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Original Article

Protective effects of metformin in non-diabetic rats with experimentally induced lower extremity ischemia-reperfusion injury

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Abstract

Aim: Lower extremity ischemia-reperfusion (IR) injury can lead to substantial skeletal muscle damage and systemic complications, primarily driven by oxidative stress and inflammation. In addition to its well-known glucose-lowering effects, metformin possesses antioxidant and anti-inflammatory properties that may confer protection against tissue damage caused by IR. This study aims to evaluate the potential protective effects of metformin on skeletal muscle injury using a rat model of lower extremity IR.

Material and Methods: A total of twenty-four male Wistar albino rats were randomly divided into four experimental groups: Control (C), Ischemia-Reperfusion (IR), IR with metformin at 4 mg/kg (IR+M4), and IR with metformin at 8 mg/kg (IR+M8). Ischemia was induced by clamping the infrarenal aorta for 45 minutes, followed by a reperfusion period of 120 minutes. In the treatment groups, metformin was administered intraperitoneally at the onset of ischemia. Gastrocnemius muscle tissues were harvested for subsequent histopathological and biochemical evaluations, including measurements of Total Antioxidant Status (TAS), Total Oxidant Status (TOS), and Oxidative Stress Index (OSI).

Results: Histopathological analysis demonstrated a significant reduction in muscle atrophy, degeneration, leukocyte infiltration, and fiber fragmentation in the IR+M8 group compared to the IR group. Biochemical assessments showed that TAS levels were considerably elevated, whereas TOS and OSI levels were markedly reduced in the metformin-treated groups, with the most prominent effects observed at the higher dosage of 8 mg/kg.

Conclusion: The findings indicate that metformin exerts a dose-dependent protective effect against skeletal muscle injury resulting from lower extremity ischemia-reperfusion in rats. These protective properties are likely due to metformin's antioxidant and anti-inflammatory mechanisms, highlighting its potential therapeutic value in mitigating IR-induced tissue damage.

Keywords: Metformin, ischemia-reperfusion, lower extremity, oxidative stress

INTRODUCTION

Acute limb ischemia is a critical condition marked by a sudden reduction in arterial blood flow to skeletal muscles, requiring immediate intervention to prevent irreversible damage. It primarily results from arterial blockages due to embolism (39.5%) or thrombosis (50.2%) [1]. Standard treatment involves urgent revascularization to restore circulation [2]. However, even after successful reperfusion, patients may develop severe complications, including muscle necrosis, compartment syndrome, and multi-organ failure [3]. Similar pathophysiological

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mechanisms are observed in other ischemic conditions, such as trauma, stroke, and myocardial infarction, where thrombolytic therapy or surgical revascularization is essential [4,5]. Paradoxically, reperfusion can exacerbate tissue damage—a phenomenon known as the "second hit" [2]. This occurs due to excessive oxidative molecule production, calcium homeostasis disruption, and inflammatory activation, collectively leading to oxidative stress, mitochondrial dysfunction, endothelial impairment, and irreversible cellular injury [6-8].

Ischemia-reperfusion (IR) injury exacerbates cellular damage beyond ischemia alone [6]. Oxygen deprivation depletes ATP, disrupts ion pumps, and causes calcium overload, leading to membrane damage and cell death [6,9]. Reperfusion generates excessive reactive oxygen species (ROS), triggering oxidative stress, lipid peroxidation, and apoptosis [6,8]. Inflammation further amplifies injury through neutrophil activation and cytokine release (TNF-α, IL-1, IL-6), worsening endothelial dysfunction and vascular permeability [6,9]. These processes contribute to tissue necrosis and systemic complications, necessitating ongoing research into therapeutic interventions [10-14].

Metformin, a biguanide primarily used for type 2 diabetes, lowers blood glucose by activating AMP-activated protein kinase (AMPK), which enhances insulin sensitivity and reduces hepatic glucose production [15,16]. Beyond glycemic control, metformin protects against IR injury via AMPK activation, boosting antioxidant defenses by upregulating superoxide dismutase (SOD) and catalase, thereby reducing ROS and preventing mitochondrial dysfunction [15,17,18]. The subsequent decline in ROS levels helps mitigate lipid peroxidation and cellular impairment, ensuring mitochondrial stability and averting the formation of mitochondrial permeability transition pores (mPTP)—a critical factor in preventing cell apoptosis and necrosis in cardiac and other tissues exposed to IR injury [17,18]. It also inhibits inflammation by downregulating nuclear factor kappa B (NF-κB) and c-Jun N-terminal kinase (JNK) signaling, limiting cytokine-mediated damage [17,19]. Additionally, metformin enhances endothelial function by stimulating nitric oxide (NO) production, improving vascular dilation and reducing ischemic injury [16].

Metformin has demonstrated protective effects against IR injury in various organs, including the heart, lungs, liver, ovaries, and testes [18,20-25]. In myocardial IR injury, it improves ventricular function, reduces infarct size, and limits apoptosis via AMPK activation [18]. Similarly, in ovarian and testicular IR models, it lowers oxidative stress markers, enhances antioxidant defenses, and prevents apoptosis, preserving tissue integrity [20-22]. While its antioxidant, anti-inflammatory, and anti-apoptotic properties are well-documented, its role in lower extremity IR injury remains underexplored. This study investigates metformin's protective potential in non-diabetic rats with IR-induced skeletal muscle injury, providing a basis for future research.

MATERIAL AND METHODS

Animals

The study utilized 12-week-old male Albino Wistar rats (250–350 g) bred in the experimental research facility of Gazi University in Ankara, Türkiye. All procedures adhered to ethical guidelines approved by the Institutional Experimental Animal Ethics Committee (G.Ü.ET-24.121). The rats were housed under controlled conditions (20–21°C, 45–55% humidity) with a 12-hour light/dark cycle and had unrestricted access to standard chow and purified water.

Chemicals

Metformin was obtained from Bilim İlaç San ve Tic. A.Ş. (Istanbul, Türkiye). Anesthesia was administered to all animals using a combination of ketamine hydrochloride (500 mg/10 ml; Ketalar; Parke-Davis; Pfizer) and xylazine hydrochloride (2%; Alfazyne; Ege Vet). All laboratory reagents utilized in the study were procured from Sigma Chemical Co. and Merck (Germany). The dosage and administration method of metformin were determined based on previous studies, with doses set at 4 mg/kg and 8 mg/kg, as referenced in the literature [26].

Experimental Protocol

Twenty-four rats were randomly divided into four groups (n=6 each): control, ischemia-reperfusion (IR), IR with 4 mg/kg metformin (IR+M4), and IR with 8 mg/kg metformin (IR+M8). The sample size for this study was determined based on previous experimental studies investigating IR injury in animal models, ensuring adequate statistical power to detect significant differences between groups [10,12-14,27]. Anesthesia was induced with intramuscular ketamine (50 mg/kg) and xylazine (10 mg/kg), with additional doses (ketamine 20 mg/kg, xylazine 5 mg/kg) administered as needed. Following a 30-minute stabilization period, surgical procedures were performed with the rats positioned supine under a warming lamp to maintain body temperature.

In the control group, only a midline laparotomy was performed, followed by intraperitoneal saline (0.3 cc of 0.9%) administration after 45 minutes. After a two-hour observation, the rats were sacrificed, and gastrocnemius muscles were collected bilaterally. In the IR group, ischemia was induced by clamping the infrarenal aorta for 45 minutes, followed by 120 minutes of reperfusion before muscle excision. In the IR+M4 and IR+M8 groups, metformin (4 mg/kg and 8 mg/kg, respectively) was administered intraperitoneally post-laparotomy before ischemia induction, followed by the same reperfusion protocol. After the experiment, gastrocnemius muscles were collected for histopathological and biochemical analysis.

All animals were euthanized by collecting 5–10 mL of blood from the abdominal aorta following ketamine (100 mg/kg) and xylazine (10 mg/kg) administration. The absence of cardiac activity and respiratory movements was monitored to confirm the completion of the procedure, with an additional two-minute observation to ensure its effectiveness.

Histopathological Evaluation

Following euthanasia, the right gastrocnemius muscle was carefully dissected and preserved in 10% formaldehyde for histopathological evaluation. To ensure unbiased analysis, a blinded pathologist assessed the samples. Standard histological processing was performed, and hematoxylin and eosin staining facilitated microscopic examination. Key pathological markers, including muscle atrophy or hypertrophy, degenerative changes, vascular congestion, nuclear internalization, centrally located oval nuclei, fiber fragmentation, hyalinization, and leukocyte infiltration, were systematically evaluated and graded by a blinded pathologist to quantify tissue damage.

Biochemical Evaluation

The left gastrocnemius muscle was immediately frozen in liquid nitrogen and stored at -80°C to maintain biochemical integrity for subsequent analysis. Oxidative stress biomarkers, including Total Antioxidant Status (TAS), Total Oxidant Status (TOS), and the Oxidative Stress Index (OSI), were measured. The methods and formulas for TAS, TOS, and OSI assessment were adopted from our previous research [27].

Statistical Analysis

The primary outcome variables included biochemical markers (TAS, TOS, OSI), as well as histopathological parameters, systematically graded by a blinded pathologist to quantify tissue damage. Histopathological scores, which were assigned numerical values, along with biochemical data were analyzed as quantitative variables. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, version 26.0 (IBM Corp., Armonk, NY, USA) for Windows. The distribution of quantitative variables was assessed using the Shapiro–Wilk test. Biochemical and histopathological parameters were tested by using one-way ANOVA test, followed by Bonferroni post-hoc analysis. A p-value of <0.05 was considered statistically significant. Results were expressed as mean±standard error of the mean (SEM), depending on the overall distribution of the variables.

RESULTS

Histopathological Results

Microscopic analysis revealed significant differences in muscle atrophy and hypertrophy among groups (Figures 1-4, Table 1, p=0.012). The IR group showed significantly greater atrophy and hypertrophy than the control (p=0.002), while the IR+M8 group exhibited a notable reduction compared to the IR group (p=0.008).

Muscle degeneration and vascular congestion also varied significantly (Table 1, p=0.031). The IR group had markedly higher degeneration and congestion than the control (p=0.006), whereas the IR+M8 group showed significant improvement (p=0.017). Histopathological analysis confirmed more severe fiber degeneration in the IR group compared to IR+M8 (Figures 1-4).

Nuclear internalization, characterized by oval and centrally located nuclei, differed among groups (Table 1, p=0.031). The IR group had significantly more internalized nuclei than the control (p=0.006), while the IR+M8 group exhibited a notable reduction compared to IR (Figures 1-4). No significant difference was observed between IR and IR+M4.

Muscle fragmentation and hyalinization were significantly elevated in the IR group (p=0.006), but IR+M8 treatment led to a significant reduction (p=0.020), indicating a dose-dependent protective effect (Table 1, Figures 1-4, p=0.033).

Leukocyte infiltration was highest in the IR group (Table 1, p=0.009) but significantly reduced in both IR+M4 and IR+M8 groups (p=0.022 and p=0.009, respectively), as confirmed histopathologically (Figures 1-4).

The IR+M4 group did not show significant differences from IR in most parameters, supporting a dose-dependent protective effect of metformin, with 8 mg/kg providing greater benefits against ischemia-reperfusion injury.

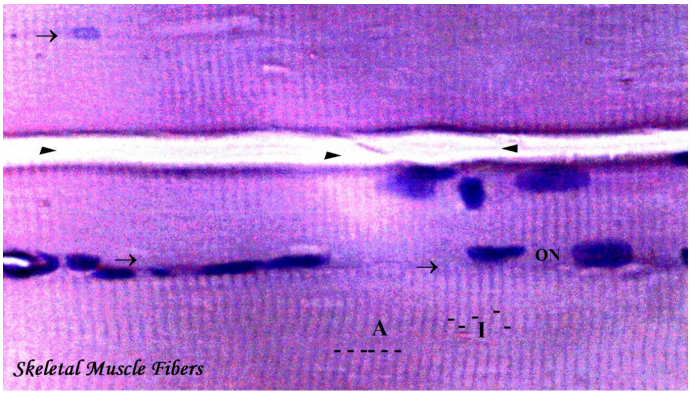

Figure 1. Histopathological examination of the gastrocnemius muscle tissue; control group; →: peripheric nucleus, ►: intercellular space, ON: oval nucleus (H&E: hematoxylin and eosin X10)

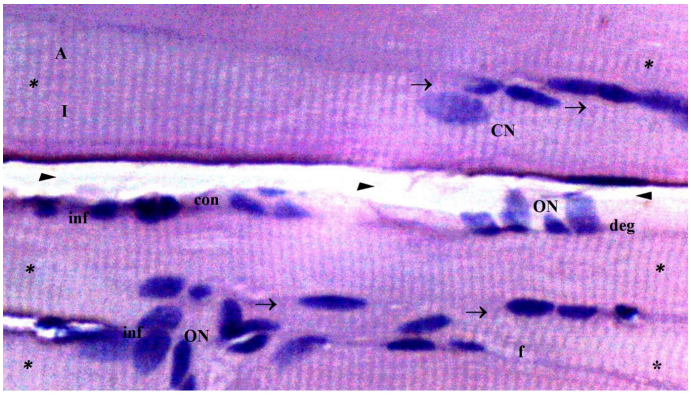

Figure 2. Histopathological examination of the gastrocnemius muscle tissue; Ischemia-Reperfusion group; →: peripheric nucleus, *: muscle fibers, ►: intercellular space, CN: central nucleus, ON: oval nucleus, f: fragmentation, deg: degeneration, con: congestion, inf: infiltration (H&E: hematoxylin and eosin X10)

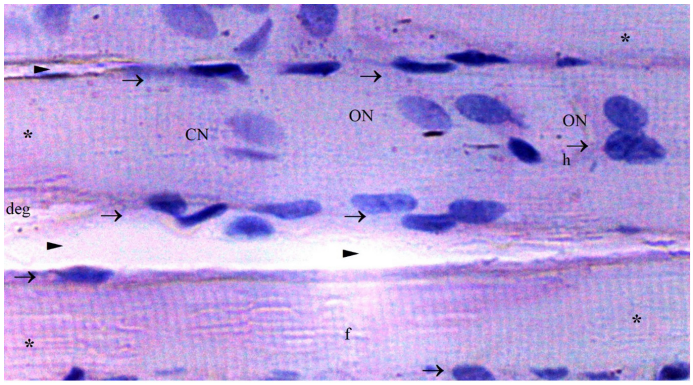

Figure 3. Histopathological examination of the gastrocnemius muscle tissue; IR+Metformin 4mg/kg Group; →: peripheric nucleus, *: muscle fibers, ►: intercellular space, CN: central nucleus, ON: oval nucleus, f: fragmentation, h: hyalinization, deg: degeneration (H&E: hematoxylin and eosin X10)

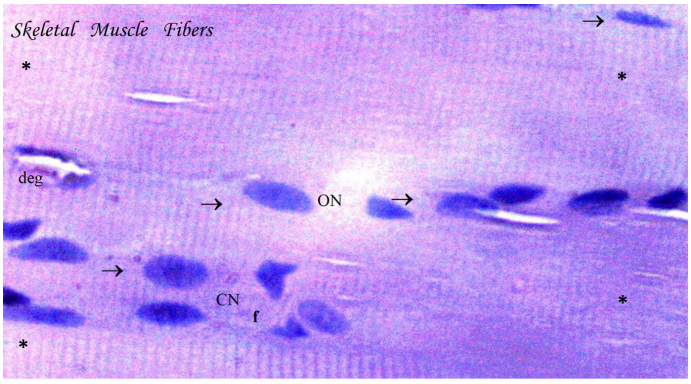

Figure 4. Histopathological examination of the gastrocnemius muscle tissue. IR+Metformin 8mg/kg Group; →: peripheric nucleus, *: muscle fibers, CN: central nucleus, ON: oval nucleus, f: fragmentation, deg: degeneraion; (H&E: hematoxylin and eosin X10)

Table 1. Histopathological analysis scores of the gastrocnemius muscle tissue [mean±SEM]

	Control group (C)	Ischemia reperfusion group (IR)	IR+metformin 4mg/kg group (IR+M4)	IR+metformin 8mg/kg group (IR+M8)	p-value
Muscle atrophy and hypertrophy	0.17±0.17	1.33±0.33*	0.67±0.21	0.33±0.21**	0.012
Muscle degeneration and congestion	0.17±0.17	1.33±0.33*	0.67±0.33	0.33±0.21**	0.031
Internalization of muscle nuclei (oval-central nuclei)	0.17±0.17	1.33±0.33*	0.67±0.33	0.33±0.21**	0.031
Fragmentation and hyalinization	0.17±0.17	1.17±0.31*	0.50±0.22	0.33±0.21**	0.033
Leukocyte cell infiltration	0.33±0.21	1.50±0.43*	0.50±0.22**	0.33±0.21**	0.024

Data are presented as Mean±Standard Error of the Mean (SEM); ANOVA test was used for statistical analysis; significance level was set at p<0.05; *p<0.05: significant difference compared to the Control Group (C); **p<0.05: significant difference compared to the Ischemia-Reperfusion Group (IR)

Biochemical Results

Biochemical analysis revealed significant differences in oxidative stress markers among groups (p<0.001, Table 2). TAS levels were significantly lower in the IR and IR+M8 groups compared to the control (p<0.001, p=0.011), but metformin treatment significantly increased TAS in both IR+M4 and IR+M8 groups relative to IR (p<0.001), indicating its antioxidant potential.

Similarly, TOS levels were significantly higher in the IR and IR+M8 groups than in the control (Table 2, p<0.001). However, metformin administration led to a substantial reduction in TOS levels in both IR+M4 and IR+M8 groups compared to IR (p<0.001), with IR+M8 showing a greater decrease than IR+M4 (p=0.018), demonstrating a dose-dependent effect.

OSI levels followed the same trend, being significantly elevated in the IR group compared to the control (p<0.001), while metformin treatment in both IR+M4 and IR+M8 groups significantly reduced OSI levels compared to IR (p<0.001), confirming its role in mitigating oxidative stress and restoring redox balance (Table 2).

Table 2. Oxidative status parameters of the gastrocnemius muscle tissue (Mean±SEM)

	Control group (C)	Ischemia reperfusion group (IR)	IR+metformin 4mg/kg group (IR+M4)	IR+metformin 8mg/kg group (IR+M8)	p-value
Total antioxidant status (TAS) (mmol/L)	2.38±0.20	0.50±0.04*	1.91±0.07*,**	2.21±0.11**	<0.001
Total oxidant status (TOS) (µmol/L)	4.58±0.52	49.73±3.65*	21.90±3.69*,**	11.42±2.42**,***	<0.001
Oxidative stress index (OSI)	0.02±0.00	1.04±0.14*	0.11±0.02**	0.05±0.01**	<0.001

Data are presented as Mean±Standard Error of the Mean (SEM); ANOVA test was used for statistical analysis; significance level was set at p<0.05; *p<0.05: Significant difference compared to the Control Group (C); **p<0.05: Significant difference compared to the Ischemia-Reperfusion Group (IR); ***p<0.05: Significant difference compared to the IR+Metformin 4mg/kg Group (IR+M4)

DISCUSSION

This study investigated the effects of metformin on gastrocnemius muscle tissue following experimentally induced IR injury. To the best of our knowledge, this is the first foundational study to examine the protective role of metformin in lower extremity IR injury. These findings provide a basis for future advanced research incorporating molecular and genetic analyses to further explore the underlying mechanisms, including the evaluation of relevant signaling pathways.

Metformin exerts significant protective effects across various organ systems by alleviating IR injury. Due to the unique oxidative damage profiles of different organ systems, the administered dose varied among studies. In ovarian and testicular IR models, doses ranging from 50 to 300 mg/kg were employed [20,22,28]. In pulmonary studies, higher doses of 200–300 mg/kg were selected, likely to investigate the systemic effects of IR injury [23,29]. For myocardial, hepatic, renal, and intestinal IR injury models, metformin doses ranged from 50 to 250 mg/kg [24,25,30-32]. However, metformin demonstrated protective effects at lower doses, such as 4 mg/kg in an infection-induced myocardial dysfunction model and 10 mg/kg in a cerebral IR model [26,33]. As no studies have directly examined lower extremity IR injury, and considering metformin's efficacy at lower doses, we selected 4 mg/kg and 8 mg/kg for administration, based on existing literature. Metformin has been administered intraperitoneally, intravenously, and orally, with all routes demonstrating effectiveness [22-26,28-33]. Although high doses did not result in metabolic or life-threatening effects due to metformin's glucose-lowering properties in previous studies, we opted for a lower dose to minimize potential interactions [20,23,24,34]. In our study, blood glucose levels were not measured; however, no physiological indicators of hemodynamic instability were observed, aligning with findings in the literature.

Metformin exhibits significant protective effects against IR injury through its antioxidant, anti-inflammatory, and anti-apoptotic properties [15-18,20-25]. A key mechanism involves AMPK activation, a crucial regulator of cellular metabolism, which enhances antioxidant defenses by upregulating enzymes such as SOD and catalase, thereby mitigating oxidative stress during reperfusion [15,19]. Studies have demonstrated metformin's efficacy in reducing oxidative stress, inflammation, and apoptosis across various IR models. For instance, Topcu et al. reported decreased MDA and TNF-α levels alongside increased glutathione (GSH) and estradiol (E2) in ovarian IR injury, preventing vascular congestion and tissue damage [22]. Similarly, An et al. found that metformin enhanced antioxidant enzyme activity while reducing TNF-α levels, alleviating hepatic IR injury [25]. Additionally, metformin improved oxygenation and reduced inflammatory and oxidative markers in lung IR models [29]. This protective effect was evidenced by a decrease in the wet-to-dry lung weight ratio and lower levels of inflammatory markers (TNF-α, IL-1, and IL-6) and oxidative stress markers (myeloperoxidase, SOD, and MDA). Our findings align with these studies, demonstrating that metformin significantly elevates TAS while reducing TOS in a dose-dependent manner. Notably, TAS levels in the IR+M8 group were comparable to those in the control group, suggesting an optimal effect at 8 mg/kg. A significant dose-dependent decrease in TOS levels was observed between the IR group and the metformin-treated groups, with a more pronounced reduction in TOS at the 8 mg/kg dose. These findings suggest that metformin exerts a dose-dependent enhancing effect on antioxidant mechanisms in lower extremity IR injury. However, OSI calculations did not reflect these dose-dependent changes. Furthermore, metformin enhances endothelial function by stimulating NO production via eNOS activation, promoting vasodilation and reducing vascular injury [15,16]. This protective effect may result from direct eNOS activation or synergy with AMPK signaling. Both our study and existing literature consistently highlight metformin's role in enhancing antioxidant defenses and reducing oxidative stress. However, further research with expanded parameters and mechanistic analyses across different doses is needed to clarify its protective pathways.

Metformin reduces ROS levels, thereby limiting lipid peroxidation, protein oxidation, and DNA damage. By preserving mitochondrial integrity, it prevents mPTP opening, a key trigger of apoptosis [17]. This protective mechanism also minimizes histopathological damage. Topcu et al. reported that metformin reduced vascular congestion, hemorrhage, and tissue degeneration [22], findings supported by Sayan et al. [28]. Similarly, metformin mitigated histopathological damage in cardiac and hepatic tissues, reducing edema, congestion, and necrosis [24,25]. In our study, histopathological evaluation of the gastrocnemius muscle revealed that metformin reduced muscle atrophy, degeneration, congestion, nuclear internalization, and fragmentation/hyalinization, aligning with previous findings. However, no significant dose-dependent differences were observed between the 4 mg/kg and 8 mg/kg groups. Since this study is the first to examine metformin's effects on lower extremity muscle using only two doses, further research with a broader dosage range and additional histopathological parameters is needed to clarify its protective mechanisms.

Metformin exerts anti-inflammatory effects by downregulating pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 through the inhibition of NF-κB and JNK signaling pathways [15,17]. This suppression reduces leukocyte adhesion and infiltration, thereby mitigating tissue damage. In our study, histopathological analysis showed a significant reduction in leukocyte infiltration in both the IR+4 mg/kg and IR+8 mg/kg groups, consistent with previous findings. However, as our study did not evaluate specific cytokine markers or signaling pathways, further research incorporating molecular analyses is necessary to fully elucidate metformin's anti-inflammatory mechanisms.

Limitations

While this study provides key evidence of metformin's protective effects against IR injury in lower extremity muscles, several limitations should be noted. The rat model, though useful for mechanistic insights, may not fully replicate human IR injury, limiting clinical translation. Additionally, 120-minute reperfusion period may not capture long-term effects, warranting extended reperfusion studies for a better understanding of sustained tissue recovery. The focus on the gastrocnemius muscle excludes other muscles and distant organs that are also susceptible to IR injury. Future research should explore systemic effects to assess metformin's broader therapeutic potential. The study also examined only two doses, limiting insight into its dose-response relationship. Expanding the dose range could help determine the optimal therapeutic window. Moreover, while oxidative stress markers were assessed, key inflammatory mediators and molecular pathways were not evaluated. Future studies incorporating cytokine profiling and molecular analyses will enhance understanding of metformin's anti-inflammatory mechanisms. Addressing these limitations will provide a more comprehensive evaluation of its therapeutic potential in IR injury.

CONCLUSION

This study highlights metformin's protective role against ischemia-reperfusion (IR) injury in skeletal muscle, primarily via its antioxidant, anti-inflammatory, and anti-apoptotic properties. Histopathological and biochemical analyses indicate that metformin, particularly at 8 mg/kg, reduces muscle degeneration, vascular congestion, leukocyte infiltration, and oxidative stress. These findings suggest its potential in mitigating IR-induced damage by regulating oxidative and inflammatory responses. Further studies should explore long-term efficacy, optimal dosing, and molecular mechanisms. Clinical trials are crucial to assess its translational value in preventing IR-related complications, reinforcing metformin's potential as a therapeutic agent for ischemic tissue protection.

Ethics Committee Approval: All experimental procedures adhered to ethical standards approved by the Gazi University Experimental Animal Ethics Committee (G.Ü.ET-24.121) and were performed at the university's animal laboratory.

Patient Consent for Publication: Patient consent form is not required.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: All authors contributed equally to the article.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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