CGA1 group was similar to that in the control group.

Conclusion: Chlorogenic acid at a dose of 12.5 mg/kg BW shows lower blood glucose level, good skeletal muscle tissue morphology and higher mRNA expressions of SOD-1, SOD-2, CN and PGC-1α compared to the DM groups.

KEYWORDS:
Muscle wasting, calcineurin, PGC-1α, SOD-1, SOD-2

INTRODUCTION

Diabetes mellitus (DM) poses a significant global public health concern, impacting an estimated 422 million adults worldwide in 2014.[1] It contributed to 1.5 million deaths globally in 2012.[2] While in Indonesia, DM ranks second as the leading cause of death, accounting for 6% of total deaths in the population.[3] DM is characterised by high oxidative stress resulting from chronic hyperglycaemia, which can affect protein metabolism.[4] An increase in antioxidant enzymes, such as superoxide dismutase-1 (SOD-1) and superoxide dismutase-2 (SOD-2), will reduce reactive oxygen species (ROS), preventing the activation of signalling pathways that could induce protein degradation.[5]

DM, associated with abnormal muscle protein metabolism, can result in decreased muscle mass and, in some cases, affect activities of daily living, leading to decreased productivity and quality of life.[6] Muscle proteins are continuously synthesised and degraded every day, typically balancing to maintain muscle mass. However, in DM, factors such as decreased synthesis or increased breakdown can result in an imbalance leading to muscle wasting.[7] Muscle wasting is a condition characterised by the loss of muscle mass, which occurs due to an imbalance between protein synthesis and degradation.[8] There are several triggers for muscle wasting, including decreased calcineurin (CN) activity, especially in cases of chronic DM. Calcineurin is involved in several adaptive responses that induce growth and regeneration of muscle fibres.[9] Decreased CN activity, as a result of suppression by conditions that trigger atrophy, leads to the reduction of various mediators that serve as key components of protein degradation in muscle.[10] Peroxisome proliferator-activated receptor gamma coactivator-1 alpha

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(PGC-1α) is associated with the regulation of skeletal muscle protein turnover.[11] Decreased CN activity will downregulate PGC-1α, resulting in decreased muscle functional capacity and muscle mass, which often accompanies DM.[12]

Chlorogenic acid (CGA) is a polyphenol compound in coffee with the highest antioxidant content.[13] Chlorogenic acid can stimulate glucose transport in skeletal muscle and offers numerous benefits in the management of DM and its complications.[14] Changes in protein metabolism due to oxidative injury and their impact on muscle mass represents one of the most challenging and poorly understood aspects of DM management. The results of this study are expected to serve as a reference for understanding the progression of DM and the potential use of CGA as an innovative therapy to maintain optimal physical capacity and functional abilities in DM patients. Therefore, it is necessary to conduct further studies to investigate the effect of CGA on muscle wasting in diabetic rats, focusing on the mRNA expressions of SOD-1, SOD-2, CN and PGC-1α.

MATERIALS AND METHODS

Design

This study was a quasi-experimental study with a post-test-only controlled group design. This study was conducted at the Department of Anatomy, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (FM-PHN UGM) starting on July 20th, 2020. This study has received approval from the Medical and Health Research Ethics Committee FM-PHN UGM/RSUP Dr. Sardjito, with the reference number KE/FK/1086/EC/2020.

Animal Model of Diabetes Mellitus

A total of 24 male Wistar rats aged 2 months old with a body weight (BW) ranging from 150 to 200 grams were obtained from the Muhammadiyah University of Yogyakarta. The animals were housed in cages (2 rats per cage) with light-dark cycle of 12 hours, controlled temperature (21 ± 2°C) and humidity (50 ± 5%), and given free access to food and water. Healthy rats were included in this study, while sick rats, characterised by reduced activity, decreased body weight and hair loss, were excluded from the study. The rats (n = 24) were divided into six groups: control, DM1.5 (DM for 1.5 months), DM2 (DM for 2 months), and three treatment groups with different dosages of CGA. The control group was injected with normal saline. Diabetic induction model was prepared by intraperitoneal injection of streptozotocin (STZ) at a dose of 60 mg/kg, dissolved in 0.1 M citric acid with a pH of 4.5. Blood samples of fasting rats were withdrawn from the tail vein at one week after diabetic induction. Diabetes condition was defined as blood glucose levels greater or equal to 250 mg/dL using a portable glucometer. Blood glucose levels were also measured before termination (Figure 1).

Chlorogenic Acid Administration

CGA was dissolved using PBS and administered via intraperitoneal injection at a total volume of 1 mL/kg BW. Three doses of CGA were used: 12.5 mg/kg BW (CGA1), 25 mg/kg BW (CGA2) and 50 mg/kg BW (CGA3). The CGA groups received treatment for 14 consecutive days (Figure 1).

Termination and Sample Harvesting

Termination was carried out on day 60 in accordance with the AVMA guidelines for the euthanasia of animals (2020 edition). Experimental animals were euthanised using ketamine at a dose of 100 mg/kg BW, administered intraperitoneally. The soleus muscle tissue was then quickly harvested and stored in a 1.5 mL tube filled with RNA preservation solution at -20°C.

Reverse Transcription PCR

Soleus muscle tissue was used for RNA extraction and the extracted RNA was then employed for cDNA synthesis. The synthesised cDNA was used in reverse transcription PCR (RT-PCR) reactions and then followed by electrophoresis. The mRNA expression levels for SOD-1 (F: GCGGTGAACCAGTTGTGGTG; R: AGCCACATTGCCCAGGTCTC), SOD-2 (F: ATGTTGTGTCGGGCGGCGTGCAGC; R: GCGCCTCGTGGTACTTCTCCTCGGTG), CN (F: AGTAACTTTCGAGCCAGCCC; R: CAACGCGACACTTTCTTCCAG), and PGC-1α (F: TCAGCGGTCTTAGCACTCA; R: TCTCTGTGGGTTTGGTGTGA) were determined through densitometric analysis using ImageJ software (NIH). The β-actin was used as a housekeeping gene (F: GCAGATGTGGATCAGCAAGC; R: GGTGTAAAACGCAGCTCAGTAA).

Immunohistochemical Staining

A formalin-fixed paraffin-embedded tissue sample was simultaneously stained using immunohistochemical (IHC) technique for CN. The sections were incubated with anti-CN antibody (rabbit polyclonal, Abcam; 1:300 dilution) and the Mouse/Rabbit Probe HRP Labelling Kit with DAB Brown (BioTnA) as the chromogen. Observation of the IHC-stained sections was carried out under a light microscope at a magnification of 400x, covering the entire field of view of the muscle tissue. The assessment was based on the observation of brown coloration in the longitudinal section of the soleus muscle tissue.

Haematoxylin-Eosin Staining

A formalin-fixed paraffin-embedded tissue sample was simultaneously stained using haematoxylin-eosin (HE) for each group. HE staining was carried out to visualise the muscle tissue morphology and the HE-stained sections were then observed under a light microscope at a magnification of 100x.

Data Analysis

The data were assessed using the Shapiro-Wilk test to determine the data distribution. One-way ANOVA test was employed and followed by post-hoc least significant difference (LSD) test for normally distributed data. While Kruskal-Wallis test was carried out and followed by post-hoc Mann-Whitney test for non-normally distributed data. A significance level of $p < 0.05$ was used as the criteria for statistical significance in all analyses.

RESULTS

Blood Glucose Level

The measurements of blood glucose level were conducted twice in each group: in the first week following diabetic induction and before termination (Figure 2). The results showed that the blood glucose level in the first week following diabetic induction in the control group (101 ± 11.9 mg/dL) was significantly lower compared to both the DM and treatment groups, namely, DM1.5 (342. 5 ± 53 mg/dL; p = 0.021), DM2 (266 ± 41.1 mg/dL; p = 0.021), CGA1 (389.5 ± 213.2 mg/dL; p = 0.021), CGA2 (459.25 ± 156.3 mg/dL; p = 0.021), and CGA3 (507.5 ± 72.7 mg/dL; p = 0.020). Furthermore, the DM2 group exhibited a significant higher blood glucose level before termination (594.5 ± 115.4 mg/dL) compared to the CGA1 (136.75 ± 4.57 mg/dL; p = 0.021) and CGA2 groups (238 ± 148.9 mg/dL; p = 0.043). Moreover, the blood glucose levels in the CGA1 and CGA2 groups were significantly lower compared to the CGA3 group. These results indicated that blood glucose levels were higher in the DM rats and CGA administration had a noticeable effect in reducing blood glucose levels.

SOD-1 and SOD-2 mRNA Expressions

The SOD-1 densitometric analysis data were initially assessed using the Shapiro-Wilk test for normality, which indicated that all groups had a normal distribution data ($p \geq 0.05$). Subsequently, a one-way ANOVA test was carried out followed by post-hoc LSD analysis, suggesting no significant difference between all groups (p = 0.450). However, mRNA expression of SOD-1 in CGA1 group was higher compared to the DM2 group. These findings indicated that CGA might have an effect on increasing mRNA expression of SOD-1 (Figure 3).

The mRNA expression of SOD-2 in the CGA1 group were significantly higher compared to the CGA3 group (p = 0.039). Moreover, the mRNA expression of SOD-2 in CGA1 group was relatively higher compared to the DM2 group. These results suggested that mRNA expression of SOD-2 was decreased in the DM groups and CGA appeared to have an effect on increasing mRNA expression of SOD-2 (Figure 3).

Calcineurin and PGC-1α mRNA Expressions

The statistical test results showed that mRNA expressions of CN in the DM2 (p = 0.016) and CGA2 (p = 0.029) groups were significantly lower compared to the control group. On the contrary, mRNA expression of CN in the CGA1 group was significantly higher compared to the DM2 group (p = 0.008). These results indicated that mRNA expression of CN was decreased in the DM rats and CGA appeared to have an effect on increasing mRNA expression of CN (Figure 3).

The statistical test results showed that mRNA expressions of PGC-1α in the DM1.5 (p = 0.028) and DM2 (p = 0.002) groups were significantly lower compared to the control group. In contrast, mRNA expression of PGC-1α in the CGA1 group was significantly higher compared to the DM2 group (p = 0.025). These results indicated that mRNA expression of PGC-1α was decreased in DM rats and CGA appeared to have an effect on increasing mRNA expression of PGC-1α (Figure 3).

Calcineurin Expression in Soleus Muscle Tissue

Immunohistochemical staining (Figure 4) of soleus muscle tissue sections revealed that CN-immunopositive expression in the DM groups were less abundant compared to the control group. In contrast, CN-immunopositive expression in the control and CGA1 groups were more abundant compared to the other groups.

Histopathological Examination of Skeletal Muscle Tissue

The histopathological examination of muscle tissue in the control group depicted a typical skeletal muscle morphology under normal condition (Figure 5). In the histopathological images of HE-stained longitudinal sections, differences in muscle structure were observed in the DM groups compared to the control group. Specifically, the muscle structure in the DM groups exhibited irregular myofibers with stacked and irregular myonuclei compared to the control group. In contrast, the CGA1 group displayed muscle structure similar to that of the control group, in which myofibers and myonuclei appeared more regular compared to the DM groups. Furthermore, the CGA1 group exhibited improved muscle morphology compared to both CGA2 and CGA3 groups.

DISCUSSION

In this study, the CGA administration is observed to enhance the skeletal muscle performance and inhibit the progression of muscle wasting in terms of reduced blood glucose level, transcriptomic signalling pathways, immunohistochemical parameters and histopathological muscle structure. Notably, in the CGA1 group with a dose of 12.5 mg/kg BW, improvements in skeletal muscle structure are more evident in its histopathological features, along with reduced blood glucose level compared to other treatment groups. Furthermore, the CGA1 group exhibits relatively higher mRNA expression of SOD-1 and SOD-2, serving as markers of antioxidant enzymes. These increases in mRNA expressions of antioxidant enzymes are in line with the potential to reduce oxidative stress. Additionally, CN and PGC-1α mRNA expressions are also higher in the CGA1 group, suggesting their roles in promoting protein synthesis and mitochondrial biogenesis to prevent muscle wasting.

Skeletal muscle consists of multinucleated myofibers (or myotubes) and satellite cells, all enclosed within the sarcolemma. A single myofiber contains the nucleus, myofilaments, sarcoplasmic reticulum and mitochondria, which performs to supply energy for movement.[11] In this study, the histopathological examination of HE-stained skeletal muscle tissue shows changes in the morphology of skeletal muscle cells in the DM groups, which are different from the morphology of skeletal muscle cells in the control group. These morphological changes are characterised by damage to the boundaries between myofibers and irregular myonuclei in the DM groups. Our result is supported by previous research that shows that skeletal muscles in DM undergo changes in its structural features, including myofibril damage, absence of Z-lines, irregular mitochondria, increased fat content, folded sarcolemma, irregular myonuclei and hyperchromatism.[12]

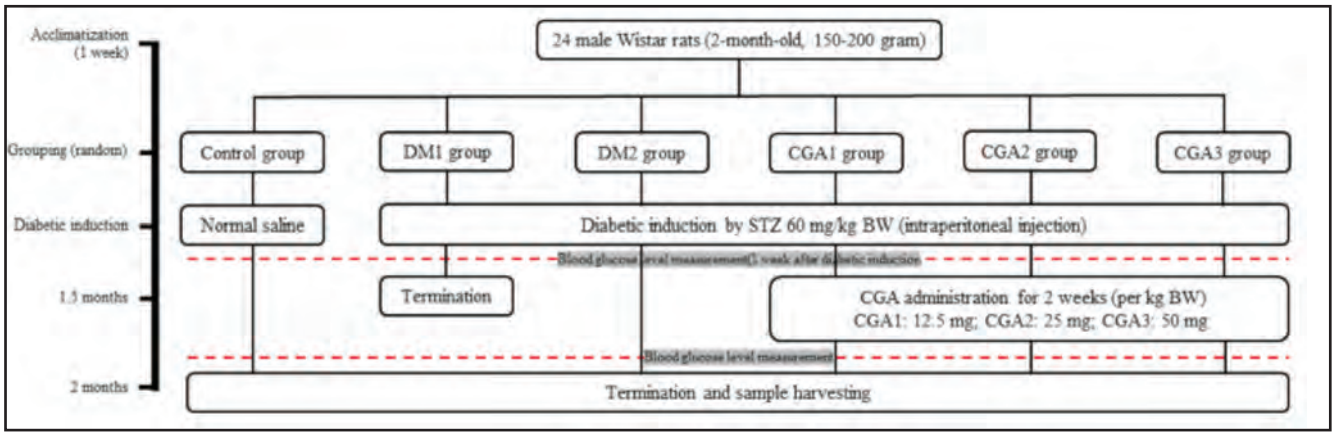

Fig. 1: Schematic diagram of research design

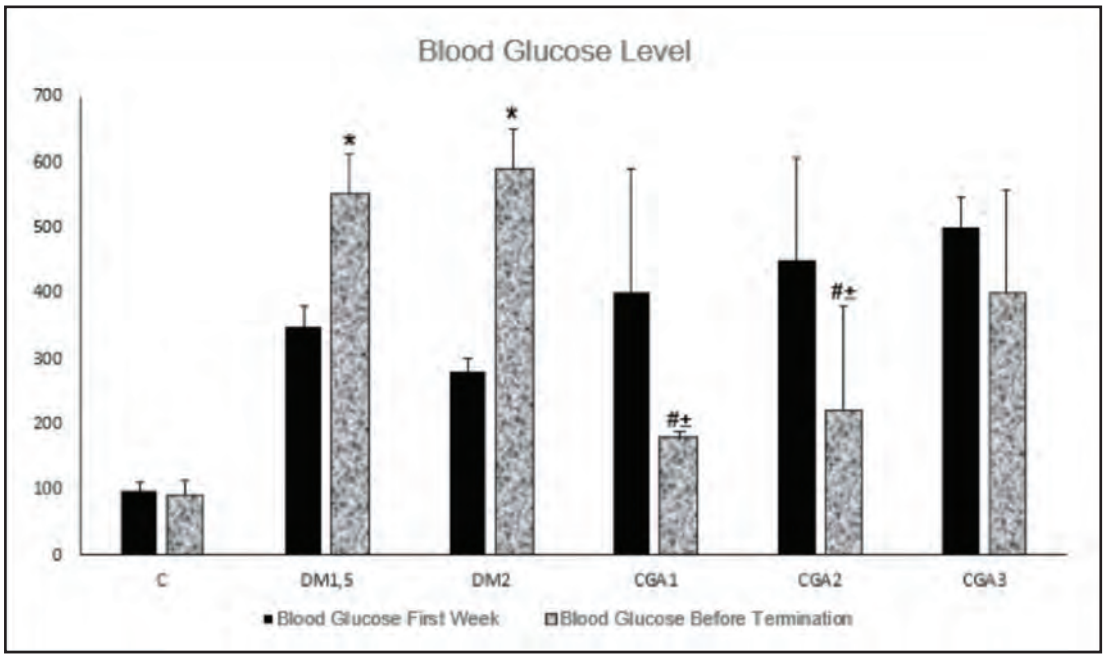

Fig. 2: Blood glucose level in the first week following diabetic induction and before termination (mean ± SD mg/dL) in the control, DM and CGA groups. *: significantly different from control group; #: significantly different from DM2 group; ±: significantly different from CGA3 group

Increased systemic ROS production in cases of DM can increase oxidative stress and lead to changes in peripheral tissues such as skeletal muscle, along with an increase level of proinflammatory transcription factors such as nuclear factor kappa B (NF-κB). The NF-κB regulates specific UPS genes that can trigger protein degradation and results in muscle wasting.[4] In the CGA1 group, the histological morphology is similar to that of the control group. The morphological changes observed in the CGA1 group are attributed to the administration of CGA, which can increase the expression of antioxidant enzymes. These enzymes play roles to stabilise redox conditions and reduce ROS levels, thereby preventing pro-inflammatory reactions and the potential damage to muscle structure and protein degradation. Our study result is supported by previous study indicating that CGA, as an antioxidant, could reduce free radicals, enhance antioxidant enzymes and inhibit oxidation reactions.[13] An increase in antioxidant enzymes such as SOD-1 and SOD-2 can effectively reduce ROS levels, preventing the activation of the NF-κB signalling pathway, which, in turn, can prevent the induction of MurF-1 expression and muscle wasting.[4]

DM induces various functional, metabolic and structural changes in the skeletal muscle.[15] In this study, DM is induced by a single intraperitoneal injection of 60 mg/kg BW of STZ. Streptozotocin, a glucosamine-nitrosourea compound, is a genotoxic methylating agent that can selectively destroy insulin-producing pancreatic β-cells through the formation of ROS and DNA alkylation.[16] Oxidative stress is known to occur

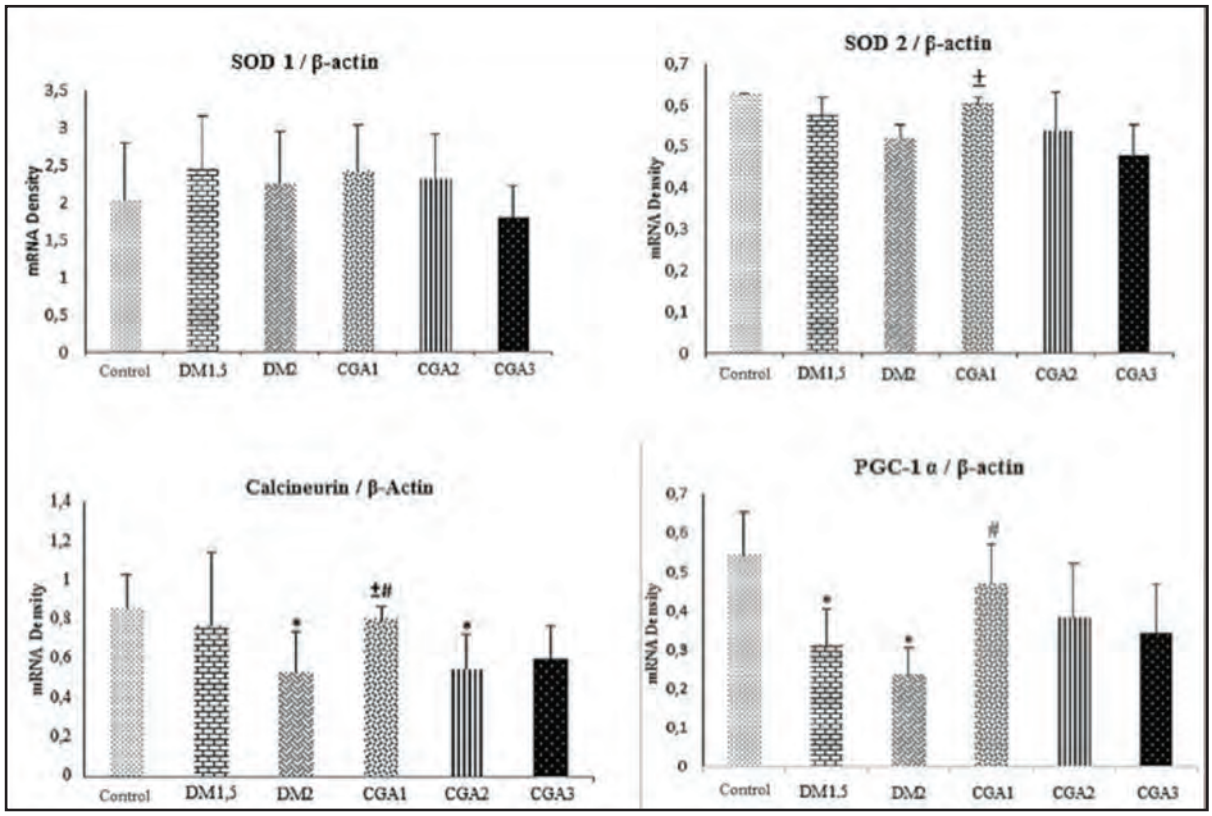

Fig. 3: The mRNA expressions of SOD-1, SOD-2, CN, and PGC-1α in the control, DM, and CGA groups. *: significantly different from control group; #: significantly different from DM2 group; ±: significantly different from CGA3 group

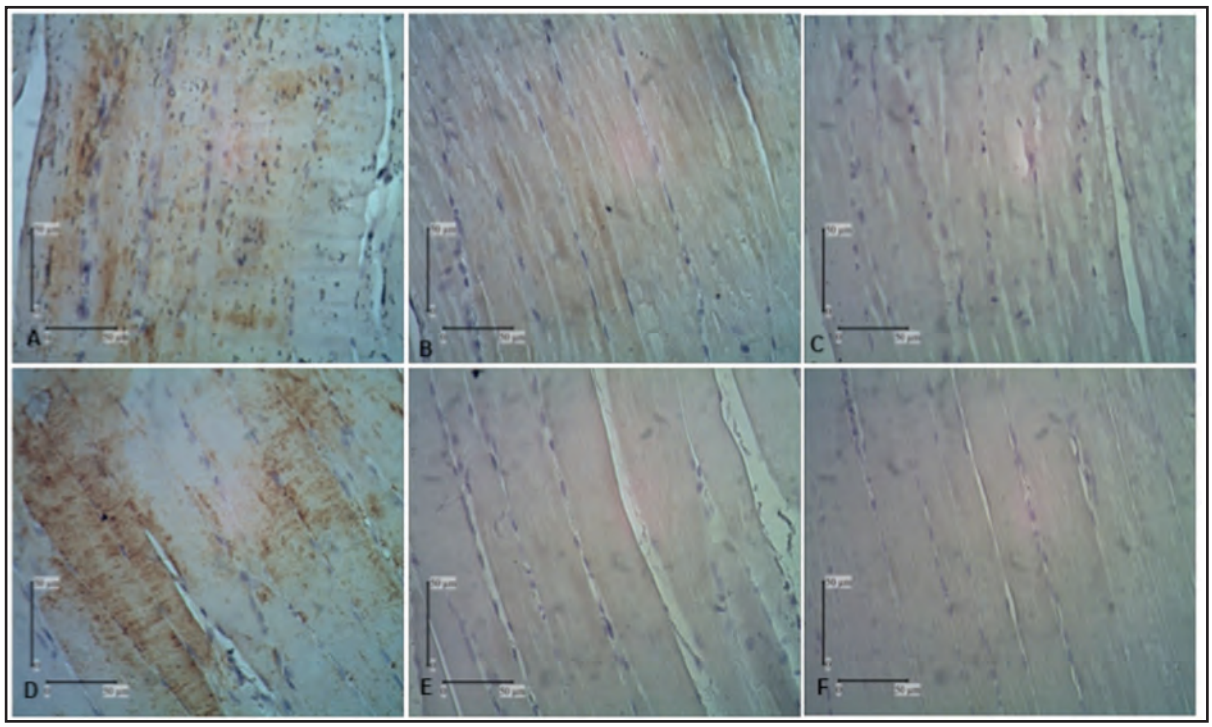

Fig. 4: Calcineurin expression in soleus muscle tissue in the control (A), DM1.5 (B), DM2 (C), CGA1 (D), CGA2 (E), CGA3 (F) groups. Longitudinal sections. Calcineurin-immunohistochemistry showed that immunopositive expression in the CGA1 group was more evident compared to the other groups. Scale bars = 50 μm

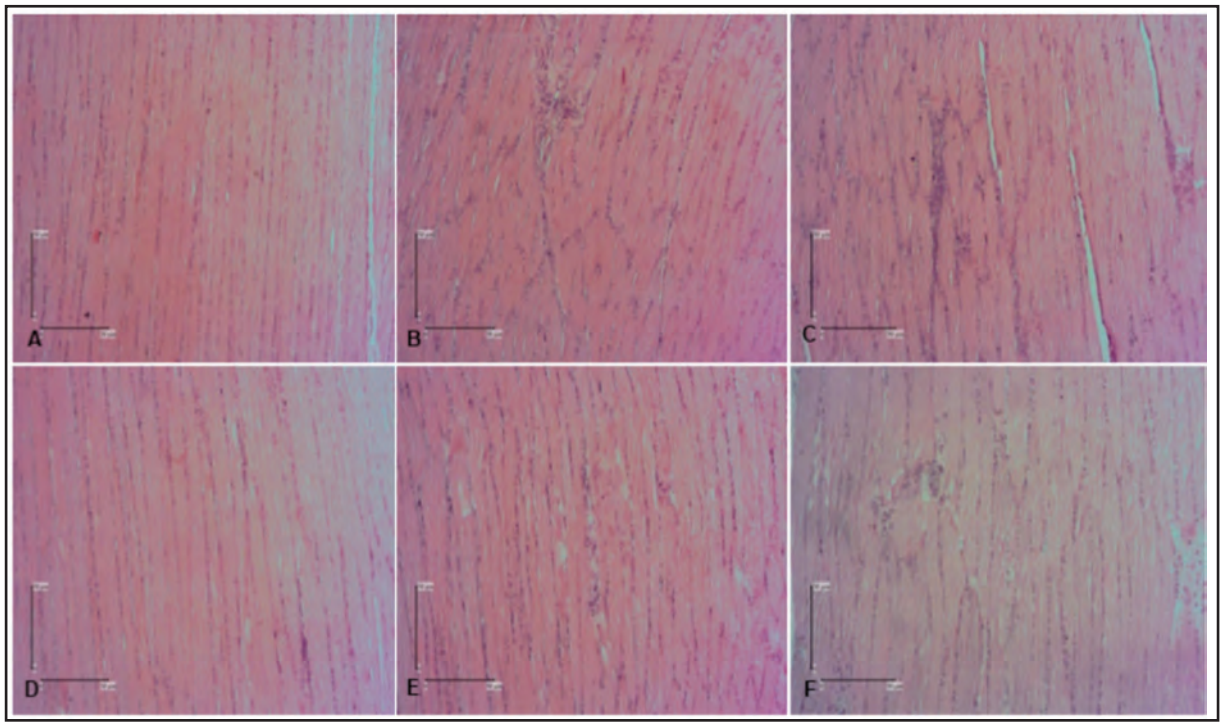

Fig. 5: HE-stained longitudinal sections through soleus muscle tissue in the control (A), DM1.5 (B), DM2 (C), CGA1 (D), CGA2 (E), CGA3 (F) groups. Muscle tissue morphology in the CGA1 group was similar to that in the control group. Scale bars = 50 μm

in both type 1 and type 2 DM and has been directly linked to elevated glucose concentrations.[2] According to the results of blood glucose analysis, it is observed that blood glucose levels in the DM2 group are higher compared to both control and CGA groups. This result is consistent with the previous study suggesting that CGA plays a role in reducing blood glucose levels, thus making it applicable for the prevention and treatment of DM.[17] Chlorogenic acid inhibits glucose uptake from the intestine by suppressing α-glucosidase activity and reducing glucose transport synergistically, ultimately resulting in a decrease in blood glucose levels.1[8]

The activity of the antioxidant defence system in response to increased levels of free radicals due to hyperglycaemia is crucial in DM. Changes in oxidative damage, whether a decrease or an increase, have a significant impact on the process of protein metabolism.[5] Antioxidant enzymes are essential for maintaining a healthy redox state, and one such enzyme that plays a critical role is SOD.[4] In our study, the mRNA expression of SOD-1 is found to be higher in the DM1 group compared to the control group. Presumably, at the onset of DM, SOD-1 increases as an adaptation response to the elevated levels of ROS. However, the mRNA expression of SOD-1 in the DM2 group tends to decrease compared to the control group. This result aligns with previous study that indicates one of the side effects of hyperglycaemia in DM is an increase in ROS production, which in turn heightens susceptibility to oxidative stress.[19]

Antioxidant enzymes such as SOD-1 and SOD-2 are essential for maintaining a healthy redox state.[20] While in the CGA1 group, there is an increase in the mRNA expression of SOD-1 compared to the DM2 group. This result is consistent with previous study showing that CGA offers health benefits by donating hydrogen atoms to reduce free radicals, inhibiting oxidation reactions, and increasing antioxidant enzymes.[21] Additionally, our study shows that mRNA expressions of SOD-2 in the DM groups are also lower compared to the control group, although not statistically significant. These results are in line with previous studies indicating that the increased ROS levels resulting from hyperglycaemia in DM leads to a decrease in antioxidant enzymes, subsequently affecting various signalling pathways.[19] In the CGA groups, there is an increase in the mRNA expression of SOD-2 compared to the DM groups. The CGA1 group exhibits better result with a significant higher level of SOD-2 mRNA expression compared to the CGA3 group. The increase in the mRNA expression of SOD-2 in the CGA groups aligns with previous studies that emphasise the antioxidant benefits of CGA, highlighting its capacity to elevate mRNA expression of SOD-2 in order to maintain a healthy redox balance and reduce ROS.[20]

DM cannot be solely characterised as a disorder of glucose dysregulation, instead, it should be recognised as a chronic inflammation that affects nearly every biological process, including protein metabolism.[13] Changes in protein metabolism and their impact on muscle mass and function are among the least comprehended aspects in the management of DM.[6] Calcineurin is a protein phosphatase 3 and a calcium-dependent serine-threonine phosphatase involved in several adaptive responses that promote muscle fibre growth and regeneration.[22] Our study shows that mRNA expression of CN in the DM2 group is significantly lower compared to the control group. This result is in accordance with previous study indicating that CN levels decrease in DM

condition, marking the initiation of disruptions in various other signalling pathways because CN plays a crucial role in the protein turnover process.[8] Decreased CN activity, resulting from suppression under atrophy-inducing conditions, leads to a reduction in various mediators that are pivotal components of protein degradation in muscle. This, in turn, may contribute to a decrease in muscle functional capacity and muscle mass, which are common companions to DM.[9] In the CGA groups, higher mRNA expression of CN is observed compared to the DM groups. Moreover, mRNA expression of CN in the CGA1 group is significantly higher compared to the DM2 and CGA2 groups. This result aligns with previous study indicating that CGA has the potential to activate and increase CN activity.[20]

In our study, immunohistochemical staining is also performed on muscle tissue sections to visualise CN expression in skeletal muscle tissue. Immunohistochemical examination shows that CN expressions in the DM groups are lower than in the control group. Specifically, CN expression in the DM1 group is lower than in the control group, while in the DM2 group it is lower than in the DM1 group, making it challenging to detect CN gene expression in the DM2 group. These results are in accordance with previous studies demonstrating that CN levels decrease in the context of DM.[8] In the CGA groups, the CGA1 group shows higher CN expression in comparison to both control group and DM groups. Furthermore, the CN expression in the CGA1 group is also higher compared to the CGA2 and CGA3 groups. This pattern of CN immunopositive expression aligns with the results related to the mRNA expression of CN. These results are supported by previous study, which confirms that CGA has the capability to activate and increase CN activity.[23]

This study not only analyses the mRNA expression of CN but also assesses the mRNA expression of PGC-1α in skeletal muscle, which is considered to be closely associated with CN. The result shows that mRNA expression of PGC-1α in the DM groups are significantly lower than in the control group. These findings align with previous study that elucidates the regulation of functional capacity and muscle mass by contractile proteins and calcium signalling activity. One of the pathways known to regulate PGC-1α involves the calcium-dependent phosphatase calcineurin.[24] In the case of DM, the decrease in CN signalling that occurs as a result of reduced PGC-1α regulation, contributing to muscle wasting.[25] In the CGA groups, the CGA1 group exhibits significantly higher mRNA expression of PGC-1α compared to the DM2 group. This result indicates that CGA has the potential to enhance the expression of PGC-1α in skeletal muscle. Our study is consistent with the previous study indicating that CGA can activate and increase CN activity.[25] Increased CN activity is known to promote higher PGC-1α expression in cases of DM.[25]

PGC-1α is a transcriptional coactivator that plays a vital role in regulating skeletal muscle metabolism, particularly energy homeostasis. It achieves this role by controlling glucose transport and is necessary for mitochondrial biogenesis, along with maintaining the oxidative phenotype of muscle fibres.[25] Among the most significant pathways for regulating muscle metabolism is mitochondrial biogenesis. In DM, the process of mitochondrial biogenesis becomes disorganised, leading to a decreased ability of cells to respond to fluctuations in nutrients and energy. This, combined with reduced mitochondrial content and alterations in mitochondrial morphology, is directly linked to the pathogenesis of the disease.[12]

Mitochondria serve various important functions, with their most prominent roles as the primary regulator of cellular metabolic activity by converting energy from macronutrient oxidation into ATP.[24] Mitochondrial activity and function in skeletal muscle are tightly regulated and the process of mitochondrial biogenesis is crucial for preserving mitochondrial integrity.[26] Elevated level of PGC-1α in muscle is linked to enhanced mitochondrial function, increased insulin sensitivity, and the capacity of PGC-1α to stimulate mitochondrial proliferation. This underlines the significance of PGC-1α in the process of mitochondrial biogenesis.[27] Most mitochondrial proteins are synthesised in the nucleus and subsequently targeted to the mitochondria. PGC-1α acts as a direct mediator of transcriptional coactivators involved in cellular energy metabolism and plays an important role in the physiological regulation of protein expression. The protein generated in this process will later be utilized for muscle regeneration, ultimately contributing to increased muscle mass.[20]

CONCLUSSION

The administration of chlorogenic acid (CGA) at a dose of 12.5 mg/kg body weight shows reduced signs of muscle wasting in diabetes mellitus (DM) rats by lowering blood glucose levels, increasing mRNA expressions of superoxide dismutase-1 (SOD-1) and -2 (SOD-2) , calcineurin (CN), and PGC-1α, and improving skeletal muscle structure which similar to that of the control group.

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