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Potential Protective Effects of Metformin on Remote Organ Injury in the Liver, Kidney, and Lung Following Experimental Lower-Extremity Ischemia–Reperfusion in Non-Diabetic Rats

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ABSTRACT

Background: Lower-extremity ischemia–reperfusion (I/R) injury triggers a systemic inflammatory and oxidative response that extends beyond the primary ischemic site, leading to remote organ damage and increased morbidity and mortality. Metformin has been reported to exert antioxidant and anti-inflammatory effects independent of its glucose-lowering properties; however, its effects on distant organ injury following limb I/R under non-diabetic conditions remain incompletely understood.

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Methods: Twenty-four male Wistar albino rats were randomly allocated into four groups (n=6 each): control, I/R, I/R + metformin 4 mg/kg, and I/R + metformin 8 mg/kg. Lower-extremity ischemia was induced by infrarenal abdominal aortic clamping for 45 minutes, followed by 120 minutes of reperfusion. Metformin was administered intraperitoneally immediately before reperfusion. Liver, lung, and kidney tissues were collected for the assessment of total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI), along with histopathological evaluation.

Results: Compared with the control group, I/R significantly increased TOS and OSI levels and decreased TAS levels in liver, lung, and kidney tissues ($p < 0.001$), accompanied by marked histopathological alterations, including hepatocellular degeneration, pulmonary edema and inflammation, and renal tubular and glomerular injury. Metformin administration was associated with significant attenuation of oxidative stress parameters and histopathological damage in all examined organs compared with the I/R group. Both doses improved TAS and reduced TOS and OSI levels; however, the 8 mg/kg dose demonstrated more consistent biochemical improvement and more pronounced histopathological findings. In particular, higher-dose metformin was associated with reduced hepatic sinusoidal dilatation and necrosis, decreased pulmonary alveolar wall thickening and total lung injury scores, and attenuated renal tubular degeneration, inflammation, and cast formation.

Conclusions: Metformin may attenuate remote liver, lung, and kidney injury following lower-extremity I/R in non-diabetic rats, potentially through mechanisms related to the modulation of oxidative stress. The observed dose-related effects suggest a possible systemic protective role; however, further experimental and clinical studies are required to confirm these findings and clarify the underlying mechanisms.

Keywords: Metformin; Ischemia–Reperfusion; Limb; Rat model; Oxidative stress

INTRODUCTION

Ischemia–reperfusion (I/R) injury is a complex biphasic pathological process in which the temporary interruption of blood flow followed by reperfusion leads to structural and functional tissue damage [1,2]. During the ischemic phase, oxygen deprivation shifts cellular metabolism from oxidative phosphorylation to anaerobic glycolysis, resulting in ATP depletion, lactate accumulation, intracellular acidosis, and dysfunction of energy-dependent ion pumps, including Na^+/K^+-ATPase and Ca^{2+}-ATPase [3,4]. These alterations lead to intracellular sodium and calcium overload, mitochondrial depolarization, cellular swelling, and activation of proteolytic and lipolytic enzymes, ultimately disrupting membrane integrity and organelle function [1,5]. In parallel, microvascular perfusion becomes heterogeneous due to endothelial swelling, leukocyte adhesion, and increased blood viscosity, further exacerbating tissue hypoxia and predisposing tissues to more severe injury upon reperfusion [5].

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Reperfusion, although essential for tissue survival, paradoxically exacerbates ischemic injury through the abrupt reintroduction of oxygen to metabolically compromised mitochondria, resulting in excessive production of reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, hydroxyl radicals, and peroxynitrite formed through interaction with nitric oxide (NO) [1,2]. Major sources of ROS in the post-ischemic environment include the mitochondrial electron transport chain, xanthine oxidase, NADPH oxidases, and uncoupled endothelial nitric oxide synthase [4]. Accumulation of ROS leads to lipid peroxidation, protein and DNA damage, activation of poly(ADP-ribose) polymerase, ATP depletion, mitochondrial permeability transition pore opening, cytochrome-c release, and ultimately apoptotic or necrotic cell death [2,3].

In addition to oxidative stress, reperfusion induces a pronounced inflammatory response characterized by endothelial activation, leukocyte recruitment, and microcirculatory dysfunction. Upregulation of adhesion molecules promotes neutrophil adhesion and transmigration, resulting in the release of proteolytic enzymes, myeloperoxidase, and additional ROS, thereby amplifying tissue injury and increasing vascular permeability [2,5]. Pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), are rapidly released into the systemic circulation and contribute to systemic inflammatory response syndrome and endothelial dysfunction in distant organs [6,7]. Consequently, I/R injury is not limited to the primary ischemic site; experimental lower-extremity ischemia has been shown to induce secondary oxidative and inflammatory damage in remote organs such as the lungs, kidneys, and liver, manifested by alveolar injury, tubular damage, and hepatocellular degeneration [6,8–10]. This multi-organ involvement significantly increases morbidity and mortality in clinical conditions such as vascular surgery, trauma, and acute limb ischemia, highlighting the need for therapeutic strategies targeting both local and systemic effects of I/R injury [5,7].

Remote organ involvement most commonly affects the lungs, kidneys, liver, and cardiovascular system, with the lungs often being affected at an early stage due to their extensive capillary network and continuous exposure to circulating inflammatory mediators [1]. If not adequately controlled, this systemic response may progress to systemic inflammatory response syndrome (SIRS) and eventually multiple organ dysfunction syndrome (MODS) [11]. Pulmonary tissue is particularly vulnerable, as accumulation of activated neutrophils within the pulmonary microvasculature increases vascular permeability, oxidative stress, and the risk of acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) [8,12]. In addition to inflammatory pathways, mitochondrial dysfunction and endothelial barrier disruption have emerged as key contributors to remote organ injury following I/R. Impaired mitochondrial function, excessive ROS production, and loss of endothelial integrity further promote tissue hypoxia and activation of apoptotic and necrotic pathways, leading to organ-specific complications such as acute kidney injury (AKI) and hepatic damage [8]. Therefore, therapeutic strategies targeting oxidative stress, inflammatory cascades, and mitochondrial dysfunction are critical for reducing morbidity and mortality associated with I/R-induced distant organ injury [5,7,13,14].

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Metformin, a biguanide widely used as a first-line treatment for type 2 diabetes mellitus, has recently gained attention for its cytoprotective and organ-protective effects beyond glycemic control [15]. A growing body of experimental evidence indicates that metformin has demonstrated protective effects against I/R injury in multiple organs, even under non-diabetic conditions, suggesting mechanisms independent of glucose metabolism [16–30]. In myocardial I/R models, metformin has been shown to reduce infarct size, improve functional recovery, and attenuate apoptosis through activation of AMP-activated protein kinase (AMPK), enhancement of antioxidant defenses, and modulation of inflammatory signaling pathways [22,23,25,27]. Similar protective effects have been demonstrated in renal I/R models, where metformin has been shown to reduce tubular necrosis, oxidative stress, and inflammatory signaling, while attenuating apoptosis and fibrotic remodeling [16,20,31–34]. In hepatic I/R injury, metformin has been reported to reduce hepatocellular damage and improve sinusoidal endothelial function through modulation of inflammatory pathways, autophagy, and mitochondrial homeostasis [21,28,29]. Emerging evidence also suggests that metformin may suppress pyroptosis and NLRP3 inflammasome activation and may exert neuroprotective effects against mitochondrial dysfunction following cardiac I/R [19,24]. In addition, experimental studies in limb I/R models have shown that metformin reduces skeletal muscle injury, vascular congestion, leukocyte infiltration, and oxidative stress [26]. Despite these well-established antioxidant and anti-inflammatory properties, the effects of metformin on remote organ injury following lower-extremity I/R, particularly in non-diabetic experimental settings, remain insufficiently investigated.

Therefore, the present study aimed to evaluate the effects of metformin on remote organ injury in the liver, lungs, and kidneys in a non-diabetic rat model of lower-extremity ischemia–reperfusion injury.

MATERIALS and METHODS

Animals

All experimental procedures were performed using male Wistar albino rats, aged approximately 12 weeks and weighing between 250 and 350 g. The animals were obtained from and maintained at the Gazi University Experimental Research Center (Ankara, Turkey). The study protocol was approved by the Gazi University Experimental Animal Ethics Committee (approval no. G.Ü.ET-25.007). All procedures were conducted in accordance with institutional and international guidelines for the care and use of laboratory animals, as well as the ARRIVE guidelines.

Throughout the experimental period, rats were housed individually under standardized laboratory conditions, with ambient temperature maintained at 20–21 °C and relative humidity at 45–55%. A controlled 12 h light/12 h dark cycle was applied, and all animals were allowed free access to standard chow and distilled water ad libitum.

Drug

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Metformin doses were selected based on previously published experimental studies demonstrating tissue-protective effects in ischemia–reperfusion and related oxidative injury models [34]. Metformin was freshly prepared on the day of the experiment and dissolved in sterile 0.9% saline without the use of additional carriers or enzyme inhibitors. Based on prior experimental evidence and the approved study protocol, metformin was administered intraperitoneally at doses of 4 mg/kg and 8 mg/kg to the respective treatment groups.

Metformin was administered immediately before reperfusion because the early reperfusion period is recognized as a critical phase during which oxidative stress generation, inflammatory activation, endothelial dysfunction, and remote organ injury are rapidly initiated. This timing was selected to evaluate the potential therapeutic effects of metformin at a clinically relevant intervention point, where treatment may be administered after ischemia has occurred but before or at the onset of reperfusion. The present study was not designed to compare alternative administration schedules, such as ischemic preconditioning, repeated dosing regimens, or delayed post-reperfusion treatment strategies, which warrant investigation in future studies.

Experimental Protocol

A total of 24 male Wistar albino rats were randomly allocated into four experimental groups (n = 6 per group): control (C), ischemia–reperfusion (IR), IR + metformin 4 mg/kg (IR+M4), and IR + metformin 8 mg/kg (IR+M8). Randomization was performed using a computer-generated random number sequence.

All surgical interventions were performed under general anesthesia induced by intramuscular administration of ketamine hydrochloride (50 mg/kg; Ketax®, Vem Ilac San. Tic. A.S., Istanbul, Turkey) and xylazine hydrochloride (10 mg/kg; Alfazyne®, 2%, Ege Vet Ltd., Izmir, Turkey). Anesthetic depth was assessed by loss of the pedal withdrawal reflex, and supplemental doses were administered as required to maintain adequate anesthesia. Following induction, animals were placed in the supine position, and body temperature was maintained at 37 ± 0.5 °C using a warming pad.

In the control group, a midline laparotomy was performed to expose the abdominal aorta without vascular occlusion. An equivalent volume (0.3 mL) of sterile 0.9% saline was administered intraperitoneally 45 minutes after laparotomy, and animals were observed for 2 hours before euthanasia.

In the IR group, lower-extremity ischemia was induced by placement of an atraumatic microvascular clamp on the infrarenal abdominal aorta for 45 minutes, followed by 120 minutes of reperfusion after clamp removal.

In the treatment groups, metformin was administered intraperitoneally at doses of 4 mg/kg or 8 mg/kg immediately before the onset of reperfusion, after completion of the 45-minute ischemic period. Reperfusion was then allowed for 120 minutes.

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At the end of the experimental period, all animals were euthanized under deep anesthesia by exsanguination via the abdominal aorta following administration of high-dose ketamine and xylazine. Death was confirmed by cessation of cardiac and respiratory activity. Subsequently, lung, kidney, and liver tissues were harvested promptly for biochemical and histopathological analyses.

Histopathological Evaluation

Tissue samples obtained from the liver, kidney, and lung were immediately fixed in 10% neutral buffered formalin at room temperature for 48 hours. Following fixation, specimens underwent routine dehydration, clearing, and paraffin embedding procedures. Serial sections (4 μm thickness) were cut from paraffin blocks using a rotary microtome (Leica RM2245, Leica Microsystems, Wetzlar, Germany) and stained with hematoxylin and eosin (H&E) for microscopic examination.

For each experimental group, tissue sections were systematically examined, and representative areas were selected for evaluation. Each microscopic field was assessed once to avoid duplicate scoring. Histopathological evaluations were performed using a light microscope (Leica DM 4000 B, Leica Microsystems, Wetzlar, Germany) equipped with a digital imaging system, and images were captured using Leica LAS V4.9 software. All evaluations were conducted at 100× magnification. Histopathological assessments were performed by an investigator blinded to the experimental groups.

Liver

Hepatic sections were evaluated for hydropic degeneration, sinusoidal dilatation, nuclear pyknosis, hepatocellular necrosis, and mononuclear inflammatory cell infiltration. Structural alterations were assessed semi-quantitatively using a scoring system defined as follows: 0 (no detectable alteration), 1 (mild), 2 (moderate), and 3 (severe).

Renal

Renal injury was assessed using a semi-quantitative scoring system based on established criteria. The evaluated parameters included glomerular vacuolization (GV), tubular dilatation (TD), vascular vacuolization and hypertrophy (VVH), tubular cell degeneration and necrosis (TCDN), Bowman's space dilatation (BSD), tubular hyaline casts (THC), lymphocyte infiltration (LI), and tubular cell shedding (TCS). Each parameter was graded on a four-point scale: 0 (no pathological finding), 1 (mild), 2 (moderate), and 3 (severe).

Lung

Pulmonary sections were examined to assess lung injury based on neutrophil infiltration and thickening of the alveolar walls. The severity of each parameter was graded as absent, mild (+), moderate (++), or severe (+++). The sum of individual scores was used to calculate the total lung injury score for each specimen.

Biochemical Evaluation

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Liver, kidney, and lung tissue samples were rapidly snap-frozen in liquid nitrogen immediately after excision and stored at −80 °C until analysis. All procedures were performed under cold conditions to prevent enzymatic degradation. Approximately 80–100 mg of tissue was weighed and homogenized in 140 mM potassium chloride solution at a ratio of 1:10 (w/v). Homogenization was performed using a tissue homogenizer, and samples were kept on ice throughout the process.

The homogenates were centrifuged at 3000 × g for 10 minutes at 4 °C, and the supernatants were collected for biochemical analyses. Total antioxidant status (TAS) and total oxidant status (TOS) were measured using commercially available colorimetric assay kits (RelAssay Diagnostics, Gaziantep, Turkey) in accordance with the manufacturer's instructions.

TAS values were calculated using Trolox equivalents, and TOS values were calculated using hydrogen peroxide equivalents. All measurements were performed in triplicate, and mean values were used for statistical analysis.

The oxidative stress index (OSI) was calculated to reflect the balance between oxidant and antioxidant status using the following formula:

OSI (arbitrary units) = [TOS (μmol H_2O_2 equivalent/L) / TAS (μmol Trolox equivalent/L)] × 100.

Statistical Analysis

All statistical analyses were conducted using SPSS software (version 26.0; IBM Corp., Armonk, NY, USA). Data normality was evaluated using the Shapiro–Wilk test. Based on the distribution characteristics of the variables, comparisons between groups were performed using one-way analysis of variance (ANOVA) for normally distributed variables or the Kruskal–Wallis multiple-comparison procedure for non-normally distributed variables, as appropriate. Following a significant overall group difference, pairwise comparisons were performed using Kruskal-Wallis test followed by Dunn's test or one-way ANOVA followed by Tukey's test. Data are expressed as mean ± standard deviation (SD) or standard error (SE), and a p-value < 0.05 was accepted as the threshold for statistical significance.

RESULTS

Biochemical Results

Liver Tissue

When liver tissue TAS levels were compared among the groups, a statistically significant difference was observed overall (Table 1, $p<0.001$). TAS levels were significantly lower in the IR and IR+M8 groups compared with the C group ($p<0.001$ for both comparisons). In contrast, TAS levels were significantly higher in the IR+M4 and IR+M8 groups compared with the IR group ($p<0.001$ for both). Moreover, TAS levels in the IR + M8 group were significantly higher than those in the IR + M4 group ($p<0.001$).

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Analysis of liver tissue TOS also revealed a significant difference among the groups (Table 1, $p<0.001$). TOS levels were significantly higher in the IR and IR+M4 groups compared with the control group ($p<0.001$ and $p=0.001$, respectively). However, TOS levels were significantly lower in the IR+M4 and IR+M8 groups compared with the IR group ($p<0.001$ for both). Additionally, TOS levels in the IR+M8 group were significantly lower than those in the IR+M4 group ($p=0.016$).

Similarly, comparison of liver tissue OSI values demonstrated a statistically significant difference among the groups (Table 1, $p<0.001$). OSI levels were significantly higher in the IR and IR + M4 groups compared with the control group ($p<0.001$ and $p=0.009$, respectively). In contrast, OSI values were significantly reduced in the IR+M4 and IR+M8 groups compared with the IR group ($p<0.001$ for both). Furthermore, OSI levels in the IR+M8 group were significantly lower than those in the IR + M4 group ($p=0.026$).

Table 1. Biochemical analyses of the liver tissue (Mean ± SD)

	Group C (n = 6)	**Group IR (n = 6)**	**Group IR+M4 (n = 6)**	**Group IR+M8 (n = 6)**	**p Value (ANOVA)**
Total antioxidant status (mmol/L)	1.96±0.10	0.68±0.06*	1.37±0.17*,&	2.02±0.21&,+	< 0.001
Total oxidative status (µmol/L)	7.22±0.59	49.79±4.03*	22.81±12.95*,&	12.08±3.61&,+	< 0.001
Oxidative Stress Index	0.37±0.03	7.31±1.16*	1.71±1.09&	0.60±0.19&,+	< 0.001

p Values indicate overall significance determined by one-way ANOVA ($p<0.05$ considered statistically significant), *$p<0.05$ vs. Control group, &$p<0.05$ vs. IR group, +$p<0.05$ vs. IR+M4 group. C, control; IR, ischemia–reperfusion; IR+M4, ischemia–reperfusion + metformin 4 mg/kg; IR+M8, ischemia–reperfusion + metformin 8 mg/kg.

Lung Tissue

Comparison of lung tissue TAS levels among the groups revealed a statistically significant difference (Table 2, $p<0.001$). TAS levels were significantly lower in the IR, IR+M4, and IR+M8 groups compared with the C group ($p<0.001$, $p<0.001$, and $p=0.015$, respectively). In contrast, TAS levels were significantly higher in the IR+M4 and IR+M8 groups compared with the IR group

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(p<0.001 for both). Moreover, TAS levels in the IR+M8 group were significantly higher than those in the IR+M4 group (p<0.001).

Analysis of lung tissue TOS demonstrated a significant difference among the groups (Table 2, p<0.001). TOS levels were significantly elevated in all experimental groups compared with the control group (p<0.001 for all). However, TOS levels were significantly lower in the IR+M4 and IR+M8 groups compared with the IR group (p=0.002 and p<0.001, respectively). In addition, TOS levels in the IR+M8 group were significantly lower than those in the IR+M4 group (p<0.001).

Similarly, lung tissue OSI values differed significantly among the groups (Table 2, p<0.001). OSI levels were significantly higher in the IR, IR+M4, and IR+M8 groups compared with the control group (p<0.001, p<0.001, and p=0.017, respectively). Conversely, OSI levels were significantly reduced in the IR+M4 and IR+M8 groups compared with the IR group (p<0.001 for both). Furthermore, OSI values in the IR+M8 group were significantly lower than those in the IR+M4 group (p<0.001).

Table 2. Biochemical analyses of the lung tissue (Mean ± SD)

	Group C (n = 6)	Group IR (n = 6)	Group IR+M4 (n = 6)	Group IR+M8 (n = 6)	p Value (ANOVA)
Total antioxidant status (mmol/L)	2.10±0.19	0.74±0.08*	1.54±0.18*,&	1.88±0.10*,&,+	< 0.001
Total oxidative status (µmol/L)	6.96±1.15	49.35±4.14*	39.90±4.83*,&	20.21±6.30*,&,+	< 0.001
Oxidative Stress Index	0.33±0.04	6.75±0.90*	2.61±0.28*,&	1.09±0.38*,&,+	< 0.001

p Values indicate overall significance determined by one-way ANOVA (p<0.05 considered statistically significant), *p<0.05 vs. Control group, &p<0.05 vs. IR group, +p<0.05 vs. IR+M4 group. C, control; IR, ischemia–reperfusion; IR+M4, ischemia–reperfusion + metformin 4 mg/kg; IR+M8, ischemia–reperfusion + metformin 8 mg/kg

Renal Tissue

Comparison of renal tissue TAS levels among the groups demonstrated a statistically significant difference (Table 3, p<0.001). TAS levels were significantly lower in the IR and IR+M8 groups compared with the C group (p<0.001 for both). In contrast, TAS levels were significantly higher in the IR+M4 and IR+M8 groups compared with the IR group (p<0.001 for both). Furthermore, TAS levels in the IR+M8 group were significantly higher than those in the IR+M4 group (p<0.001).

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Analysis of renal tissue TOS revealed a significant difference among the groups (Table 3, $p<0.001$). TOS levels were significantly elevated in the IR and IR+M4 groups compared with the control group ($p<0.001$ and $p=0.002$, respectively). However, TOS levels were significantly reduced in the IR+M4 and IR+M8 groups compared with the IR group ($p<0.001$ for both).

Similarly, renal tissue OSI values differed significantly among the groups (Table 3, $p<0.001$). OSI levels were significantly higher in the IR and IR + M4 groups compared with the control group ($p<0.001$ and $p=0.023$, respectively). Conversely, OSI levels were significantly lower in the IR+M4 and IR+M8 groups compared with the IR group ($p<0.001$ for both).

Table 3. Renal tissue oxidative status parameters (Mean ± SD)

	Group C (n = 6)	Group IR (n = 6)	Group IR+M4 (n = 6)	Group IR+M8 (n = 6)	p Value (ANOVA)
Total antioxidant status (mmol/L)	2.17±0.21	0.54±0.13*	1.38±0.15*,&	2.13±0.23&,+	< 0.001
Total oxidative status (µmol/L)	6.72±0.59	39.77±7.80*	21.29±11.92*,&	14.82±3.08&	< 0.001
Oxidative Stress Index	0.31±0.05	7.58±1.51*	1.60±1.00*,&	0.71±0.19&	< 0.001

p Values indicate overall significance determined by one-way ANOVA ($p<0.05$ considered statistically significant), *$p<0.05$ vs. Control group, &$p<0.05$ vs. IR group, +$p<0.05$ vs. IR+M4 group. C, control; IR, ischemia–reperfusion; IR+M4, ischemia–reperfusion + metformin 4 mg/kg; IR+M8, ischemia–reperfusion + metformin 8 mg/kg.

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Histopathological Results

Liver Tissue

Evaluation of liver tissue morphology demonstrated statistically significant differences among the groups with respect to hepatocyte degeneration, sinusoidal dilatation, pyknotic nuclei, cells undergoing necrosis, and mononuclear (MN) cell infiltration in the parenchyma ($p=0.019$, $p=0.014$, $p=0.020$, $p=0.020$, and $p=0.020$, respectively) (Table 4; Figures 1 (a-d)).

Hepatocyte degeneration was significantly more pronounced in the IR group than in the C group ($p=0.004$). In addition, hepatocyte degeneration was significantly reduced in both the IR+M4 and IR+M8 groups compared with the IR group ($p=0.040$ and $p=0.012$, respectively). Sinusoidal dilatation was significantly greater in the IR group compared with the control group ($p=0.002$). Furthermore, sinusoidal dilatation was significantly reduced in the IR+M8 group compared with the IR group ($p=0.020$). The presence of pyknotic nuclei was significantly higher in the IR group than in the control group ($p=0.003$). This parameter was significantly lower in the IR + M8 group

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compared with the IR group (p=0.033). The number of cells undergoing necrosis was significantly greater in the IR group compared with the control group (p=0.003). In contrast, this finding was significantly reduced in the IR+M8 group relative to the IR group (p=0.024). Similarly, mononuclear cell infiltration in the hepatic parenchyma was significantly increased in the IR group compared with the control group (p=0.003), while a significant reduction was observed in the IR+M8 group compared with the IR group (p=0.024).

Table 4. Histopathological analysis scoring of the renal tissue (Mean ± SE)

	Group C (n = 6)	Group IR (n = 6)	Group IR+M4 (n = 6)	Group IR+M8 (n = 6)	p Value (Kruskal –Wallis)
Hepatocyte degeneration	0.33±0.21	1.33±0.21*	0.67±0.21&	0.50±0.22&	0.019
Sinusoidal dilatation	0.17±0.17	1.33±0.21*	0.83±0.31	0.50±0.22&	0.014
Pyknotic nucleus	0.17±0.17	1.67±0.42*	1.00±0.37	0.67±0.21&	0.020
Cells undergoing necrosis	0.33±0.21	1.50±0.22*	0.83±0.31	0.67±0.21&	0.020
MN cell infiltration in the parenchyma	0.17±0.17	1.33±0.21*	0.67±0.33	0.50±0.22&	0.020

p Value indicates significance determined using the Kruskal–Wallis test ($p<0.05$).

*$p<0.05$ vs. Control group, &$p< 0.05$ vs. IR group. C, control; IR, ischemia–reperfusion; IR+M4, ischemia–reperfusion + metformin 4 mg/kg; IR+M8, ischemia–reperfusion + metformin 8 mg/kg.

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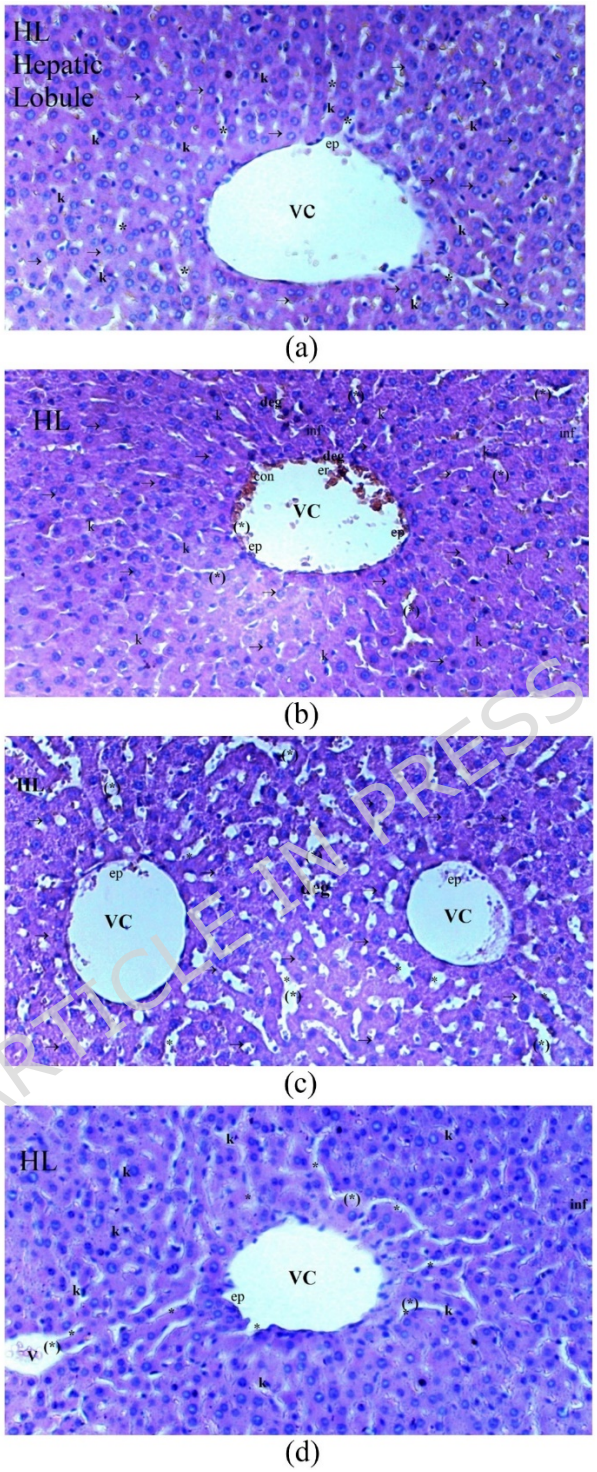


Figure 1 (a-d). Histopathological examination of liver tissues in the C, IR, IR + M4, and IR + M8 groups. (a): Normal liver architecture in the control (C) group, showing preserved hepatic lobular structure and intact hepatocytes, (b): Liver tissue from the IR group, demonstrating marked hepatocyte degeneration, congestion sinusoidal dilatation, pyknotic nuclei, and increased necrotic cells, (c): Liver tissue from the IR + M4 group, showing partial improvement with reduced

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hepatocyte degeneration and less pronounced sinusoidal changes compared with the IR group, (d): Liver tissue from the IR + M8 group, exhibiting near-normal hepatic architecture with minimal hepatocyte degeneration and significantly reduced sinusoidal dilatation and necrotic cells compared with the IR group. Abbreviations: vc—vena centralis; hl—hepatic lobule; →—hepatocytes; k—Kupffer cells; *—*sinusoids;* (*)—sinusoidal dilatation; cong—capillary congestion; ep—epithelium.

Lung Tissue

Evaluation of lung tissue morphology revealed statistically significant differences among the groups in terms of interstitial edema, interstitial hemorrhage, leukocyte infiltration (neutrophil/lymphocyte), thickness of the alveolar walls, and total damage score (p=0.005, p=0.019, p=0.043, p=0.020, and p=0.001, respectively) (Table 5; Figures 2 (a-d)).

Interstitial edema was significantly more pronounced in the IR group compared with the C group (p<0.001). In contrast, interstitial edema was significantly reduced in both the IR+M4 and IR+M8 groups compared with the IR group (p=0.032 and p=0.009, respectively). Interstitial hemorrhage was observed significantly more frequently in the IR group than in the control group (p=0.003). This finding was significantly reduced in both the IR+M4 and IR+M8 groups compared with the IR group (p=0.026 for both). Leukocyte infiltration (neutrophil/lymphocyte) was significantly greater in the IR group compared with the control group (p=0.006).The thickness of the alveolar walls was significantly increased in the IR group compared with the control group (p=0.003). Furthermore, alveolar wall thickness was significantly lower in the IR+M8 group than in the IR group (p=0.033). The total lung damage score was significantly higher in the IR and IR + M4 groups compared with the control group (p<0.001 and p=0.020, respectively). In contrast, total damage scores were significantly lower in both the IR+M4 and IR+M8 groups compared with the IR group (p=0.036 and p=0.011, respectively).

Table 5. Histopathological analysis scoring of the lung tissue (Mean ± SE)

	Group C (n = 6)	**Group IR (n = 6)**	**Group IR+M4 (n = 6)**	**Group IR+M8 (n = 6)**	**p Value (Kruskal –Wallis)**
Interstitial edema	0.33±0.21	1.50±0.22*	0.83±0.17&	0.67±0.21&	0.005
Interstitial hemorrhage	0.17±0.17	1.33±0.33*	0.50±0.22&	0.50±0.22&	0.019
Alveolar congestion	0.33±0.21	0.67±0.21	0.67±0.21	0.67±0.21	0.607
Leukocyte infiltration (neutrophil/lymphocyte)	0.17±0.17	1.50±0.34*	1.00±0.37	0.83±0.31	0.043

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Thickness of the alveolar walls	0.17±0.17	1.67±0.42*	1.00±0.37	0.67±0.21&	0.020
Total damage score	1.00±0.82	6.67±0.71*	4.00±0.73*,&	3.33±1.05&	0.001

p Value indicates significance determined using the Kruskal–Wallis test ($p<0.05$).

*$p<0.05$ vs. Control group, &$p<0.05$ vs. IR group. C, control; IR, ischemia–reperfusion; IR+M4, ischemia–reperfusion + metformin 4 mg/kg; IR+M8, ischemia–reperfusion + metformin 8 mg/kg.

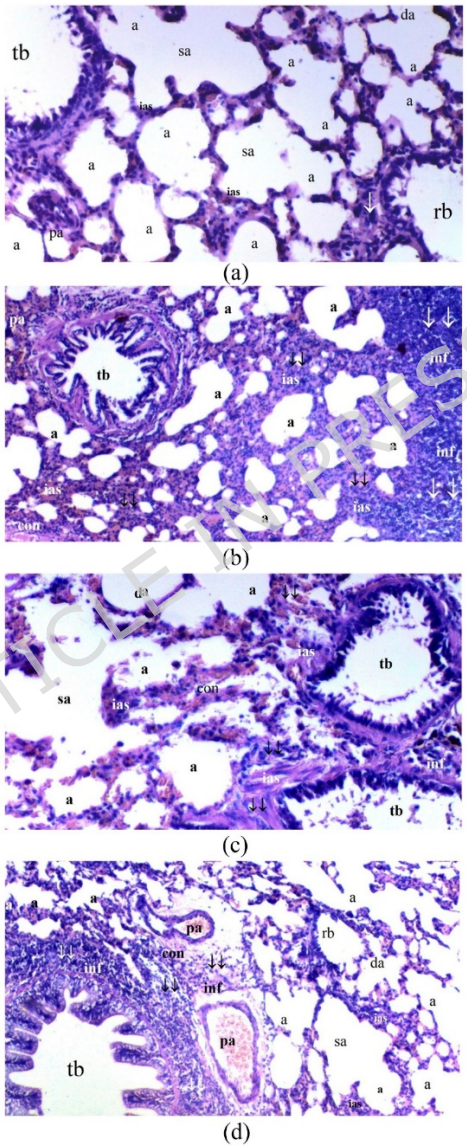


Figure 2. Histopathological examination of lung tissues in the C, IR, IR + M4, and IR + M8 groups. (a): Normal lung tissue in the control (C) group, showing preserved alveolar architecture and thin interalveolar septa, (b): Lung tissue from the IR group, demonstrating increased leukocyte

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infiltration, interstitial edema, capillary congestion, and marked thickening of the alveolar walls, (c): Lung tissue from the IR + M4 group, showing partial improvement with reduced leukocyte infiltration and less pronounced alveolar wall thickening compared with the IR group, (d): Lung tissue from the IR + M8 group, exhibiting near-normal lung architecture with minimal leukocyte infiltration and significantly reduced alveolar wall thickening compared with the IR group. Abbreviations: a—alveolus; sa—saccus alveolaris; da—ductus alveolaris; ias—interalveolar septum; pa—pulmonary artery; tb—terminal bronchiole; rb—respiratory bronchiole; cong—capillary congestion; ↓↓—pulmonary parenchyma.

Renal Tissue

Evaluation of renal tissue morphology demonstrated statistically significant differences among the groups with respect to glomerular vacuolization, tubular dilatation, vascular vacuolization and hypertrophy, tubular cell degeneration and necrosis, Bowman's space dilatation, tubular hyaline casts, lymphocyte infiltration, and tubular cell shedding ($p=0.015$, $p=0.006$, $p=0.047$, $p=0.005$, $p=0.020$, $p=0.045$, $p=0.010$, and $p=0.045$, respectively) (Table 6; Figures 3 (a-d)).

Glomerular vacuolization was significantly more pronounced in the IR group compared with the C group ($p=0.003$). This finding was significantly reduced in the IR+M8 group compared with the IR group ($p=0.009$). Tubular dilatation was significantly greater in the IR group than in the control group ($p<0.001$). In contrast, tubular dilatation was significantly reduced in both the IR+M4 and IR+M8 groups compared with the IR group ($p=0.014$ and $p=0.006$, respectively). Vascular vacuolization and hypertrophy were observed significantly more frequently in the IR group than in the control group ($p=0.007$). Tubular cell degeneration and necrosis were significantly increased in the IR group compared with the control group ($p<0.001$). This parameter was significantly reduced in both the IR+M4 and IR+M8 groups relative to the IR group ($p=0.032$ and $p=0.009$, respectively). Bowman's space dilatation was significantly greater in the IR group than in the control group ($p=0.003$). Furthermore, Bowman's space dilatation was significantly reduced in the IR+M8 group compared with the IR group ($p=0.024$). The presence of tubular hyaline casts was significantly higher in the IR group than in the control group ($p=0.007$). This finding was significantly reduced in the IR+M8 group compared with the IR group ($p=0.044$). Lymphocyte infiltration was significantly increased in the IR group compared with the control group ($p=0.004$). In contrast, lymphocyte infiltration was significantly reduced in both the IR+M4 and IR+M8 groups compared with the IR group ($p=0.012$ and $p=0.004$, respectively). Similarly, tubular cell shedding was significantly greater in the IR group than in the control group ($p=0.009$), while a significant reduction was observed in the IR+M8 group compared with the IR group ($p=0.026$).

Table 6. Renal tissue histopathological findings (Mean ± SE)

	Group C	Group IR	Group IR+M4	Group IR+M8	p Value

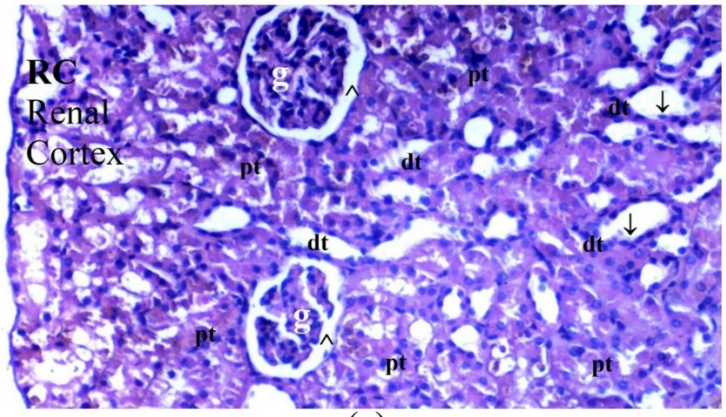
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	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(Kruskal–Wallis)
Glomerular vacuolization	0.33±0.21	1.50±0.22*	0.83±0.31	0.50±0.22&	0.015
Tubular dilatation	0.50±0.22	2.17±0.40*	1.00±0.26&	0.83±0.31&	0.006
Vascular vacuolization and hypertrophy	0.33±0.21	1.33±0.21*	1.00±0.26	1.00±0.26	0.047
Tubular cell degeneration and necrosis	0.17±0.17	1.33±0.21*	0.67±0.33&	0.50±0.22&	0.005
Bowman's space dilatation	0.17±0.17	1.33±0.21*	0.67±0.33	0.50±0.22&	0.020
Tubular hyaline casts	0.17±0.17	1.33±0.33*	0.67±0.33	0.50±0.22&	0.045
Lymphocyte infiltration	0.33±0.21	1.33±0.21*	0.50±0.22&	0.33±0.21&	0.010
Tubular cell shedding	0.50±0.22	1.50±0.22*	0.83±0.31	0.67±0.21&	0.045

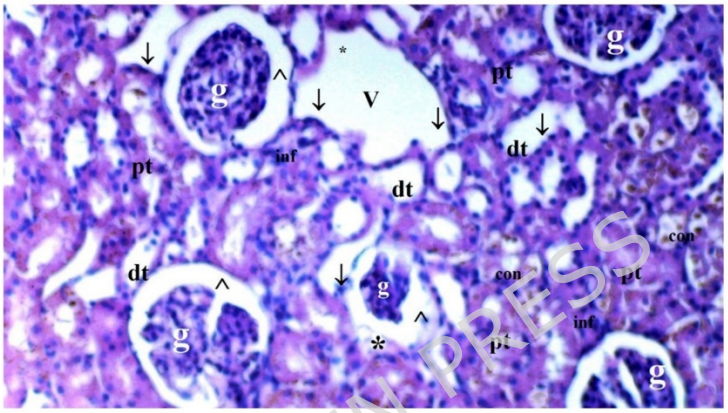
p Value indicates significance determined using the Kruskal–Wallis test ($p<0.05$), *$p<0.05$ vs. Control group, &$p<0.05$ vs. IR group. C, control; IR, ischemia–reperfusion; IR+M4, ischemia–reperfusion + metformin 4 mg/kg; IR+M8, ischemia–reperfusion + metformin 8 mg/kg.

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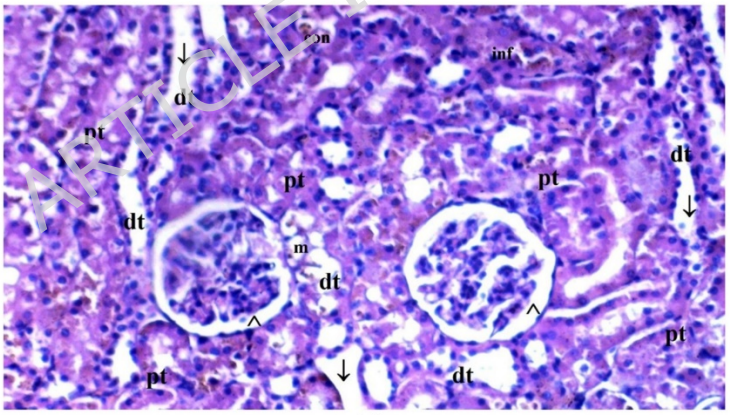
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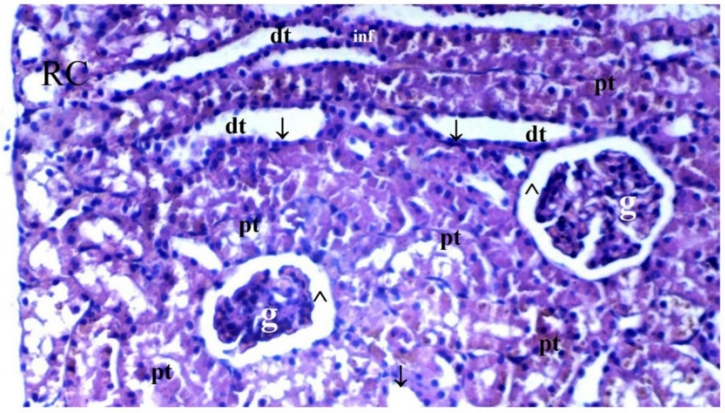
(a)



(b)



(c)



(d)

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Figure 3. Histopathological examination of renal tissues in the C, IR, IR + M4, and IR + M8 groups. (a): Normal renal tissue in the control (C) group, showing preserved glomerular and tubular architecture, (b): Renal tissue from the IR group, demonstrating marked tubular degeneration and necrosis, glomerular vacuolization, dilatation of Bowman's space, tubular dilatation, inflammatory cell infiltration, and vascular congestion, (c): Renal tissue from the IR + M4 group, showing partial improvement with reduced tubular degeneration and inflammatory changes compared with the IR group, (d): Renal tissue from the IR + M8 group, exhibiting near-normal renal architecture with mild residual degeneration and significantly reduced glomerular and tubular pathological changes compared with the IR group. Abbreviations: dt—distal tubule; pt—proximal tubule; g—glomerulus; *—degenerated glomerulus; ^—Bowman's space; ↓—dilated tubules; inf—inflammation; cong—vascular congestion; m—macula densa.

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DISCUSSION

The systemic consequences of ischemia–reperfusion (I/R) injury extend well beyond the primary ischemic site, frequently involving distant organs such as the lungs, kidneys, and liver, and contributing to systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction (MOD) [2,10,12,35]. Experimental and clinical studies of lower-limb I/R consistently demonstrate elevations in circulating pro-inflammatory cytokines, neutrophil activation, and biochemical markers of distant organ injury, supporting the concept that limb I/R initiates a systemic inflammatory cascade rather than a localized event [5,6,8].

Among remote organs, the lungs are particularly vulnerable. Hindlimb I/R models reproducibly induce acute lung injury (ALI) or acute respiratory distress syndrome (ARDS), characterized by increased microvascular permeability, interstitial and alveolar edema, impaired gas exchange, and pulmonary inflammation [36,37]. The attenuation of post-ischemic lung injury by antioxidant and anti-inflammatory agents such as N-acetylcysteine, melatonin, and quercetin underscores the central role of oxidative stress and neutrophil-mediated inflammation in pulmonary damage following limb I/R [38–42]. Conditioning strategies, including remote ischemic preconditioning, have also been shown to mitigate pulmonary injury by modulating inflammatory pathways and enhancing endogenous antioxidant defenses [35,43].

The kidneys are similarly susceptible, frequently developing acute kidney injury (AKI) characterized by tubular epithelial swelling, vacuolization, and necrosis, accompanied by

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activation of inflammatory pathways such as Toll-like receptor-4 and nuclear factor-κB [10,44]. In addition, ischemic postconditioning has been shown to reduce both histopathological damage and inflammatory responses in renal tissue, suggesting that renal injury arises from the interplay of inflammatory and microcirculatory disturbances [8,44]. Although not directly ischemic, the liver is also affected by systemic I/R, exhibiting sinusoidal congestion, hepatocellular degeneration, cytoplasmic vacuolization, and necrosis, together with biochemical evidence of oxidative stress following lower-extremity I/R [10].

While direct evidence linking limb I/R to hepatic injury remains limited, hepatic dysfunction is thought to result from circulating inflammatory mediators and oxidative stress. High-mobility group box-1 (HMGB1) has been identified as a key early mediator, acting through pattern-recognition receptors such as Toll-like receptor-4 to amplify systemic inflammation [5,9,45,46]. Collectively, these findings indicate that limb I/R induces a systemic pathophysiological response that affects distant organs through convergent mechanisms involving oxidative stress, cytokine release, neutrophil recruitment, and endothelial dysfunction. This multi-organ involvement substantially increases morbidity and mortality, highlighting the importance of therapeutic strategies targeting both local and systemic components of I/R injury.

Metformin, a widely used biguanide and first-line agent for type 2 diabetes mellitus, has attracted increasing attention for its cytoprotective effects beyond glycemic control. Experimental evidence indicates that metformin demonstrates antioxidant, anti-inflammatory, and mitochondria-protective properties, largely mediated through activation of AMP-activated protein kinase (AMPK) and modulation of downstream stress-response pathways [15,22,23]. Through both AMPK-dependent and independent mechanisms, metformin reduces excessive ROS generation, enhances endogenous antioxidant capacity, and preserves mitochondrial integrity, thereby limiting oxidative injury during I/R [15,22].

Previous experimental studies have suggested that metformin may modulate inflammatory signaling pathways, including TLR4/NF-κB signaling and NLRP3 inflammasome activation, resulting in reduced cytokine release, leukocyte infiltration, and apoptotic cell death [20,23,24,28]. Experimental studies have also reported that metformin may improve endothelial function, enhance microcirculatory perfusion, and regulate nitric oxide signaling, collectively contributing to reduced vascular injury and tissue edema during reperfusion [20,27]. Protective effects have been described across multiple organs—including the heart, lungs, liver, kidneys, and skeletal muscle—in both diabetic and non-diabetic conditions [15,20,26–30]. However, these molecular and cellular mechanisms were not directly evaluated in the present study and are discussed only as potential explanations based on previously published evidence.

Although the protective effects of metformin against ischemia–reperfusion injury have been reported previously in various organs and experimental settings, studies simultaneously evaluating oxidative stress parameters and histopathological alterations in multiple remote organs following lower-extremity ischemia–reperfusion under non-diabetic conditions remain limited. By

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combining biochemical assessment of oxidative stress (TAS, TOS, and OSI) with histopathological evaluation of the liver, lung, and kidney within a single experimental model, the present study provides additional descriptive evidence regarding the potential systemic protective effects of metformin following lower-extremity ischemia–reperfusion injury.

Effects of Metformin on Liver Tissue After Limb I/R

Lower-extremity I/R initiates a systemic inflammatory and oxidative cascade capable of inducing secondary injury in distant organs, including the liver. Reperfusion is associated with a "second-hit" phenomenon characterized by mitochondrial dysfunction, calcium overload, endothelial activation, neutrophil recruitment, complement activation, and excessive ROS generation [1,2]. The dissemination of these mediators into the systemic circulation enables propagation of injury beyond the primary ischemic site [8,47].

Within this context, the liver is susceptible to remote injury despite the absence of direct ischemia in infrarenal aortic clamping models. Experimental studies have demonstrated that limb I/R induces hepatic oxidative stress and histopathological alterations such as hepatocyte degeneration and necrosis [9,10]. However, the extent of hepatic involvement appears to be model- and time-dependent [8].

Mechanistically, hepatic injury following limb I/R is driven by excessive ROS production, mitochondrial dysfunction, endothelial impairment, and inflammatory signaling. These processes promote lipid peroxidation, protein and DNA damage, and activation of apoptotic and necrotic pathways within hepatocytes [4,8]. Histopathological alterations are often accompanied by impaired antioxidant defense systems, including reduced glutathione and glutathione peroxidase activity [48].

In the present study, lower-extremity I/R was associated with a marked oxidative imbalance in liver tissue, as evidenced by decreased TAS and increased TOS and OSI levels. These biochemical changes were accompanied by histopathological findings including hepatocyte degeneration, sinusoidal dilatation, nuclear pyknosis, necrosis, and inflammatory cell infiltration, indicating remote hepatic injury.

Metformin administration was associated with attenuation of these alterations. Both doses improved oxidative stress parameters, with more consistent normalization observed at the higher dose. Histopathological findings showed a similar pattern, with broader structural preservation at the higher dose. These findings are consistent with previous studies demonstrating that metformin reduces hepatic oxidative stress and inflammatory activation in I/R models [21,28,49].

Although molecular pathways were not directly evaluated in this study, the observed findings are compatible with previously described mechanisms involving modulation of oxidative stress, inflammatory signaling, and mitochondrial function. Overall, these results suggest that metformin may attenuate remote hepatic injury following limb I/R, with evidence of a dose-related effect.

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Effects of Metformin on Lung Tissue After Limb I/R

Pulmonary tissue is highly susceptible to remote injury following lower-extremity I/R due to its extensive capillary network and exposure to circulating inflammatory mediators [12,36]. Remote lung injury is characterized by oxidative stress, endothelial dysfunction, neutrophil infiltration, and increased vascular permeability, leading to interstitial edema, alveolar wall thickening, and impaired gas exchange [37,38].

In the present study, lung tissue in the I/R group demonstrated significant oxidative stress, reflected by decreased TAS and increased TOS and OSI levels, together with histopathological damage. Metformin treatment was associated with significant improvement in oxidative stress parameters and attenuation of histopathological injury, with more pronounced effects observed at the higher dose.

Histological evaluation showed reductions in interstitial edema, leukocyte infiltration, and alveolar wall thickening in metformin-treated groups, suggesting improved endothelial integrity and reduced inflammatory response. These findings are consistent with previous reports demonstrating that metformin reduces pulmonary oxidative stress and inflammatory injury in I/R models [15,50,51].

Overall, the present findings indicate that metformin may confer pulmonary protection following limb I/R, possibly through mechanisms related to oxidative stress modulation, with a dose-related effect.

Effects of Metformin on Renal Tissue After Limb I/R

Renal tissue is particularly vulnerable to remote injury following limb I/R due to systemic oxidative stress, inflammatory activation, and microcirculatory disturbances [8,10]. These processes contribute to tubular epithelial injury, mitochondrial dysfunction, and structural damage [44,52].

More broadly, contemporary studies suggest that metformin may act as a modifier of ischemia–reperfusion injury through attenuation of oxidative stress, preservation of cellular energy balance, and modulation of inflammatory pathways [54].

In the present study, I/R was associated with significant oxidative stress in renal tissue, as indicated by reduced TAS and increased TOS and OSI levels, along with marked histopathological alterations. Metformin treatment attenuated these changes, with improvements observed in both biochemical and structural parameters.

Histopathological analysis demonstrated reductions in tubular degeneration, inflammatory infiltration, and several injury parameters in metformin-treated groups, with broader protective effects observed at the higher dose. These findings are consistent with previous studies demonstrating that metformin exerts renoprotective effects through modulation of oxidative stress and inflammatory pathways [20,53].

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Taken together, these findings suggest that metformin may mitigate remote renal injury following limb I/R, with evidence of a dose-dependent protective effect.

Limitations

This study has several limitations that should be considered. First, it was conducted in a rat model of lower-extremity ischemia–reperfusion (I/R), which, although appropriate for mechanistic evaluation of remote organ injury, may not fully replicate the complexity of human I/R pathophysiology; therefore, extrapolation to clinical settings should be approached with caution.

Second, the analysis was limited to an acute reperfusion period of 120 minutes, precluding assessment of long-term organ injury, functional recovery, tissue remodeling, fibrosis, and sustained organ protection. Consequently, the present findings reflect only the early phase of ischemia–reperfusion injury. Future studies incorporating longer follow-up periods and serial histopathological and functional assessments will be necessary to determine whether the protective effects of metformin persist beyond the acute phase and influence subsequent tissue repair and remodeling processes.

Third, although comprehensive biochemical and histopathological evaluations were performed in the liver, lungs, and kidneys, key molecular mechanisms implicated in metformin's protective effects—such as AMPK activation, TLR4/NF-κB signaling, cytokine responses, HMGB1 release, apoptosis, and mitochondrial function—were not directly investigated. Accordingly, mechanistic interpretations in the present study are based on integration with existing literature rather than direct experimental confirmation. Furthermore, molecular mediators potentially involved in metformin-mediated protection, including AMPK-related signaling pathways, inflammatory cytokines, apoptosis-associated proteins, and inflammasome-related markers, were not evaluated. Therefore, the present findings should be interpreted as descriptive evidence of tissue protection rather than direct evidence of specific molecular mechanisms.

Fourth, the assessment of oxidative stress relied on global oxidative status parameters (TAS, TOS, and OSI), which provide an overall evaluation of oxidant–antioxidant balance but do not identify specific molecular sources or targets of oxidative injury. More specific biomarkers of oxidative damage, including lipid peroxidation products, protein oxidation markers, DNA oxidation markers, and mitochondrial oxidative stress indicators, were not evaluated. Therefore, the precise nature and cellular origin of oxidative injury could not be determined. Future studies incorporating these complementary biomarkers would provide a more detailed characterization of metformin-mediated redox modulation following ischemia–reperfusion injury.

Fifth, another limitation is the relatively small sample size (n = 6 per group), which is commonly used in exploratory experimental ischemia–reperfusion studies but may limit statistical power, particularly for semi-quantitative histopathological assessments that are inherently subject to biological variability. Nevertheless, statistically significant and internally consistent findings were observed across multiple biochemical and histopathological endpoints in all examined organs,

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supporting the overall reliability of the observed treatment effects. Future studies with larger sample sizes would be valuable to further validate and refine these findings. In addition, some biochemical parameters demonstrated relatively greater variability within certain treatment groups, particularly in the lower-dose metformin group. This variability may reflect biological heterogeneity in individual responses to ischemia–reperfusion injury and treatment, which is commonly encountered in exploratory experimental animal studies. Nevertheless, the overall direction of the biochemical findings remained consistent with the histopathological results and the observed dose-related protective trend.

Sixth, the histopathological scoring systems used in this study were semi-quantitative in nature. Although all tissue evaluations were performed by an experienced investigator blinded to group allocation using predefined scoring criteria, inter-observer variability and formal reproducibility analyses were not assessed. Therefore, a degree of observer-dependent subjectivity cannot be completely excluded. Future studies incorporating independent observers and formal agreement analyses would further strengthen the robustness of histopathological assessments.

Seventh, functional markers of organ injury were not evaluated. Although biochemical oxidative stress parameters and histopathological analyses consistently demonstrated tissue protection, clinically relevant functional indices such as renal, hepatic, and pulmonary function parameters were not assessed. Therefore, the relationship between the observed structural protection and organ function could not be directly determined. Future studies incorporating both histopathological and functional outcome measures would provide a more comprehensive evaluation of metformin-mediated organ protection.

Eighth, circulating systemic inflammatory and oxidative stress markers were not evaluated. Although the study demonstrated consistent biochemical and histopathological changes across multiple distant organs, systemic mediators such as cytokines, acute-phase reactants, and circulating oxidative stress parameters were not measured. Therefore, the systemic mechanisms underlying remote organ protection could not be directly characterized. Future studies incorporating both tissue-specific and circulating biomarkers would provide a more comprehensive understanding of the systemic effects of metformin following ischemia–reperfusion injury.

Ninth, direct assessments of local skeletal muscle injury were not performed, and systemic biomarkers potentially linking lower-extremity ischemia–reperfusion injury to remote organ damage were not evaluated. Therefore, although biochemical and histopathological evidence of remote organ injury was demonstrated, the present study could not directly distinguish local from systemic mechanisms or establish causal biological links between limb ischemia–reperfusion injury and distant organ alterations. Future studies incorporating local tissue injury markers, systemic inflammatory mediators, and functional outcome measures would help clarify the pathophysiological relationship between local and remote organ injury.

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Tenth, only two doses of metformin were evaluated. Although a dose-related trend was observed, the absence of a broader dosing range limits precise identification of the optimal therapeutic window.

Eleventh, only a single administration schedule was evaluated. Alternative treatment paradigms, including preconditioning, repeated dosing, or delayed post-reperfusion administration, were not investigated. Therefore, the optimal timing and therapeutic window of metformin could not be determined from the present study.

Twelfth, metformin was administered as a single intraperitoneal dose immediately before reperfusion, which differs substantially from the chronic oral administration commonly used in clinical practice. Pharmacokinetic parameters, including plasma metformin concentrations, blood lactate levels, and blood gas measurements, were not evaluated. Therefore, the relationship between the experimental dosing strategy and clinically relevant systemic exposure could not be determined. Consequently, the present findings should be interpreted primarily as evidence of acute experimental effects during the early reperfusion period rather than direct evidence of clinical efficacy under standard therapeutic conditions.

Finally, the study was conducted exclusively in non-diabetic animals. While this design allowed assessment of glucose-independent effects of metformin, it limits understanding of how metabolic comorbidities, particularly diabetes mellitus, may influence its organ-protective actions.

Despite these limitations, the present study provides consistent biochemical and histopathological evidence suggesting that metformin may attenuate remote organ injury following lower-extremity I/R and offers a basis for future mechanistic and translational investigations.

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Conclusion

In conclusion, the findings of this study indicate that metformin may exert protective effects against remote liver, lung, and renal injury following lower-extremity ischemia–reperfusion in a non-diabetic rat model. Lower-extremity I/R was associated with marked systemic oxidative stress and significant histopathological alterations across all examined organs. Metformin administration was associated with attenuation of these effects, reflected by improvements in oxidant–antioxidant balance (increased TAS and reduced TOS and OSI) and partial preservation of tissue architecture, including reduced hepatocellular degeneration, pulmonary inflammation, and renal tubular and glomerular injury.

Notably, these protective effects demonstrated a dose-related pattern, with the 8 mg/kg dose showing more consistent biochemical improvement and broader histopathological preservation compared with the 4 mg/kg dose.

Taken together, these findings suggest that metformin may mitigate I/R-induced remote organ injury, potentially through mechanisms related to the modulation of oxidative stress. However, the

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molecular pathways underlying these protective effects were not directly investigated in the present study and therefore require further mechanistic evaluation.

Further studies incorporating longer reperfusion periods, detailed molecular analyses, and translational clinical models are warranted to clarify the underlying mechanisms and to determine the potential clinical relevance of metformin in ischemia–reperfusion–associated multi-organ injury.

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Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Author Contributions: Conceptualization, A.Ö., A.K.; methodology, H.D., A.Ö., A.K.Y., A.D.D., Ş.C.S., and M.A.; validation, H.D. and A.Ö.; formal analysis, M.A.; investigation, A.K.Y., A.Ö., H.D., and M.A.; resources, A.Ö., A.K.Y., H.D., A.K., Ş.C.S., A.D.D., and M.A.; writing—original draft preparation, A.K.Y. and H.D.; writing—review and editing, A.Ö. and M.A.; supervision, A.Ö., A.K., and M.A.; project administration, A.Ö. All the authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

Ethical approval for the study was obtained from Animal Research Committee of Gazi University (Ankara, Turkey; approval no. G.Ü.ET-25-007).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Not applicable

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