International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 17, Special Issue 2, 2025

Original Article

COFFEE LOWERS BLOOD GLUCOSE LEVELS BUT FAILS TO IMPROVE SKELETAL MUSCLE MASS IN ALLOXAN-INDUCED RATS

RIANDINI AISYAH[1,2,5*], SAFARI WAHYU JATMIKO[1,3,5], NUR MAHMUDAH[1,4,5]

[1]Department of Biomedical Science, Faculty of Medicine, Universitas Muhammadiyah Surakarta, Surakarta-57169, Central Java, Indonesia. [2]Molecular Biology Laboratory, Faculty of Medicine, Universitas Muhammadiyah Surakarta, Surakarta-57169, Central Java, Indonesia. [3]Clinical Pathology Laboratory, Faculty of Medicine, Universitas Muhammadiyah Surakarta, Surakarta-57169, Central Java, Indonesia. [4]Cell Histology Laboratory, Faculty of Medicine, Universitas Muhammadiyah Surakarta, Surakarta-57169, Central Java, Indonesia. [5]Family Medicine Research Center, Faculty of Medicine, Universitas Muhammadiyah Surakarta, Surakarta-57169, Central Java, Indonesia

[*]Corresponding author: Riandini Aisyah; [*]Email: riandini.aisyah@ums.ac.id

Received: 14 Mar 2025, Revised and Accepted: 18 May 2025

ABSTRACT

Objective: This study aimed to evaluate the potency of coffee as a non-pharmacological alternative treatment for diabetes melitus by measuring blood glucose levels, skeletal muscle diameter, and the number of skeletal muscle cell nuclei.

Methods: This study used 24 *Rattus norvegicus* weighing 150-250g and divided them into 6 groups: non-diabetic control, diabetic control, insulin, Lampung Robusta coffee at doses of 0.054g/200g b. w, 0.108 g/200g b. w, 0.162g/200g b. w, respectively. Rats were acclimatized for 7 days before the treatment. All groups except the non-diabetic control were injected with alloxan at a dose of 125 mg/kg b. w. Coffee was made by dissolving each dose with hot water without using any sweeteners. Coffee was administered for 14 d, and blood sugar levels were measured before and after the treatment. The gastrocnemius muscle was taken for histopathological test to measure the diameter and quantify the number of cell nuclei. The data was analyzed with the One-Way Anova for body weight and muscle diameter and Kruskal-Wallis tests performed to analyze blood glucose and myonuclear number.

Results: Kruskal-Wallis test showed the significance of blood sugar levels p=0.01, Dunn's Post Hoc comparisons of animal group test showed significant differences between groups 1-3, 2-3, and 4-6. Body weight, muscle diameter, and muscle cell nuclei number exhibited no significant differences within the groups. The Spearman's rho test indicated no significant correlation between muscle diameter and myonuclear number (0.474).

Conclusion: On the 14th day of treatment, coffee was able to lower blood sugar levels but was unable to improve skeletal muscle diameter as well as myonuclear number.

Keywords: Coffee, Diabetes melitus, Blood glucose, Histopathological test, Muscle diameter, Myonuclear, Gastrocnemius muscle

DOI: https://dx.doi.org/10.22159/ijap.2025.v17s2.05 Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

Diabetes melitus (DM) is a chronic disease caused by impaired insulin secretion, insulin action, and/or both. This results in elevated blood glucose levels in diabetes mellitus patients, along with all associated implications [1]. Diabetes has risen steadily. The number of diabetes melitus sufferers in the world reached 382 million in 2013. This fig. is estimated to be 592 million in 2035. In Indonesia, 8.5 million DM sufferers were found in 2013 and this is estimated to be 14.1 million in 2035 [2]. Non-diabetics' insulin ineffectiveness causes metabolic diseases like hyperglycemia. It alters lipid, carbohydrate, and protein metabolism, which may increase vascular disease risk [3]. The anti-hyperglycemic drugs that are widely used do not protect against diabetes melitus well enough. As a result, a lot of clinical experience and research studies show that diabetic complications are complicated and that only treating one part of the disease's pathophysiology is not enough to stop it from getting worse and causing problems [4]. As no sole treatment is entirely beneficial, investigating the pathophysiological underpinning of the disease may facilitate the development of novel therapeutics to mitigate disease progression and its potential implications.

Diabetes melitus causes various complications that can be categorized into two large groups, macroangiopathy and microangiopathy. The most common complications of DM cases are microangiopathy, which includes diabetic neuropathy, diabetic nephropathy, diabetic retinopathy, and mixed types [5, 6]. Diabetes melitus also has an impact on osteogenesis, vascular thickening, and skeletal muscle defects [7]. The significant prevalence of diabetes melitus, coupled with its complications and economic implications, renders it a critical issue necessitating a solution [2]. Diabetes management therapy combines both non-pharmacological and pharmacological interventions, reinforced by familial support [8]. Non-pharmacological therapy encompasses dietary regulation, particularly a black rice diet [9] and ketogenic [10]. Pharmacological treatment for diabetes melitus involves insulin or non-insulin medications. Both treatments induce a range of adverse effects, including short-term complication like hypoglycemia and long-term effects such as renal failure. Consequently, alternative therapy methods are required that are more secure, readily accessible, and cost-effective.

Coffee offers protection against oxidative stress and metabolic syndrome due to its substances of caffeine, chlorogenic acid, and diterpenes. Studies in humans indicate that coffee consumption influences lipid oxidation, protein integrity, DNA damage repair, modification of antioxidant capacity, and the activity of antioxidant enzymes via elevated glutathione levels. The polyphenol content in coffee enhances endothelial function and mitigates metabolic syndrome by inhibiting oxidative stress. Antioxidative substances in coffee impede the oxidation of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) in mitochondria and diminish Reactive Oxygen Species (ROS) [11].

Diabetes hinders muscle regeneration, potentially affected by food ingredients such as coffee [12]. Limited research has particularly investigated the impact of coffee on skeletal muscle mass in diabetic people, resulting in unclear results [13, 14]. Demographic representation, especially regarding diabetes, is essential for comprehending the wider implications of coffee dietary intake [12, 13]. Skeletal muscle mass is becoming important in the management of diabetes because reduced muscle mass impairs glucose clearance and increases diabetes risk [15]. Dietary interventions, including coffee consumption, are understudied. This gap suggests more research on how coffee affects muscle mass and diabetes control.

Caffeine, chlorogenic acids, trigonelline, and diterpenes in coffee affect metabolic pathways related to muscle physiology and glycemic management in a pleiotropic manner. Importantly, coffee is widely drunk, culturally accepted, and could be prolonged. It differs from other specialized nutritional treatments that may be expensive or involve complicated behavioral changes. These characteristics suggest using coffee as a dietary intervention to improve skeletal muscle performance and glycemic management in diabetic patients.

The research aimed to investigate the impact of coffee on blood glucose levels and alterations in skeletal muscle. The beneficial effects of coffee in reducing blood sugar levels are widely recognized; nevertheless, the effectiveness of coffee in this context for improved muscle mass remains unclear. This research adds new value to the scientific understanding of the potential of coffee as a multifunctional agent in DM management, particularly focusing on muscle mass as a therapeutic target. According to the literature review completed, no similar research has been undertaken. We hypothesized that coffee would reduce blood glucose levels without impacting skeletal muscle mass due to its short-term intervention.

MATERIALS AND METHODS

Material

The research utilized the Wistar strain of *Rattus norvegicus*. Induction of diabetes in rats with alloxan at a dose of 125 mg/kg body weight (b. w) intraperitoneally. The coffee utilized is Lampung Robusta. Blood glucose measurement using an Accu-check glucometer with blood sugar sticks. Staining of skeletal muscle cells using the hematoxylin-eosin method following the protocols. The prepared specimen was identified with a light microscope, and microphotographs were captured using optical microscope *Optilab* (Olympus corporation, Tokyo, Japan). The number of muscle cell nuclei was counted using Image J software (National Institutes of Health, United States). This study is part of a project of study conducted by the same investigators, yet with a different focus on research variables.

Method

Experimental animal

Male *Rattus norvegicus*, weighing between 150-250g and aged 2-3 mo were included, while those who were ill and stressed were omitted. Rats were acclimatized in the cage for 7 d before treatment to avoid stress. Acclimatization is carried out at room temperature with lighting according to the day-night cycle, feeding using Br1 (Japfa Comfeed, Indonesia)±75 g/d for each group with ad libitum drinking.

Experimental design

This study performed a laboratory experiment with a post-test only control group design. A total of 24 rats that met the criteria were divided into 6 groups as follows: negative control non-diabetic: given distilled water (group 1), negative control–diabetic: given alloxan and distilled water (group 2), positive control, given alloxan and insulin (group 3), group 4: given alloxan and coffee 0.054g/200g b. w, group 5: given alloxan and coffee 2: 0.108 g/200g b. w, and group 6: given alloxan and coffee 3: 0.162g/200g b. w. Alloxan was administered intraperitoneally at a dose of 125 mg/kg b. w to rats in groups 2-6 on day 0 after acclimatization, while insulin was injected to rats subcutaneously at a dose of 1 IU/kg b. w [16] in group 3 on day 4 after alloxan induction. Alloxan is a typical diabetogenic agent used in diabetes research to investigate substance that may treat diabetes. Compared to other diabetogenic drugs like streptozotocin, alloxan is more commonly employed in diabetes research because to its availability and low cost, especially for creating hyperglycemic conditions in a shorter period of time. Administering alloxan to experimental animals results in a multiphasic blood glucose response. Alloxan could be given intraperitoneally, intravenously, and subcutaneously, with intraperitoneal being the most common route [17].

Experimental procedures

Subsequent to completing the drying and grinding process into powder, coffee is prepared by dissolving each portion of coffee in hot water, devoid of any sugars or other sweeteners. Coffee was administered at three dosage levels: 0.054g/200g b. w, 0.108g/200g b. w, and 0.162g/200g b. w. Every dose of coffee was dissolved in 25cc of heated water. Once the coffee has cooled sufficiently, it is administered orally to the rats. Coffee was given daily for a duration of 14 d. Group 4 (coffee with a dose of 0.054g/200g b. w) is equivalent to 3g of coffee in humans, group 5 (coffee dose of 0.108g/200g b. w is equivalent to 6g in humans, group 6 (coffee dose of 0.162g/200g b. w) is equivalent to a dose of 9g in humans [18].

On day 0, all test animals were measured for their initial blood sugar levels. On day 1, rats in groups 2 to 6 were induced with hyperglycemia using alloxan at a dose of 125 mg/kg b. w intraperitoneally. On day 4, blood sugar levels were measured again as diabetic blood sugar levels. On days 5 to 14, *rats* in group 3 were given treatment in the form of insulin injection of 1 IU/kg b. w per day, coffee was given at a dose of 0.054 g/200g b. w for group 4, coffee dose of 0.108 g/200g b. w for group 5, and group 6 with a coffee dose of 0.162/200g b. w. Blood glucose levels are measured by an Accu-Chek glucometer. On day 14 of treatment (day 19 of the study), blood sugar levels were measured again as post-treatment blood sugar and the rats were terminated for histopathological analysis of gastrocnemius muscle.

Histopathology

Histological preparation based on Culling (1974) and Luna (1968) methods [19, 20]. The right gastrocnemius muscle tissue was excised, then have been fixed with 10% formalin, dehydrated and successively cleaned with one solution session (10% formalin I, 10% formalin II, 10% formalin III, 70% alcohol, 96% alcohol, absolute alcohol I, absolute alcohol II, absolute alcohol III, xylol I, xylol II, xylol III, liquid paraffin I, liquid paraffin II) within 23 h. The tissue was positioned transversally, then blocked with liquid paraffin, after being cooled for 30 min cut by a microtome with a thickness of 4-5 µm. Before mounting, staining was carried out using the Harris-hematoxylin eosin (HE) method by soaking in xylol I, II, III for 5 min each and then soaked in absolute alcohol I and II for 5 min. Before being soaked in HE (15 min), it was soaked in distilled water for 1 min. The sample was soaked again in distilled water (1 min), then 5-7 min in 10% acid alcohol, twice in distilled water for 1 min and 15 min. After that, it was stained with eosin. The stained preparation was then soaked in 96% alcohol I and 96% alcohol II for 3 min each. Then it was soaked again in absolute alcohol III, followed by absolute alcohol IV for 3 min each. Subsequently, it was cleansed in xylol I and xylol II for 5 min.

The prepared specimen was identified with a light microscope, and microphotographs were captured using optical microscope *Optilab*. The microscopic images observed were whether there were microscopic changes in muscle fibers and nuclei and cross-fiber patterns or not. The gastrocnemius muscle is one of the locomotor muscles frequently used in rats and is easiest to obtain from euthanized rats. The diameter of the muscle is measured at the largest diameter of the gastrocnemius muscle using a vernier caliper. The gastrocnemius muscle is more accessible than the quadriceps, and due to its pennate structure, it facilitates the evaluation of structural parameters including fascicle length and pennation angle [21]. The slide observed with a microscope magnification of 400x by counting the nuclei of skeletal muscle cells that are basophilic and located at the cell periphery. The number of muscle cell nuclei was counted using Image J software in the cell counter section with 5 fields of view and then averaged.

Statistical analysis

Blood glucose levels and myonuclear counts were statistically analyzed using the non-parametric Kruskal-Wallis test due to the data did not satisfy the assumptions of normality, whereas body weight and muscle diameter data were analyzed using One-Way ANOVA to determine group differences. Pairwise comparisons of animal group test was conducted to identify groups that differed significantly. The non-parametric Spearman's rho correlation test was performed to analyze data on muscle diameter and the quantity of cell nuclei.

RESULTS AND DISCUSSION

Coffee antidiabetic test via blood sugar level monitoring

All the rats experienced an increase in blood sugar levels on the 4th day after the induction of alloxan 125 mg/kg b. w (table 1).

The antidiabetic effect and improvement of muscle mass of rats on the 14th day after hyperglycemia induction (day 19) with treatment using coffee obtained the following results as illustrated in table 2.

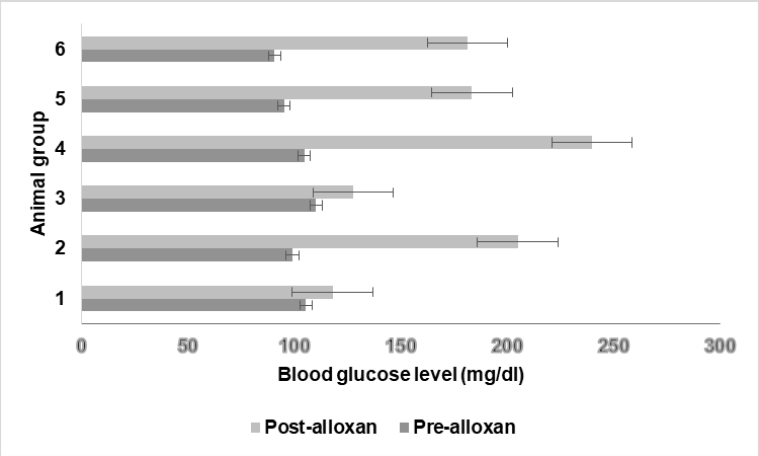

Fig. 1: Increase in blood glucose level day 4 after alloxan induction (125 mg/kg b. w), 1: group 1 (non-diabetic negative control), 2: group 2 (diabetic negative control), 3: group 3 (insulin, positive control), 4: group 4 (coffee dose 0.054g/200g b. w), 5: group 5 (coffee dose 0.108g/200g b. w), 6: group 6 (coffee dose 0.162g/200g b. w)

Table 1: The results of measuring body weight (g), blood sugar levels (mg/dl), muscle diameter (mm), and myonuclear number in rats after 14 d of coffee adminitration

Variables	Group						p-value	95% CI	
	1	2	3	4	5	6		Lower	Upper
BW (g)a	218±23.8	182.6±36.2	185.2±57.6	173.3±31.1	202.2±17.7	195.8±11.7	0.487*	0.000	0.000
Delta FPG (mg/dl)b	15(19.5)	46.5(223.5)	44(127.5)	13.5(220.75)	34(157.7)	28(193.5)	0.01**	0.533	0.915
Muscle diameter (mm)a	0.675±0.2	0.675±0.17	0.650±0.19	0.625±0.17)	0.575±0.95	0.550±0.1	0.840*	0.000	0.000
Myonuclear (n)b	54.5(22.9)	63.6(30.3)	50.5(23.4)	81.6(40.7)	56.1(18.5)	61.1(58.7)	0.192**	0.260	0.758

Description: BW: body weight, delta FPG: delta fasting plasma glucose pre and post-coffee administration, Group 1: group 1 (non-diabetic negative control), 2: group 2 (diabetic negative control), 3: group 3 (insulin-positive control), 4: group 4 (coffee dose 0.054g/200g b. w, 5: group 5 (coffee dose 0.108g/200g b. w), 6: group 6 (coffee dose 0.162g/200g b. w). [a]: mean±SD; [b]: median (IQR). *: p-value of One way Anova; **: p-value of Kruskal-Wallis. A p-value<0.05 indicates statistical significance. The outcomes of the difference test with a p-value<0.05 were confirmed by a Dunn's Post Hoc comparisons of animal groups.

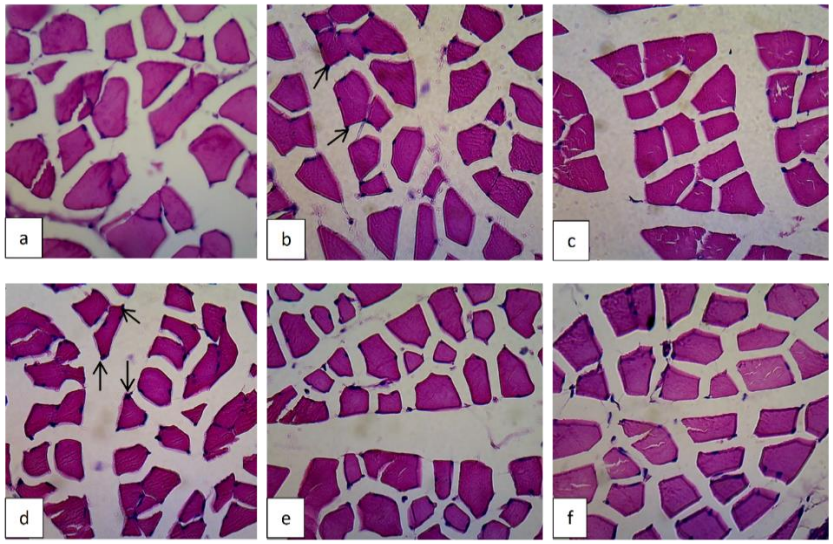

Fig. 2: Transversal section of gastrocnemius muscle tissue staining. Skeletal muscle cells (myocytes) are stained pink with eosin, while myonuclear cells are stained blue with hematoxylin, as indicated by black arrows. No microscopic differences were observed between the study groups. a. Group 1 (non-diabetic negative control), b. Group 2 (diabetic negative control), c. Group 3 (insulin, positive control), d. Group 4 (coffee dose 0.054g/200g b. w), e. Group 5 (coffee dose 0.108g/200g b. w), f. Group 6 (coffee dose 0.162g/200g b. w). Hematoxylin-eosin (HE) staining at 400x magnification

Table 2: Dunn's post hoc comparisons of animal group

Group comparison	p-value*
1-3	0.009
2-3	0.004
4-6	0.028

*= p-value<0.05 indicates a significant difference between groups. 1: negative control non-diabetic, 2: negative control diabetic, 3: positive control (insulin), 4: coffee dose 0.054g/200g b. w, 6: coffee dose 0.162g/200g b. w

Table 3: Correlation between muscle diameter and myonuclear number

	n	Spearman			
		rho	p	95% CI	
				Lower	Upper
Muscle diameter-myonuclear number	24	0.154	0.474	-0.278	0.534

The Spearman rank correlation coefficient (rho), p-value, and CI results indicate that there is no significant correlation between muscle diameter and myonuclear number.

Skeletal muscle histopathology

The histopathological staining of gastrocnemius muscle cells in test rats following diabetes induction and 14 d of coffee administration are showed in fig. 2.

The results Spearman's rho correlation test between muscle diameter and muscle cell nuclei are presented in table 2.

DISCUSSION

According to table 1, all rats administered by alloxan at a dose of 125 mg/kg b. w exhibited hyperglycemia. Alloxan is a commonly employed diabetogenic agent used to induce diabetes in experimental animals. Its mechanism involves an increase in cytosolic-free calcium ions within pancreatic β-cells. The influx of calcium triggered by alloxan leads to depolarization of the β-cell membrane. This condition results in a rapid elevation of insulin levels accompanied by high Fasting Blood Glucose (FBG), ultimately causing significant disturbances in peripheral insulin sensitivity within a short period [22]. Alloxan that enters the bloodstream is disseminated throughout the body and subsequently penetrates the pancreatic β cells via Glucose Transporter-2 (GLUT-2). Upon entering the pancreatic β cells, alloxan induces damage that leads to necrosis and subsequent destruction by scavenger receptors. Damage to pancreatic β cells impedes insulin production, leading to elevated blood glucose levels, as shown in insulin-dependent diabetes [23-25]. It is also involved two pathogenic effects: selective suppression of glucose-stimulated insulin production and induced ROS formation, cause selective beta cell necrosis. Cells develop insulin-dependent or type 1-like diabetes from both actions. The former involves alloxan's particular suppression of glucokinase, a pancreatic glucose sensor enzyme, while the latter involves its redox cycling, which generates ROS. Significantly, alloxan's chemical and structural features have been related to both impacts. Selective uptake by pancreatic beta cells and accumulation in these cells demonstrate alloxan's diabetogenicity [17].

Table 1 shows that coffee decreases blood sugar levels in diabetic rats in a dose-dependent manner. This result is in line with existing publications that coffee improves blood sugar and serum insulin levels [26]. The mechanism of coffee in lowering blood sugar levels is not fully understood. Chronic coffee consumption can lower blood sugar levels. The interplay of coffee consumption, blood glucose reduction, and muscle mass preservation is intricate and affected by multiple molecular pathways, including those involving adenosine monophosphate-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR) signaling. Coffee helps reduce blood glucose levels by activating AMPK, which improves glucose transport in skeletal muscle without negatively impacting muscle hypertrophy. This occurrence may be ascribed to the brief period of interventions in studies, which frequently do not let substantial changes in muscle mass build up. AMPK functions antagonistically to mTOR, which is essential for the growth of muscles. Elevated AMPK activity can suppress mTOR signaling, hence obstructing muscle growth while concurrently promoting glucose uptake [27]. Caffeine causes an increase in the release of calcium from the sarcolemma. Increased calcium ions cause the activation of Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) and AMPK [28, 29]. Both cause increased GLUT4 mRNA formation and GLUT4 translocation to the cell membrane, thereby mediating glucose entry into muscle cells. In line with this, another study showed that CGA decreased fasting blood sugar levels in db/db rat using a glucose tolerance test and stimulated glucose transport in skeletal muscle through GLUT4 translocation mediated by AMPK activation [30].

The results of this research differ from previous publications, which showed that caffeinated coffee increases blood sugar [31], and the results of the study reported that coffee supplementation was not associated with fasting blood sugar levels [32]. The results of this study show that giving caffeinated coffee significantly reduces fasting blood sugar levels in test rats (p=0.01). The results of studies regarding coffee's impact on muscle mass are incongruous, with certain research indicating a preventive benefit against decreased muscle mass [14]. This disparity may arise from differences in study design, demographic characteristics, and measuring methodologies.

Diabetes melitus is associated with various disorders of the locomotor system, including decreased skeletal muscle mass and function. Inappropriate insulin action in DM contributes to decreased skeletal muscle mass and physical performance [33]. Based on this, an analysis was conducted on the relationship between decreased blood sugar levels and muscle diameter. Table 1 shows that there is no difference in muscle diameter between groups. This indicates that decreased blood sugar levels due to coffee do not affect muscle diameter. Muscle diameter is one way to measure muscle size in addition to muscle volume and anatomical cross-sectional area [34]. Muscle size has a non-linear correlation with body mass index [35]. Based on this, this research is in line with study, which state that a decrease in blood sugar in DM is not followed by a decrease in body mass index [36]. The cause of this condition is due to the duration of treatment is not long enough so that the treatment has not been able to improve the formation of Advanced Glycation End Product (AGE) and proinflammatory cytokines in experimental animals, which results in no increase in muscle diameter.

High blood sugar levels for a long time cause the formation of AGE [37]. In addition, AGE causes decreased insulin production and secretion. The AGE formed causes the activation of Nuclear Factor Kappa B (NF-κB), which then causes the formation of proinflammatory cytokines such as Interleukin-1 (IL-1), Interleukin-6 (IL-6), and Transforming Growth Factor-α (TNF-α) [38]. Proinflammatory cytokines and AGEs lead to more severe insulin resistance [37, 38]. Human insulin, which falls into the short-acting and intermediate-acting categories, tends to increase the risk of

hypoglycemia due to its longer duration of action, potentially causing a significant decrease in glucose levels. This results in a higher risk of hypoglycaemia [39]. Insulin resistance causes a reduction in glucose entering the cells so that glucose cannot be converted into glycogen [40]. Unchanged glycogen in muscle cells causes the muscle diameter to remain unchanged [41].

Muscle mass affects body weight; the greater the muscle mass, the more weight increases [42]. The research results in table 1 show that there is no difference in muscle mass between groups. This research is in line with publications stating that decreasing blood sugar levels is not associated with weight loss and does not change muscle mass [36, 43]. Table 2 shows that there is no correlation between muscle diameter and the number of myonuclear. This is in accordance with the myonuclear domain theory which states that the number of myonuclear is relatively constant and proportional to muscle volume [44]. Stability of the number of myonuclear is necessary to maintain muscle function [45]. Even the decrease in the number of nuclei in atrophied muscles is not due to a decrease in the number of myonuclear, but rather a decrease in the number of satellite cells [46, 47]. The reduction of body weight owing to diabetogenic induction is linked to increased muscle wasting and depletion of tissue proteins [48]. The results of this study showed that muscle diameter did not differ between groups, according to the number of nuclei in the muscles, which also did not differ.

Overall, based on blood glucose level data and histopathological examination of skeletal muscle, this study shows that giving coffee for 14 d can reduce blood sugar levels but does not improve skeletal muscle mass. This study has limitations by the relatively short treatment duration. This interval may be enough to observe initial pharmacodynamic responses, but it may not represent long-term glycemic control efficacy and sustainability. To attain improved outcomes, it is essential to establish circumstances for type 2 diabetes melitus and to implement a prolonged coffee intervention. Tissue-level adaptations such pancreatic beta-cell regeneration, insulin sensitivity modification in skeletal muscle and liver, lipid metabolism, and oxidative stress require prolonged monitoring, which short-term research cannot provide. Baseline glucose levels, handling or environment-induced stress reactions, and animal metabolism's related diurnal oscillation may be confounders. Acute symptoms like hypoglycemia or hunger suppression may obscure the long-term potential of anti-diabetic treatments.. Future research with longer treatment durations and larger sample groups are needed to confirm these findings and assess intervention durability and safety. Coffee may help manage blood glucose levels, but its long-term effects on muscle mass and metabolic health are unknown, requiring further research. To better understand these connections and what they mean for metabolic health, we need longitudinal studies with a range of intervention lengths and mechanistic research into how AMPK and mTOR work together. On the other hand, coffee may help control blood sugar levels, but we still don't fully understand how it affects muscle mass and metabolic health over time, which means we need to learn more about the biological processes behind these effects. The combination of coffee consumption with prolonged weight training could be utilized as an alternative intervention in future studies.

CONCLUSION

Coffee can lower blood glucose levels but does not improve muscular mass. An extended research duration is required to ascertain the impact of elevated blood sugar levels on muscle mass, hence enabling the evaluation of coffee's effects on muscle mass. Further study is suggested concerning the impact of coffee to improve muscle mass in type 2 diabetes melitus setting.

ACKNOWLEDGEMENT

Authors would like to thanks to the Research and Innovation Center Universitas Muhammadiyah Surakarta for financial support by regular competitive grant research No. 213.4/A.3-III/lPPM/V/2019 and Faculty of Medicine Universitas Muhammadiyah Surakarta for all supports.

ETHICAL CONSIDERATION

This study has obtained ethical approval from the Health Research Ethics Committee Faculty of Medicine Universitas Muhammadiyah Surakarta with ethical clearance letter No. 2310/A.2/KEPK-FK UMS/VII/2019.

AUTHORS CONTRIBUTIONS

RA: design of the work, conducting research, processing data, manuscript preparations, writing original draft. SWJ: conceptualization, data analysis, critical for important intellectual content, writing original draft. NM: collected data, data interpretation, supervision, manuscript preparations. All authors have reviewed and approved the final version the manuscript.

CONFLICT OF INTERESTS

The author declares no conflict of interest

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