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Protective Effects of BPC 157 in Rats with Experimentally Induced Lower Extremity Ischemia-Reperfusion Injury

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Abstract

Objective:

Ischemia–reperfusion (I/R) injury remains a major complication in peripheral arterial disease, characterized by oxidative stress, inflammation, and apoptosis. Body Protection Compound-157 (BPC 157), a stable gastric pentadecapeptide, has demonstrated cytoprotective properties in multiple tissues. This study aimed to evaluate the protective effects of BPC 157 in a rat model of lower limb I/R injury.

Methods:

Twenty-four male Wistar albino rats were randomized into four groups (n=6): SHAM, B (BPC 157 only), IR (I/R), and IRB (I/R + BPC 157). I/R was induced by abdominal aortic clamping for 45 minutes followed by 2 hours of reperfusion. BPC 157 (20 µg/kg, intraperitoneal) was administered at the 45th minute of ischemia in B and IRB groups. Biochemical markers (MDA, SOD, TAS, TOS)

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were measured in serum. Gene expression of Il-6, Hif-1α, p53, Bcl-2, Bax, and Casp3 was assessed by qRT-PCR, while immunohistochemistry evaluated VEGF, eNOS, IL-6, and Caspase-3 expression. Histopathological changes were scored with hematoxylin–eosin and Masson's trichrome staining.

Results:

I/R significantly increased MDA, TOS, p53, Bax, Casp3, Hif-1α, Il-6, and histopathological injury scores, while reducing SOD, TAS, and VEGF expression. Bcl-2 mRNA was not significantly reduced by I/R compared with SHAM; however, BPC 157 significantly increased Bcl-2 expression compared with IR. In the IRB group, BPC 157 reduced MDA and TOS, restored SOD and TAS, downregulated p53, Bax, and Casp3, reduced IL-6 and Caspase-3 immunoreactivity, and partially restored VEGF expression. Histological analysis confirmed improved muscle architecture and reduced collagen deposition in IRB compared with IR.

Conclusion:

BPC 157 appears to exert protective effects against skeletal muscle I/R injury by attenuating oxidative stress, modulating apoptosis, reducing inflammation, and supporting angiogenic activity. These findings suggest that BPC 157 may represent a potential therapeutic candidate for mitigating reperfusion injury; however, further studies with larger cohorts and dose–response evaluations are required to confirm these effects and establish clinical relevance.

Keywords: Ischemia-Reperfusion Injury, Lower extremity, BPC157, Oxidative Stress, Apoptosis, Inflammation

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INTRODUCTION

Chronic non-communicable diseases—including cardiovascular, metabolic, renal, and oncologic conditions—are leading causes of global morbidity and mortality, with peripheral artery disease (PAD) representing a major contributor due to its progressive nature, high prevalence, and cardiovascular risk equivalence to coronary artery disease.[1–3] PAD, most commonly caused by atherosclerotic obstruction of the lower extremity arteries, impairs oxygen and nutrient delivery, resulting in clinical manifestations ranging from intermittent claudication to critical limb-threatening ischemia.[4,5] It affects more than 200 million individuals worldwide and constitutes a substantial global health burden.[6,7] In advanced stages, revascularization via bypass surgery, endovascular intervention, or thromboembolectomy is often required.[8,9] However, despite restoring perfusion, these procedures frequently

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trigger ischemia–reperfusion (I/R) injury, in which reperfusion exacerbates oxidative stress, inflammation, endothelial dysfunction, and cell death, ultimately compromising therapeutic outcomes.[10–13]

I/R injury is not merely the consequence of the initial ischemic insult but arises from a complex cascade of biochemical and inflammatory processes triggered upon restoration of oxygenated blood flow. During ischemia, mitochondrial dysfunction leads to adenosine triphosphate (ATP) depletion, ionic imbalance, and metabolic acidosis, which compromise cellular integrity.[11,13,14] Upon reperfusion, a rapid surge in reactive oxygen species (ROS) occurs via multiple enzymatic sources, including xanthine oxidase, NADPH oxidase, the mitochondrial electron transport chain, and uncoupled nitric oxide synthase.[15–18] These ROS—such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$), and peroxynitrite ($ONOO^-$)—induce lipid peroxidation, protein oxidation, and DNA damage, thereby activating cell death pathways including apoptosis, necrosis, necroptosis, and autophagy.[13,19–21]

In parallel, reperfusion initiates robust inflammatory responses characterized by interleukin-6 (IL-6) upregulation, neutrophil infiltration, and cytokine release, alongside endothelial dysfunction, leukocyte adhesion, and complement activation.[18,20,22] These processes involve key molecular mediators—including hypoxia-inducible factor 1-alpha (HIF-1α), vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), p53, B-cell lymphoma 2 (Bcl-2), Bcl-2–associated X protein (Bax), and cysteine-aspartate protease-3 (Caspase-3)—which regulate oxidative stress responses, angiogenesis, and apoptosis, with their imbalance determining tissue survival or loss.[23–25] Furthermore, the interplay between oxidative stress, inflammation, and apoptosis is closely linked to activation of signaling pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a central regulator in the pathogenesis of tissue injury. Similar mechanisms have been demonstrated across multiple experimental models, underscoring the role of NF-κB–mediated signaling in coordinating oxidative damage, inflammatory responses, and apoptotic pathways.[26–29] Macroscopically, skeletal muscle I/R injury manifests as edema, degeneration, necrosis, and compartment syndrome, and may progress to systemic complications.[10,30,31]

Numerous pharmacological and experimental strategies have been explored to mitigate I/R injury; however, none has achieved consistent clinical efficacy or routine adoption in vascular practice.[31–37] Mannitol, administered prior to

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revascularization, has been used clinically as an osmotic agent and free-radical scavenger in acute limb ischemia and has been associated with improved limb salvage and reduced mortality.[20] However, the supporting evidence remains observational, and effective dosing carries a significant risk of acute kidney injury. Edaravone, a free-radical scavenger approved in Japan for acute ischemic stroke, has demonstrated protective effects in tourniquet-induced muscle I/R in murine models but is not approved for limb I/R indications outside Japan.[38] Mitochondrial permeability transition pore inhibitors reduce skeletal muscle and renal injury in rat lower-limb I/R models but are limited in clinical translation due to immunosuppressive and pharmacokinetic constraints.[39] Nitric oxide donors and L-arginine improve microvascular perfusion, enhance endothelial function, and reduce oxidative stress; however, their effects are often modest and short-lived, with hypotension limiting sustained clinical use.[32] Anti-inflammatory and NF-κB–targeted therapies can attenuate cytokine-mediated injury.[33,34,40] Calcium channel blockers reduce intracellular calcium overload and cellular injury but demonstrate variable clinical efficacy and limited impact on inflammatory and oxidative pathways.[41] Similarly, statins, dexmedetomidine, and antioxidant therapies—including N-acetylcysteine, allopurinol, and hydrogen-rich saline—target reactive oxygen species and have shown protective effects in preclinical models; however, clinical translation remains inconsistent due to limited bioavailability and the inability to simultaneously modulate multiple pathogenic mechanisms.[37,42,43] These limitations underscore the need for novel therapies offering multimodal protection by simultaneously targeting oxidative stress, inflammation, apoptosis, and microvascular dysfunction.

Body Protection Compound-157 (BPC 157) is a synthetic, stable gastric pentadecapeptide composed of 15 amino acids, originally isolated from the Body Protecting Compound in human gastric juice. It is highly resistant to degradation in gastric fluid and has demonstrated a favorable safety profile in both experimental and clinical studies, including trials in inflammatory bowel disease in which no lethal dose was identified.[44,45] Although its mechanisms of action are not fully elucidated, experimental models consistently demonstrate broad cytoprotective and regenerative effects across multiple organ systems, including the gastrointestinal tract, skin, tendon, ligament, skeletal muscle, bone, myocardium, and vascular tissues.[46–59] At the molecular level, BPC 157 promotes angiogenesis through upregulation of VEGF and angiopoietin-1 and enhances nitric oxide (NO) signaling via activation of endothelial nitric oxide synthase.[60,61] It also exhibits potent antioxidant activity, evidenced by reduced malondialdehyde (MDA) levels,

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thereby limiting apoptosis and contributing to cellular homeostasis.[50,59,62] Its anti-apoptotic effects are further associated with downregulation of NF-κB and caspase activity, alongside upregulation of Bcl-2, as demonstrated in gastrointestinal and neuronal I/R models.[63,64] In addition, BPC 157 modulates inflammatory signaling by reducing the expression of pro-inflammatory cytokines, including IL-6 and tumor necrosis factor-alpha (TNF-α).[65,66] Furthermore, it preserves vascular integrity by decreasing leukocyte adhesion, attenuating platelet aggregation, and supporting microvascular stability.[62,67] Collectively, these mechanisms are particularly relevant in I/R injury, where reperfusion induces bursts of reactive oxygen species and inflammatory mediators.

Despite promising preclinical findings across multiple organ systems, evidence regarding the protective role of BPC 157 in skeletal muscle during lower extremity I/R injury remains limited, particularly within the clinically relevant context of PAD and acute limb ischemia. BPC 157 was selected for this study based on its multimodal cytoprotective properties—including antioxidant, anti-inflammatory, anti-apoptotic, and pro-angiogenic effects—which directly target the key mechanisms underlying I/R injury. The analysis focused on oxidative stress markers, apoptosis-related gene expression, the hypoxia-regulated mediator Hif-1α, and the inflammatory cytokine Il-6. In addition, histopathological and immunohistochemical assessments were performed to evaluate tissue architecture, inflammatory infiltration, collagen deposition, and the expression of VEGF, eNOS, IL-6, and caspase-3. Accordingly, this study aimed to evaluate the potential of BPC 157 to attenuate lower-limb ischemia–reperfusion injury in rats through an integrated biochemical, molecular, and morphological approach. Based on these considerations, we hypothesized that BPC 157 would exert a protective effect against skeletal muscle ischemia–reperfusion injury by simultaneously modulating oxidative stress, apoptosis, inflammation, and angiogenic signaling pathways. To our knowledge, this is one of the few studies to simultaneously investigate these interconnected mechanisms in skeletal muscle tissue, thereby providing a more holistic understanding of the potential therapeutic role of BPC 157 in ischemia–reperfusion injury.

MATERIAL AND METHOD

Animals

This study was performed using twenty-four 12-week-old male Wistar albino rats weighing 250–350 g. All rats were housed in individual cages under controlled environmental conditions (temperature: 20–21 °C; relative

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humidity: 45–55%; 12-hour light/dark cycle) with ad libitum access to standard laboratory chow and distilled water. The animals were obtained from and housed at the Animal Laboratory of the Gazi University Experimental Research Center (Ankara, Turkey), a certified facility dedicated to experimental animal research. All experimental procedures were approved by the Gazi University Animal Experiments Local Ethics Committee (Approval No: G.Ü.ET-24.049; date: 27.05.2024). All methods were conducted in accordance with relevant institutional and international guidelines and regulations, including the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1986) and the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020). The study was conducted and reported in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments, https://arriveguidelines.org) guidelines.

Drug

BPC 157, a stable gastric pentadecapeptide derived from the Body Protecting Compound in human gastric juice, was administered in accordance with previously established protocols.[31,62,63,68–70] The peptide (sequence: GEPPPGKPADDAGLV; molecular weight 1419.54 g/mol) was obtained from Sigma-Aldrich (Taufkirchen, Germany) and synthesized at >95% purity, verified by high-performance liquid chromatography (HPLC). It is readily soluble in saline and aqueous solutions at physiological pH (7.0). No carrier agents or peptidase inhibitors were used. Based on prior pharmacological reports, the intraperitoneal dose was set at 20 μg/kg, consistent with established experimental applications. [31,62,63,68–70]

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Experimental Protocol

Twenty-four male Wistar albino rats were randomly assigned to four groups (n=6 per group): Sham-operated control (SHAM), BPC 157 only (B), Ischemia-Reperfusion (IR) and Ischemia-Reperfusion with BPC 157 treatment (IRB). SHAM group underwent midline laparotomy without induction of ischemia and received 0.3 mL of 0.9% sodium chloride (NaCl) intraperitoneally. B group underwent same procedure but received BPC 157 (20 μg/kg, intraperitoneally). IR group was subjected to 45 minutes of bilateral hindlimb ischemia induced by abdominal aortic clamping, followed by 2 hours of reperfusion. IRB group underwent same ischemia–reperfusion protocol as IR group but received BPC 157 (20 μg/kg, intraperitoneally) at 45th minute of ischemia, immediately prior to reperfusion. The duration of ischemia (45 minutes) and reperfusion (120 minutes) was selected based on previously validated experimental models demonstrating that this time frame reliably

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induces significant skeletal muscle injury characterized by oxidative stress, inflammation, and apoptosis, while maintaining model stability and survival.[10,12,31,36,71]

Anesthesia was induced with ketamine hydrochloride (45 mg/kg; Ketalar®, Pfizer, USA) and xylazine hydrochloride (5 mg/kg; Xylazinbio®, Bioveta, Czech Republic). To maintain anesthetic depth, half-doses were administered every 90 minutes, with adequacy confirmed by the absence of withdrawal and auricular reflexes, as previously described in experimental animal models.[31,72,73] The abdomen was shaved, disinfected with 10% povidone-iodine (Batticon®, Adeka, Turkey), and a midline laparotomy was performed. In IR and IRB groups, the abdominal aorta was clamped distal to the renal arteries using an atraumatic bulldog clamp (Aesculap, Germany) to induce ischemia, followed by reperfusion after clamp removal. Abdominal aortic clamping was selected over femoral artery occlusion to ensure uniform and complete bilateral hindlimb ischemia, as femoral models may be influenced by collateral circulation, resulting in variable and incomplete ischemia. This approach provides a more reproducible and clinically relevant model of global lower extremity I/R injury.[31,35,71–73] SHAM and B groups underwent vessel exposure without clamping. Throughout the procedure, body temperature was maintained at 37 ± 0.5 °C on a thermostatically controlled heating pad.

At the end of the reperfusion period, all animals were euthanized by exsanguination under deep anesthesia induced with ketamine (45 mg/kg) and xylazine (5 mg/kg), administered intramuscularly into the forelimb muscles to ensure complete loss of pain and reflexes. To avoid potential interference with experimental outcomes, injections were not performed in the hindlimbs from which gastrocnemius muscle samples were obtained. Blood was collected from the inferior vena cava, allowed to clot, and centrifuged at 3000 rpm for 10 minutes (NF 200, NÜVE, Turkey); serum was stored at –80 °C for biochemical assays.

Skeletal muscle samples were obtained from the gastrocnemius muscles of both hind limbs of male Wistar albino rats included in the experimental protocol. To ensure sufficient and undisturbed tissue sampling for different analyses, a bilateral collection strategy was used: the right gastrocnemius muscle was fixed in 10% neutral-buffered formalin and reserved for histopathological and immunohistochemical evaluations, while the left gastrocnemius muscle was used for quantitative real-time polymerase chain reaction (qRT-PCR) analyses. In addition, serum samples collected from the inferior vena cava were utilized for biochemical assays.

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Biochemical Analysis

Serum malondialdehyde (MDA), an index of lipid peroxidation, was quantified using a rat-specific ELISA kit (Sunred Biological Technology, Shanghai, China) and expressed as nmol/mL. Superoxide dismutase (SOD) activity, reflecting endogenous antioxidant defense, was measured with a rat SOD ELISA kit (Sunred Biological Technology, Shanghai, China) and expressed as ng/mL. Total oxidant status (TOS) was determined using a rat TOS ELISA kit (Elabscience Biotechnology, USA) and reported as μmol H_2O_2 equivalents/L, while total antioxidant status (TAS) was measured with a rat TAS ELISA kit (Elabscience Biotechnology, USA) and expressed as mmol Trolox equivalents/L.

All assays were performed according to the manufacturers' instructions, and optical density was measured using a microplate reader. The methodology was applied in the same manner as in previous lower-extremity I/R studies.[31,35,37,73,74]

RNA Isolation and Quantitative Real-Time PCR

Skeletal muscle samples from the gastrocnemius muscles were homogenized in 1.5 mL Eppendorf® tubes with tungsten carbide and micro-glass beads using a TissueLyser LT system (Qiagen, USA) pre-cooled to –80 °C. Homogenization was performed in three 1-min cycles until complete tissue disruption.

Total RNA was isolated using TRIzol™ Reagent in combination with the PureLink™ RNA Mini Kit (Thermo Fisher Scientific, USA), according to the manufacturer's protocol. RNA purity and concentration were determined with a NanoQ Lite spectrophotometer (Mecasys, South Korea), and integrity was assessed by A260/A280 ratios. RNA concentrations were normalized to 750 ng/10 μL with RNase-free water.

Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Thermo Fisher Scientific, USA) in a ProFlex™ thermal cycler under standard conditions (25 °C, 10 min; 37 °C, 120 min; 85 °C, 5 min). cDNA samples were stored at –20 °C until use.

qRT-PCR was performed with a StepOnePlus™ Real-Time PCR System (Applied Biosystems™, Thermo Fisher Scientific, USA) using gene-specific primers (Micromolecules Biotechnology, Turkey). Target genes included Il-6, Hif-1α, p53, Bcl-2, Bax, and Casp3, with GAPDH and β-actin as reference genes. Primer sequences are provided in Table 1.

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Table 1. Forward and reverse primer sequences used for quantitative real-time PCR analysis of target genes.

Gene	Forward primer	Reverse primer
Il-6	GAGAAAAGAGTTGTGCAATGGC	ACTAGGTTTGCCGAGTAGAC
Hif-1α	CCACCGCAACTGCCACCACT	AGGGGCACGGTCACCTGGTT
p53	GCACAAACACGCACCTCAAAGC	CTTGCATTCTGGGACAGCCAAG
Bcl-2	GCAGAGATGTCCAGTCAGC	CCCACCGAACTCAAAGAAGG
Bax	GCGAATTGGAGATGAACTGG	GTGAGCGAGGCGGTGAGGAC
Casp3	TGAGCATTGACACAATACAC	AAGCCGAAACTCTTCATC
GAPDH	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTCAAA
β-actin	CGCTAACATCAAATGGGGTG	ACAACCTGGTCCTCAGTGTA

The PCR protocol consisted of an initial enzyme activation step at 95 °C for 10 minutes, after which 40 amplification cycles were performed, including denaturation at 95 °C for 15 seconds and combined annealing/extension at 60 °C for 1 minute. Amplification specificity was verified by melt curve analysis, and gene expression levels were quantified using the $2^{-\Delta\Delta C_T}$ method. qRT-PCR analysis was performed in accordance with previously established and validated protocols.[23–25,75,76]

Histopathology and Immunohistochemistry

Skeletal muscle samples were fixed in 10% neutral-buffered formalin, dehydrated through graded ethanol series (60%, 70%, 80%, 90%, 96%, and 100%), cleared in xylene, and embedded in paraffin. Transverse sections (4 µm) were cut using a rotary microtome (Leica RM2235, Germany). Following deparaffinization and rehydration, sections were stained with hematoxylin–eosin (H&E; Bio-Optica, Italy) for general morphology or Masson’s trichrome (MT; Bio-Optica, Italy) to assess collagen deposition. Histological damage was semi-quantitatively scored (1 = minimal change, 5 = severe change) for muscle fiber degeneration, edema, inflammatory infiltration, and vascular congestion using a light microscope (Leica DM4000B, Germany) according to previously described methods.[31,73] All histopathological and immunohistochemical evaluations were performed by a pathologist blinded to the experimental groups. For each specimen, at least three transverse sections were obtained, and three non-overlapping microscopic fields per section were evaluated. Representative images were captured from these fields for analysis. Histopathological assessment was conducted according to previously established standard methods.[31,73,77,78]

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For immunohistochemistry, deparaffinized and rehydrated sections underwent antigen retrieval in citrate buffer (pH 6.0; Thermo Fisher Scientific, USA) using a PT Module (LabVision™). Endogenous peroxidase was blocked with 3% hydrogen peroxide, and non-specific binding was prevented with Protein Block (TA-125-PBQ, Thermo Fisher Scientific, USA). Sections were incubated for 2 h at room temperature with primary antibodies against VEGF (orb227746), eNOS (orb228585), IL-6 (orb682863), and Caspase-3 (orb153764) (Biorbyt, UK). After PBS washing, HRP Polymer Quanto (TL-125-QPH, Thermo Fisher Scientific, USA) was applied, and immunoreactivity was visualized with DAB chromogen (TA-125-HA, Thermo Fisher Scientific, USA), followed by hematoxylin counterstaining, in accordance with previously described standard immunohistochemical procedures.[79]

Immunohistochemical expression was evaluated using the H-score method, in which staining intensity (weak = 1, moderate = 2, strong = 3) and the percentage of positive cells were assessed in three random fields.[80,81] The H-score was calculated as H-score = ΣPi (i + 1), where *i* denotes staining intensity and *Pi* the percentage of cells at each intensity.

Statistical Analysis

All data are presented as mean ± standard deviation (SD). Biochemical, histopathological, and immunohistochemical analyses were performed using SPSS software (version 26.0; IBM Corp., Armonk, NY, USA), while gene expression data were analyzed with GraphPad Prism (version 8.1; GraphPad Software, Boston, MA, USA). Statistical comparisons were conducted using one-way analysis of variance (ANOVA) with Bonferroni correction, followed by Tukey's post hoc test for multiple comparisons. A p-value ≤ 0.05 was considered statistically significant.

Although no formal a priori power calculation was performed, the sample size of six animals per group (total n = 24) was selected based on commonly used group sizes in comparable rodent models of skeletal muscle I/R injury, in which cohorts of six to eight animals are frequently employed for the combined evaluation of biochemical, molecular, histopathological, and immunohistochemical endpoints. [31,37,71] The final sample size was also determined in accordance with ethical considerations under the 3Rs principle (Replacement, Reduction, Refinement) and was approved by the Gazi University Experimental Animal Ethics Committee.

RESULTS

Biochemical Findings

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MDA levels were significantly elevated in IR group compared with SHAM group ($p<0.0001$). B group also showed a mild but statistically significant increase compared with SHAM ($p=0.0230$). Administration of BPC 157 in IRB group resulted in a significant reduction in MDA levels compared with group IR ($p=0.0002$), reaching values comparable to the SHAM group ($p=0.8497$).

SOD activity was markedly decreased in IR group compared with both SHAM ($p=0.0020$) and B ($p=0.0019$) groups. Administration of BPC 157 following ischemia (IRB group) significantly restored SOD activity relative to IR group ($p<0.0001$). Moreover, SOD levels in IRB group were significantly higher than those observed in SHAM ($p=0.0352$) and B ($p=0.0376$) groups.

TOS was markedly elevated in IR group compared with SHAM group ($p=0.0019$), while the increase relative to B group did not reach statistical significance ($p=0.0960$). Administration of BPC 157 significantly reduced TOS levels in IRB group compared with both IR ($p<0.0001$) and B ($p=0.0092$) groups, resulting in values comparable to those observed in the SHAM group ($p=0.3201$).

TAS was significantly reduced in IR group compared with SHAM ($p=0.0385$). In IRB group, TAS was significantly higher than both IR ($p=0.0021$) and B ($p=0.0062$) groups, approaching SHAM levels ($p=0.5625$). These findings, along with the results for MDA, SOD, TOS, and TAS, are collectively summarized in Table 2.

Table 2. Biochemical analyses of blood serum in experimental groups.

	SHAM group	B group	IR group	IRB group	p value (ANOVA)
MDA (nmol/ml)	5.55±1.17	7.91±0.60*	10.02±1.57$^{*, +}$	6.16±1.56$^{\#}$	<0.001
SOD (ng/ml)	20.14±2.42	20.18±1.50	14.98±2.33$^{*, +}$	23.74±2.04$^{*, +, \#}$	<0.001
TOS (µmol/L)	225.50±27.19	275.90±26.10	344.30±23.44*	176.80±84.97$^{\#, +}$	<0.001
TAS (mmol/L)	47.66±3.54	36.79±13.54	34.64±4.51*	53.53±4.65$^{+, \#}$	0.003

Notes: Data are presented as mean ± standard deviation (SD); *$p<0.05$: Compared to SHAM group; +$p<0.05$: Compared to B group; #$p<0.05$: Compared to IR group; One-way ANOVA followed by Tukey's post hoc test was Applied; SHAM group, laparotomy only; B group, laparotomy + intraperitoneal BPC 157 (20 µg/kg); IR group, ischemia (45 min) + reperfusion (120 min); IRB

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group, ischemia (45 min) + BPC 157 (20 μg/kg, intraperitoneal) + reperfusion (120 min).

Abbreviations: MDA, malondialdehyde; SOD, superoxide dismutase; TOS, total oxidant status; TAS, total antioxidant status.

Histopathological Findings

Hematoxylin–Eosin

In SHAM and B groups, muscle fibers demonstrated preserved histoarchitecture, characterized by polygonal morphology, peripheral localization of nuclei, and intact cross-striations, with no evidence of inflammatory infiltration or vascular alterations. By contrast, IR group revealed pronounced pathological changes, including marked myofiber degeneration, cytoplasmic vacuolization, interstitial edema, dense neutrophil infiltration, and prominent vascular congestion, consistent with severe ischemia–reperfusion injury. In IRB group, administration of BPC 157 mitigated these alterations, with substantially reduced degenerative changes and partial preservation of muscle fiber organization compared with IR group. Semi-quantitative histopathological scoring further supported these findings, demonstrating significantly higher injury scores in IR group compared with both SHAM and B ($p<0.0001$), while IRB group exhibited significantly lower injury scores than IR ($p=0.0007$). These results are illustrated in Figure 1 and summarized in Table 3.

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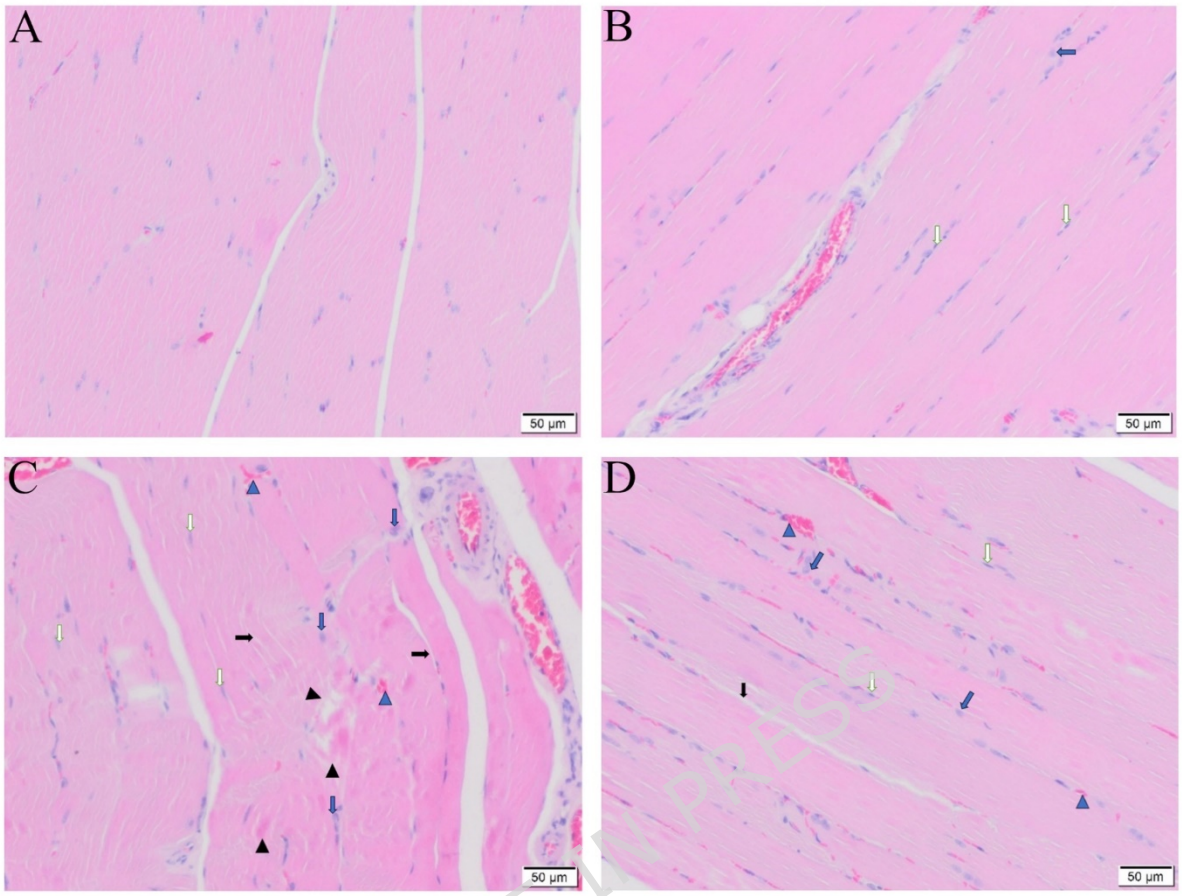


Figure 1. Histopathological evaluation of the right gastrocnemius muscle in sham-operated control (SHAM), BPC 157 only (B), ischemia–reperfusion (IR), and ischemia–reperfusion with BPC 157 treatment (IRB) groups (n = 6 per group) using hematoxylin and eosin (H&E) staining. Black arrows indicate interstitial edema; blue arrows indicate neutrophil infiltration; black arrowheads indicate muscle fiber degeneration; blue arrowheads indicate vascular congestion; and white arrows indicate nuclear condensation. A: SHAM group, laparotomy only; B: B group, laparotomy + intraperitoneal BPC 157 (20 µg/kg); C: IR group, ischemia (45 min) + reperfusion (120 min); D: IRB group, ischemia (45 min) + BPC 157 (20 µg/kg, intraperitoneal) + reperfusion (120 min). Images were obtained at 200× magnification. Scale bars, 50 µm.

Masson's Trichrome

MT staining highlighted preserved connective tissue architecture in SHAM and B groups, with minimal collagen deposition and well-defined muscle–connective tissue boundaries. In contrast, IR group exhibited extensive pathological alterations, characterized by pronounced collagen accumulation, disruption of the muscle–connective tissue interface, and increased interstitial

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fibrosis, as evidenced by intense blue staining. Administration of BPC 157 in IRB group markedly attenuated these changes, with reduced collagen deposition and improved preservation of muscle fiber organization compared with IR group. Semi-quantitative scoring further confirmed these observations, demonstrating significantly higher fibrosis scores in IR group relative to SHAM and B ($p < 0.0001$), whereas IRB group exhibited significantly lower fibrosis scores than IR ($p = 0.0005$). These findings are illustrated in Figure 2 and summarized in Table 3.

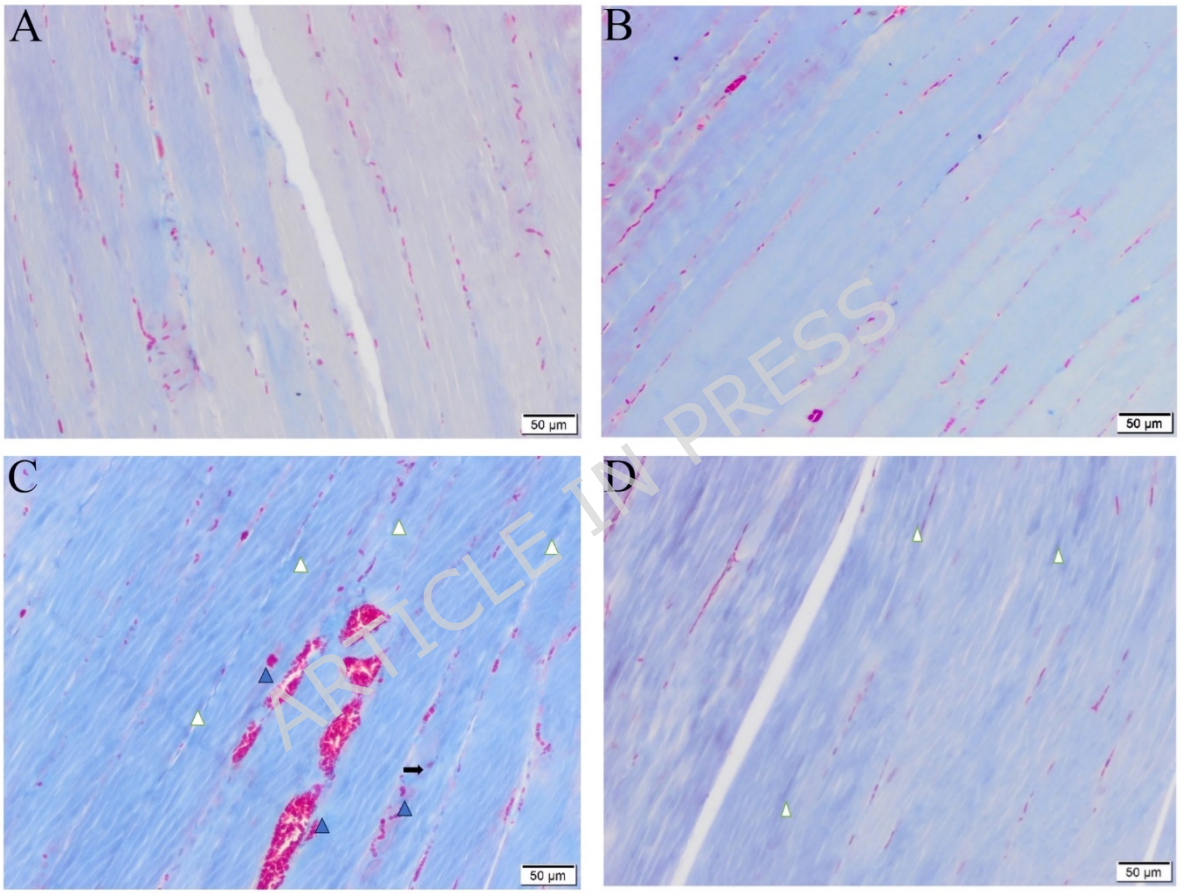


Figure 2. Histopathological evaluation of the right gastrocnemius muscle in sham-operated control (SHAM), BPC 157 only (B), ischemia–reperfusion (IR), and ischemia–reperfusion with BPC 157 treatment (IRB) groups (n = 6 per group) using Masson's trichrome (MT) staining. White arrowheads indicate collagen deposition; black arrows indicate muscle fiber degeneration; and blue arrowheads indicate vascular congestion. A: SHAM group, laparotomy only; B: B group, laparotomy + intraperitoneal BPC 157 (20 µg/kg); C: IR group, ischemia (45 min) + reperfusion (120 min); D: IRB group, ischemia (45 min) +

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BPC 157 (20 µg/kg, intraperitoneal) + reperfusion (120 min). Images were obtained at 200× magnification. Scale bars, 50 µm.

Table 3. Histopathological scoring of right gastrocnemius muscle injury in experimental groups.

	SHAM group	B group	IR group	IRB group	p value (ANOVA)
H&E	1.67±0.41	2.00±0.45	3.50±0.45*, +	2.33±0.41#	<0.001
MT	1.17±0.26	1.92±0.38*	3.58±0.38*, +	2.42±0.58*, #	<0.001

Notes: Scores are presented as mean ± standard deviation (SD), based on semi-quantitative evaluation (1 = minimal change, 5 = severe change); *p<0.05: Compared to SHAM group; +p<0.05: Compared to B group; #p<0.05: Compared to IR group; One-way ANOVA followed by Tukey's post hoc test was performed; SHAM group, laparotomy only; B group, laparotomy + intraperitoneal BPC 157 (20 µg/kg); IR group, ischemia (45 min) + reperfusion (120 min); IRB group, ischemia (45 min) + BPC 157 (20 µg/kg, intraperitoneal) + reperfusion (120 min).

Abbreviations: H&E, Hematoxylin–Eosin staining; MT, Masson's Trichrome staining.

Gene Expression Analysis (qRT-PCR)

qRT-PCR analysis demonstrated significant alterations in gene expression profiles among the experimental groups (Figure 3). IL-6 expression was markedly increased in IR group (7.020±1.550) compared with SHAM (1.043±0.332, p=0.001) and B (0.995±0.467, p=0.001). Although IRB group (3.836±4.322) exhibited lower Il-6 expression than IR, the reduction was not statistically significant (p=0.113). Hif-1α expression was significantly elevated in IR (12.626±4.429) compared with SHAM (1.060±0.429, p<0.001) and B (1.697±0.745, p<0.001), while BPC 157 treatment (IRB, 5.087±2.437) significantly reduced expression relative to IR (p<0.001). There was also no significant difference between SHAM and IRB groups in terms of Hif-1α expression (p=0.059). The pro-apoptotic marker p53 was significantly upregulated in IR (11.628±3.931) compared with SHAM (1.047±0.336, p<0.001) and B (2.681±1.101, p<0.001) but was significantly decreased in IRB (5.679±4.875) compared with IR (p=0.02).

In terms of anti-apoptotic marker, Bcl-2 expression showed no significant difference among SHAM (1.047±0.595), B (1.513±1.445), and IR groups

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(0.190±0.121). However, BPC 157 administration in IRB group (2.074±0.999) significantly increased Bcl-2 expression compared with IR group (p=0.011). By contrast, the pro-apoptotic marker Bax expression was significantly higher in IR group (6.614±2.806) compared with SHAM (1.023±0.234, p<0.001), B (0.278±0.093, p<0.001), and IRB (1.128±0.624, p<0.001), with no significant difference between IRB and SHAM. Finally, Casp3 expression was markedly upregulated in IR group (5.561±2.453) compared with SHAM (1.039±0.276, p<0.001) and B (1.786±1.395, p=0.001). In contrast, treatment with BPC 157 in IRB group (1.890±0.728) significantly reduced Casp3 expression relative to IR group (p=0.002).

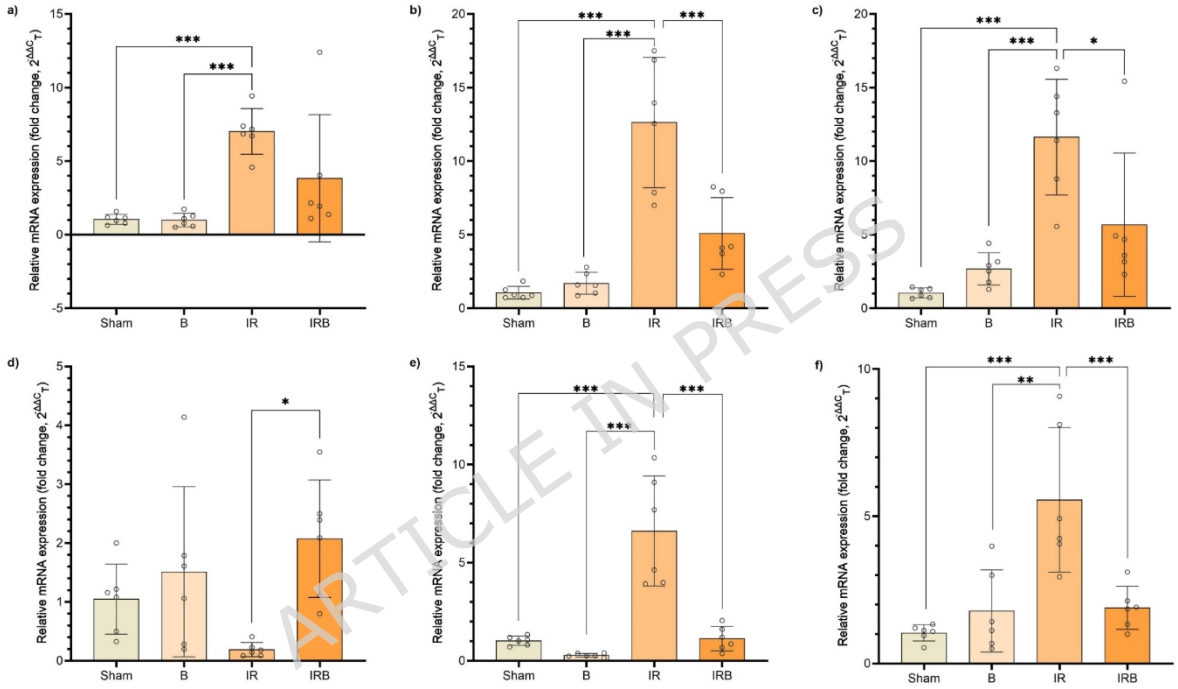


Figure 3. Relative mRNA expression levels of target genes in the left gastrocnemius muscle across experimental groups. Expression levels of (a) interleukin-6 (Il-6), (b) hypoxia-inducible factor-1 alpha (Hif-1α), (c) tumor protein p53 (p53), (d) B-cell lymphoma 2 (Bcl-2), (e) Bcl-2–associated X protein (Bax), and (f) cysteine-aspartate protease-3 (Casp3) were assessed by quantitative real-time polymerase chain reaction (qRT-PCR). Groups were defined as sham-operated control (SHAM), BPC 157 only (B), ischemia–reperfusion (IR), and ischemia–reperfusion with BPC 157 treatment (IRB). Data are expressed as mean ± standard deviation (SD) (n = 6 per group). Each dot represents an individual animal. Gene expression was normalized to GAPDH and β-actin and calculated using the $2^{-\Delta\Delta C_T}$ method. Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by

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Tukey's HSD post hoc test. Significance between indicated groups is shown by brackets: *p ≤0.05, **p ≤0.01, ***p ≤0.001.

Immunohistochemical Findings

Immunohistochemical analysis demonstrated distinct alterations in marker expression among the experimental groups (Table 4). eNOS expression was significantly elevated in the IR condition compared with SHAM ($p<0.0001$) and B ($p=0.0023$) groups. In contrast, IRB group exhibited significantly lower eNOS levels than IR ($p=0.0014$), although values remained higher than SHAM ($p=0.0189$). No significant difference was observed between B and IRB ($p=0.9960$) groups. These results indicate that ischemia–reperfusion markedly upregulates eNOS, whereas BPC 157 treatment attenuates this increase, partially restoring values toward baseline. Caspase-3 expression was significantly elevated in IR group compared with SHAM ($p<0.0001$) and B ($p=0.0431$). BPC 157 treatment (IRB) significantly reduced Caspase-3 levels relative to IR ($p=0.0094$), with values not significantly different from SHAM ($p=0.0504$) or B ($p=0.8948$). IL-6 immunoreactivity was significantly elevated in IR group compared with SHAM ($p<0.0001$) and B ($p=0.0014$). Treatment with BPC 157 significantly reduced IL-6 levels in IRB group relative to IR ($p=0.0026$). No significant differences were observed between SHAM and B ($p=0.4784$), SHAM and IRB ($p=0.3420$), or B and IRB ($p=0.9940$). VEGF immunoreactivity was significantly reduced in the IR group compared with SHAM ($p<0.0001$) and B ($p=0.0012$). In contrast, IRB group exhibited a significant increase in VEGF levels compared with IR ($p=0.0086$), though values remained lower than SHAM ($p=0.0018$). VEGF expression in IRB group did not differ significantly from B ($p=0.8259$). A significant difference was also observed between SHAM and B ($p=0.0125$), with higher VEGF levels in SHAM.

Table 4. Semi-quantitative immunohistochemical H-scores of right gastrocnemius muscle in experimental groups.

	SHAM group	B group	IR group	IRB group	p value (ANOVA)
eNOS	46.10±16.43	91.95±23.23*	147.20±18.25*,+	89.01±30.64*,#	<0.001
Caspase-3	39.17±12.99	68.54±9.22*	92.65±10.55*,+	62.64±21.93#	<0.001
IL-6	126.61±15.65	147.31±22.80	209.61±25.17*,+	150.91±31.78#	<0.001
VEGF	209.60±25.17	170.60±19.77*	119.90±9.57*,+	160.90±20.58*,#	<0.001

Notes: Data are expressed as mean ± standard deviation (SD); One-way ANOVA followed by Tukey's HSD post hoc test was used for group comparisons; *p<0.05: Compared to SHAM group; +p<0.05: Compared to B group; #p<0.05: Compared to IR group; SHAM group, laparotomy only; B group, laparotomy + intraperitoneal BPC 157 (20 µg/kg); IR group, ischemia (45 min) + reperfusion (120 min); IRB group, ischemia (45 min) + BPC 157 (20 µg/kg, intraperitoneal) + reperfusion (120 min).

Abbreviations: eNOS, endothelial nitric oxide synthase; Caspase-3, cysteine-aspartic protease-3; IL-6, interleukin-6; VEGF, vascular endothelial growth factor.

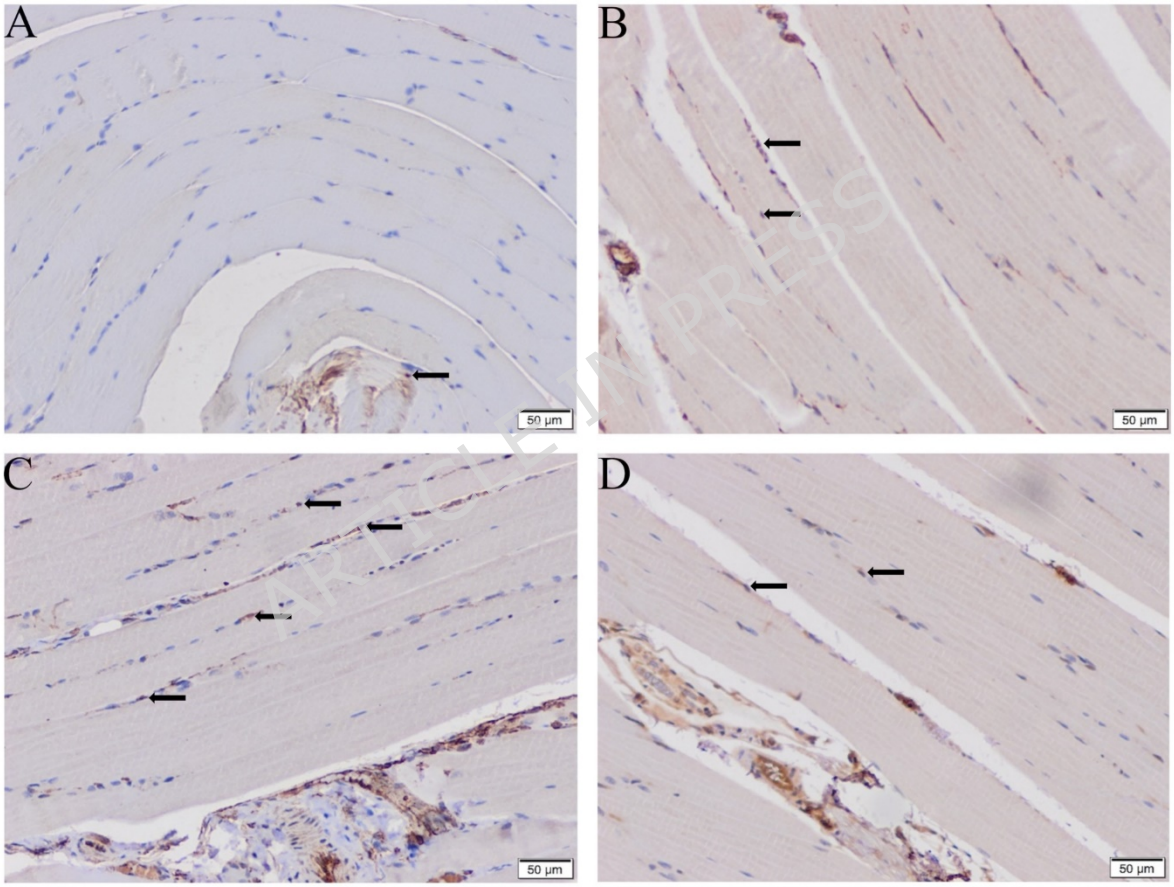


Figure 4. Representative micrographs showing endothelial nitric oxide synthase (eNOS) immunoreactivity (3,3′-diaminobenzidine (DAB), brown; hematoxylin counterstain, blue) in sham-operated control (SHAM), BPC 157 only (B), ischemia–reperfusion (IR), and ischemia–reperfusion with BPC 157 treatment (IRB) groups (n = 6 per group). A: SHAM group, weak, focal staining; B: B group, mild–moderate positivity; C: IR group, widespread perivascular labeling consistent with reperfusion-related upregulation; D: IRB group,

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staining reduced versus IR, trending back toward baseline. Black arrows denote eNOS-positive profiles. Images were obtained at 200× magnification. Scale bars, 50 μm. Quantitative H-scores mirrored this pattern, as reported in Table 4.

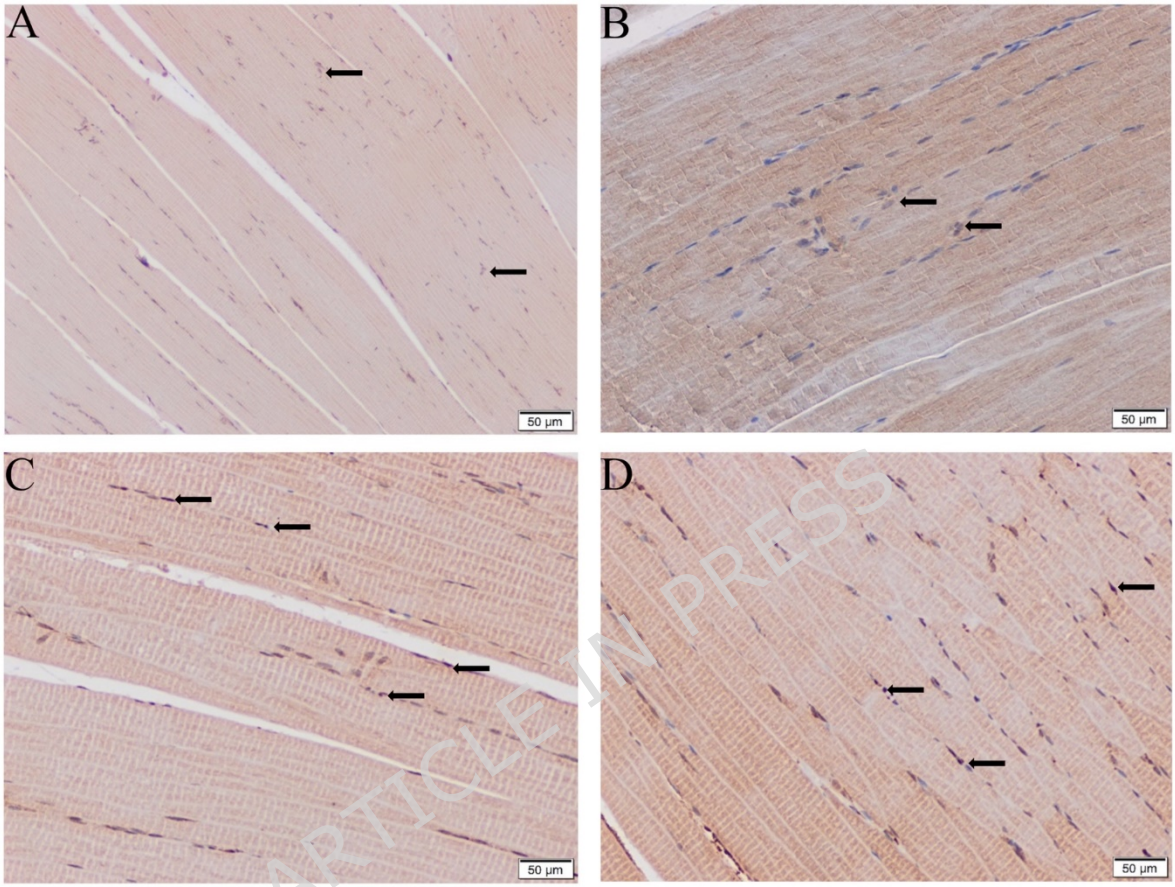


Figure 5. Representative micrographs showing cysteine-aspartate protease-3 (Caspase-3) immunoreactivity (3,3′-diaminobenzidine (DAB), brown; hematoxylin counterstain, blue) in sham-operated control (SHAM), BPC 157 only (B), ischemia–reperfusion (IR), and ischemia–reperfusion with BPC 157 treatment (IRB) groups (n = 6 per group). A: SHAM group, weakly positive fibers/cells; B: B group, low–moderate scattered positivity; C: IR group, strong cytoplasmic labeling in degenerating myofibers and interstitial cells, consistent with apoptosis after I/R; D: IRB group, marked attenuation of staining relative to IR. Black arrows indicate caspase-3–positive myofibers/cells. Images were obtained at 200× magnification. Scale bars, 50 μm. H-score analysis confirmed significant elevation in IR and reduction with BPC 157 (IRB), as detailed in Table 4.

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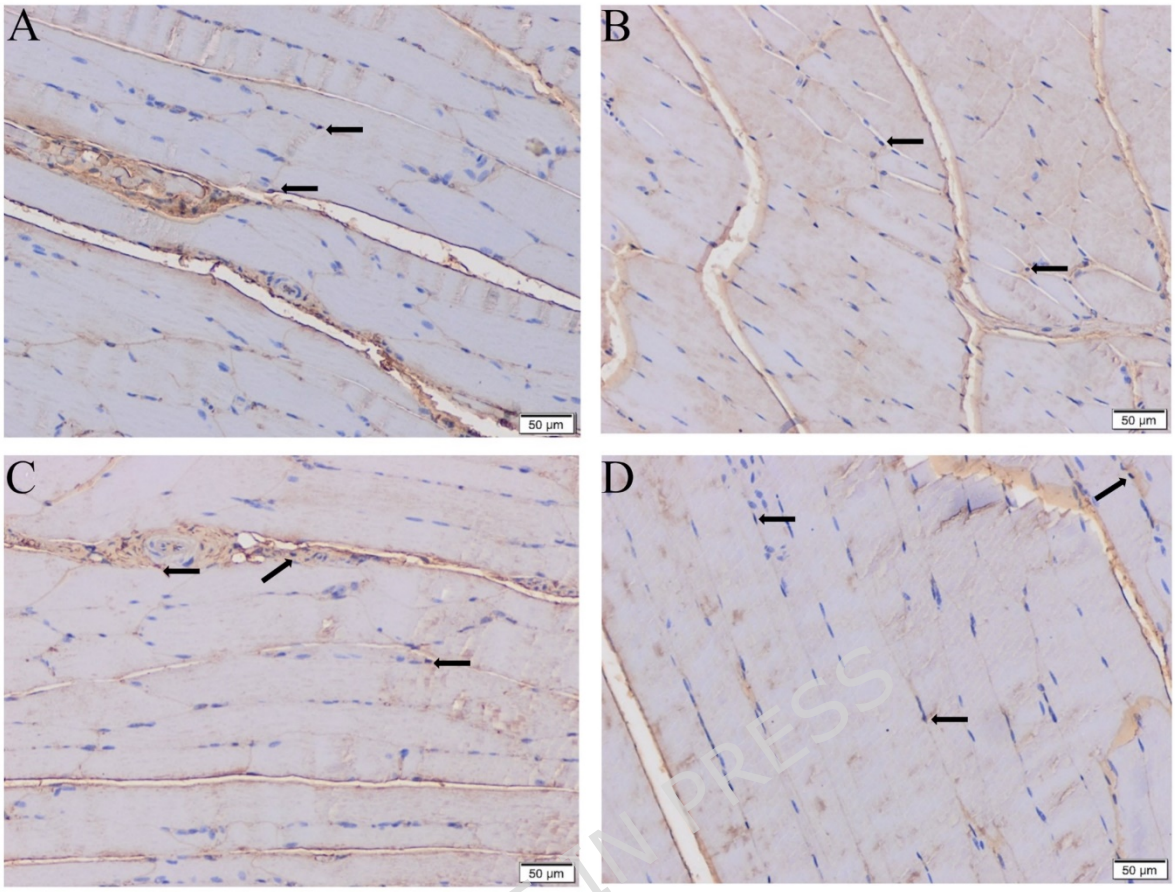


Figure 6. Representative micrographs showing interleukin-6 (IL-6) immunoreactivity (3,3′-diaminobenzidine (DAB), brown; hematoxylin counterstain, blue) in sham-operated control (SHAM), BPC 157 only (B), ischemia–reperfusion (IR), and ischemia–reperfusion with BPC 157 treatment (IRB) groups (n = 6 per group). A: SHAM group and B: B group, low baseline staining; C: IR group, robust cytoplasmic labeling within myofibers, indicating an amplified inflammatory response; D: IRB group, visibly decreased IL-6 immunolabeling compared with IR. Black arrows denote IL-6 immunoreactive cells. Images were obtained at 200× magnification. Scale bars, 50 µm. Semi-quantitative H-scores showed significant IL-6 elevation in IR and reduction with BPC 157 (IRB), consistent with Table 4.

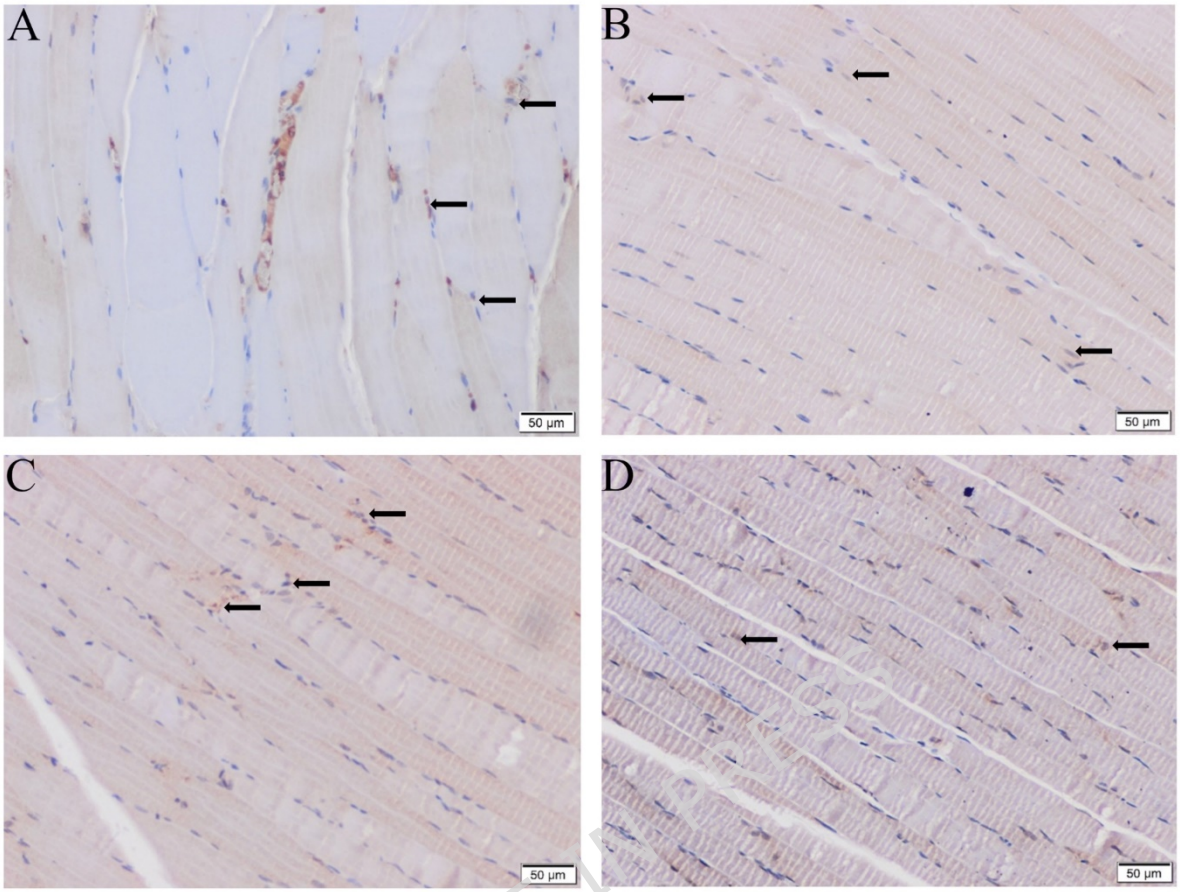


Figure 7. Representative micrographs showing vascular endothelial growth factor (VEGF) immunoreactivity (3,3′-diaminobenzidine (DAB), brown; hematoxylin counterstain, blue) in sham-operated control (SHAM), BPC 157 only (B), ischemia–reperfusion (IR), and ischemia–reperfusion with BPC 157 treatment (IRB) groups (n = 6 per group). A: SHAM group, strong in myofibers and endothelium; B: B group, moderate staining; C: IR group, markedly reduced VEGF labeling, consistent with suppressed angiogenic signaling after I/R; D: IRB group, partial restoration of VEGF staining compared with IR. Black arrows indicate VEGF-positive profiles. Images were obtained at 200× magnification. Scale bars, 50 µm. H-score quantification confirmed IR-associated VEGF loss and its partial recovery with BPC 157 (IRB), as summarized in Table 4.

DISCUSSION

I/R injury is a major complication of PAD, which affects hundreds of millions globally. Although revascularization restores limb perfusion, it paradoxically induces oxidative stress, inflammation, endothelial dysfunction, and apoptosis, leading to tissue damage.[10,14] BPC 157, a stable gastric

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pentadecapeptide first identified for gastroprotection[44,45,82], has shown cytoprotective effects in various tissues.[48,51,58,60–63,65,67,69,83] From a pharmacological perspective, BPC 157 exhibits several features that support its use in experimental I/R models. It is highly stable, resistant to degradation in gastric fluid, and readily soluble in saline and aqueous solutions at physiological pH, enabling reproducible administration.[70] Pharmacokinetic studies in rats and dogs have demonstrated systemic absorption and distribution across multiple tissues, with elimination primarily via urinary and biliary pathways, supporting its bioavailability and translational relevance.[70] Pharmacodynamically, BPC 157 appears to exert pleiotropic cytoprotective effects rather than acting through a single pathway. In previous I/R models, these effects include attenuation of oxidative stress, preservation of endothelial and microvascular integrity, modulation of nitric oxide signaling, suppression of inflammatory cytokine expression, and regulation of apoptotic pathways.[55,62,63] These properties suggest that BPC 157 may represent a potential therapeutic candidate for I/R injury. The present study evaluated its protective effects in skeletal muscle using integrated biochemical, molecular, and histological analyses.

MDA and SOD are established biomarkers of oxidative stress in I/R injury, with MDA reflecting ROS-induced lipid peroxidation and SOD representing a key antioxidant enzyme that neutralizes superoxide radicals. [27–29] Previous studies have demonstrated that BPC 157 attenuates oxidative stress primarily by reducing MDA levels, as shown in Budd–Chiari syndrome and isoprenaline-induced myocardial I/R models.[50,59] Similar reduction in MDA following BPC 157 treatment have also been demonstrated in colitis-associated I/R model.[62] In hepatic ischemia–reperfusion induced by Pringle's maneuver, BPC 157 reduced MDA levels together with preserved histological integrity, supporting the consistency of its antioxidative effects.[55] Although direct evidence of its effects on SOD in I/R is limited, BPC 157's antioxidant activity has been linked to radical scavenging via its carboxyl groups and restoration of glutathione- and enzyme-based defense systems.[51,56] Additionally, BPC 157 has been shown to restore NO bioavailability and attenuate its pathological overproduction in cecum perforation and peritoneal adhesion models.[84,85] Given the interplay between O2•−, NO, and ONOO−, SOD activity is critical, as it reduces superoxide levels, enhances NO bioavailability, and limits peroxynitrite formation.[86] Accordingly, BPC 157 may regulate NO homeostasis not only through nitric oxide synthase–dependent pathways but also via SOD-mediated mechanisms, although direct evidence remains limited. In the present study, IR group exhibited markedly increased MDA levels and reduced

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SOD activity compared with the SHAM and B groups, confirming severe oxidative damage and impaired antioxidant defense. BPC 157 treatment (IRB group) significantly reduced MDA levels relative to IR group, reaching values comparable to SHAM and B groups, indicating effective suppression of lipid peroxidation. Moreover, SOD activity in IRB group not only recovered but exceeded that observed in SHAM and B groups, suggesting an enhanced antioxidant response beyond baseline. Collectively, these findings suggest that BPC 157 may contribute to preserving membrane integrity through MDA reduction and enhancing antioxidant defenses via SOD augmentation; however, as these parameters were measured in serum rather than directly in skeletal muscle tissue, they should be interpreted with caution, as they may not fully reflect local oxidative changes within the injured tissue.

Oxidative stress is a central mechanism in I/R injury, reflecting an imbalance between ROS production and endogenous antioxidant defenses. TAS and TOS are well-established biomarkers used to quantify this balance in experimental models.[31,35,87] Although previous studies have not directly evaluated TAS and TOS, BPC 157 has demonstrated consistent antioxidative effects across different models. In myocardial I/R, BPC 157 reduced oxidative stress markers and MDA levels while preserving cardiac tissue.[50] In hippocampal I/R, it attenuated neuronal injury by modulating key regulatory genes —early growth response 1 (Egr1), AKT serine/threonine kinase 1 (Akt1), Kirsten rat sarcoma viral oncogene homolog (Kras), proto-oncogene tyrosine-protein kinase Src (Src), vascular endothelial growth factor receptor 2 (VEGFR2), nitric oxide synthase 3 (NOS3), and nitric oxide synthase 1 (NOS1)—and suppressing nitric oxide synthase 2 (NOS2) and NF-κB signaling, highlighting its ability to influence multiple ROS-related pathways.[63] Experimental study in *Drosophila melanogaster* also demonstrated that BPC 157 significantly improved survival under hydrogen peroxide–induced oxidative stress, suggesting that its protective effects extend to hydrogen peroxide–dependent pathways of ROS toxicity.[88] Our findings extend this evidence by directly demonstrating modulation of TAS and TOS in skeletal muscle I/R injury. IR group exhibited significantly elevated TOS and reduced TAS compared with SHAM, confirming severe oxidative imbalance. BPC 157 treatment (IRB group) markedly decreased TOS and restored TAS toward SHAM levels, with TAS values significantly higher than those in both IR and B groups. These results indicate that BPC 157 not only suppresses excessive ROS production but also enhances systemic antioxidant capacity beyond baseline. However, as TAS and TOS were measured in serum rather than directly in skeletal muscle tissue, these

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findings should be interpreted with caution, as they may not fully reflect local redox changes within the injured tissue.

Notably, the BPC 157-only group demonstrated a modest but statistically significant increase in MDA levels compared with SHAM, accompanied by a slight elevation in TOS. Although these changes were limited and not associated with consistent alterations in other oxidative markers or histopathological evidence of tissue injury, they may reflect a transient or adaptive modulation of redox homeostasis rather than a direct pro-oxidant effect. Importantly, no structural tissue damage was observed. These findings suggest that the effects of BPC 157 on oxidative balance may be context-dependent and warrant further investigation under non-injured conditions.

Beyond oxidative imbalance, apoptotic signaling represents a key mechanism of tissue damage in I/R injury. Mitochondria-dependent pathways—regulated by p53, Bcl-2, Bax, and Caspase-3—play a central role in this process. Under cellular stress, p53 induces expression of the pro-apoptotic protein Bax, leading to mitochondrial membrane disruption, cytochrome c release, and subsequent activation of Caspase-3, thereby amplifying apoptotic cell death. In contrast, Bcl-2 preserves mitochondrial integrity and inhibits Bax activity; however, I/R shifts this balance toward apoptosis, characterized by increased Bax and Caspase-3 expression and reduced Bcl-2 levels.[14,24,25] Prior studies provide evidence that BPC 157 modulates these apoptotic cascades. Huang et al. demonstrated that BPC 157 reduced radiation-induced hepatic apoptosis through upregulation of Kruppel-like factor 4 (KLF4), which suppressed p53 and attenuated Caspase-3 activity.[64] Vukojević et al. further showed that in hippocampal I/R, BPC 157 regulated apoptotic gene networks by upregulating Egr1, Akt1, Kras, VEGFR2, NOS3, and NOS1, while simultaneously suppressing NOS2 and NF-κB.[63] Of particular relevance, increased Akt1/Kras activity suppresses BAX, whereas NF-κB downregulation favors Bcl-2 expression, collectively counteracting apoptosis.[89] Additional studies support these protective effects in other organs. Duzel et al. reported that BPC 157 reduced apoptosis and preserved mucosal integrity in colitis-associated injury, whereas Gojkovic et al. demonstrated enhanced vascular repair and decreased apoptotic cell death in tendon healing.[59,62] Our findings in skeletal muscle I/R are consistent with and extend previous reports. In IR group, p53, Bax, and Casp3 were significantly elevated, indicating a pronounced pro-apoptotic state. Notably, BPC 157 treatment (IRB group) significantly reduced p53, Bax, and Casp3 levels toward those observed in SHAM group. Immunohistochemical analysis further supported these molecular findings, demonstrating intense Caspase-3 activity in IR group that was markedly

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attenuated following BPC 157 treatment. Collectively, these results may highlight BPC 157's role as a modulator of apoptosis by suppressing key pro-apoptotic mediators, thereby shifting the balance toward cell survival.

Importantly, Bcl-2 mRNA expression did not show a significant decrease in the IR group compared to the SHAM group, suggesting that anti-apoptotic signaling at the transcriptional level may not be markedly suppressed in this experimental model. Therefore, the observed increase in Bcl-2 expression in the BPC 157-treated group is more likely to reflect an upregulation or enhancement of anti-apoptotic pathways rather than a restoration of a diminished baseline. This distinction is important for accurate interpretation of the underlying mechanisms. Although Bax and Bcl-2 mRNA expression patterns provide insight into apoptotic signaling, the absence of protein-level validation limits the interpretation of the Bax/Bcl-2 balance. In particular, the lack of Bax protein assessment and the absence of a calculated Bax/Bcl-2 ratio restrict the ability to fully characterize apoptotic regulation. Therefore, these findings should be interpreted with caution, and further studies with larger cohorts incorporating protein-level analyses are warranted to confirm these observations.

Modulation of inflammation is central to limiting I/R injury, as reperfusion triggers cytokine release, neutrophil recruitment, endothelial activation, and secondary microvascular dysfunction.[18,20,22,90] Excessive IL-6 signaling may amplify both local tissue injury and systemic inflammatory responses. BPC 157 has demonstrated anti-inflammatory properties in diverse models, including a bile duct ligation model of liver injury where it reduced IL-6, TNF-α, and interleukin-1 beta (IL-1β) levels, and a formalin-induced pain model where it attenuated IL-6 expression while exerting antinociceptive effects.[52,91] Besides lowering IL-6 levels, treatment attenuated mucosal inflammation and preserved vascular integrity in colitis-associated I/R.[62] Anti-inflammatory effects have also been confirmed in other disease contexts, such as periodontitis and cancer cachexia, underscoring its systemic immunomodulatory potential beyond I/R.[65,66] In our study, immunohistochemical analysis demonstrated a significant reduction in IL-6 protein levels, with H-scores markedly lower in IRB group than in IR group, supporting an anti-inflammatory effect at the protein level. Therefore, attenuation of IL-6 immunoreactivity by BPC 157 may contribute not only to reduced inflammatory infiltration but also to preservation of microvascular and structural integrity in reperfused skeletal muscle. Il-6 expression was markedly elevated in IR group, reflecting a strong inflammatory response. Notably, BPC 157 treatment (IRB group) did not significantly reduce Il-6 gene

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expression compared with IR, although a downward trend was observed. This apparent discrepancy may reflect the complex relationship between gene expression and protein synthesis, which is influenced by post-transcriptional regulation, mRNA stability, translational efficiency, and protein turnover rates. In addition, the acute nature of the experimental model may contribute to temporal differences between transcriptional and protein-level responses. Therefore, these findings should be interpreted with caution, and further validation using quantitative protein-level analyses such as Western blotting is warranted.

Alongside oxidative stress, apoptosis, and inflammation, vascular and hypoxia-adaptive pathways also play a decisive role in I/R injury. VEGF, eNOS, and HIF-1α constitute key mediators of angiogenesis, vascular protection, and metabolic adaptation under ischemic stress. VEGF is typically upregulated under hypoxia, driving endothelial proliferation and migration to restore perfusion and limit tissue damage.[92] HIF-1α stabilizes in hypoxic environments, where it directly induces VEGF expression and regulates glycolytic genes to support cellular survival.[93] eNOS-derived NO contributes to vascular homeostasis by promoting vasodilation, limiting platelet aggregation, and reducing oxidative stress, thereby counteracting endothelial dysfunction.[94] Collectively, these molecules act in concert, with HIF-1α–driven VEGF signaling enhancing eNOS activity and synergistically improving angiogenesis, oxidative balance, and reperfusion outcomes.[95] Experimental evidence suggests that BPC 157 interacts with this axis. It has been shown to enhance VEGF expression and endothelial proliferation and to stimulate eNOS phosphorylation via Src–Caveolin-1 signaling.[60,61,64] Additional studies indicate that BPC 157 supports VEGFR2 internalization and VEGFR2–Akt–eNOS activation, independent of shear stress or ligands, while also exerting context-specific control over angiogenesis, enhancing vascularization in ischemic muscle and tendon but suppressing pathological corneal neovascularization.[46] Although direct associations with HIF-1α remain limited, its reported effects on VEGF and eNOS are consistent with modulation of hypoxia-regulated pathways.[51,67] Our findings provide new insights into skeletal muscle I/R injury. Hif-1α expression was significantly elevated in IR group, consistent with hypoxia-driven stress, and was reduced in IRB group, although remaining above SHAM levels, suggesting that BPC 157 limits excessive Hif-1α activation while preserving adaptive signaling. VEGF, which was markedly suppressed in IR group, increased following BPC 157 treatment, although not to SHAM levels, indicating partial restoration of angiogenic activity. By contrast, eNOS expression was paradoxically highest in IR group and was reduced by BPC 157

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in IRB group, although it remained elevated compared with SHAM group. The apparently paradoxical pattern of eNOS expression observed in this study may reflect the complex and context-dependent role of eNOS in ischemia–reperfusion injury. Under conditions of oxidative stress, eNOS can become functionally “uncoupled,” leading to the production of superoxide and reactive nitrogen species instead of nitric oxide, thereby contributing to tissue injury rather than protection. In this setting, increased eNOS immunoreactivity in the IR group may represent a compensatory but dysfunctional response. Conversely, the relative reduction of eNOS expression in the BPC 157-treated group may indicate partial restoration of endothelial homeostasis and reduced oxidative stress–induced dysregulation, rather than diminished protective signaling. However, further studies are required to clarify the paradoxical eNOS findings and to define the precise molecular intermediaries involved.

Histopathological manifestations of I/R injury include necrosis, apoptosis, edema, inflammation, and microvascular disruption, processes largely driven by oxidative bursts, excessive ROS production, inflammatory activation, and endothelial dysfunction, culminating in severe structural and functional impairment.[96] In the present study, hematoxylin–eosin staining revealed significantly higher injury scores in IR group, characterized by structural disruption and vascular congestion, whereas BPC 157 treatment (IRB group) markedly attenuated these changes, restoring values toward those observed in the SHAM group. Masson’s trichrome staining further demonstrated pronounced collagen deposition, disruption of muscle–connective tissue architecture, fiber degeneration, interstitial edema, and cytoplasmic damage in IR group, all of which were significantly reduced in IRB group. These results are in line with previous reports demonstrating histological protection by BPC 157 across multiple I/R models. In hippocampal I/R, treatment normalized VEGF expression, downregulated NF-κB, preserved tissue architecture, and reduced apoptotic neurons and inflammatory infiltration.[63] In a rat cecum perforation model, BPC 157 promoted angiogenesis while reducing hemorrhagic necrosis and adhesions.[85] In hepatic I/R induced by Pringle’s maneuver, BPC 157 preserved sinusoidal structure, limited necrosis, and attenuated inflammatory infiltration.[55] In colitis-associated ischemia–reperfusion injury, BPC 157 was shown to restore vascular integrity, enhance re-epithelialization, and reduce mucosal inflammation.[62] BPC 157 also promoted rapid revascularization and decreased adhesion formation in a duodenocolic fistula model.[49] In tendon and muscle injury models, it also accelerated healing and preserved structural alignment.[47,53] Collectively, these findings reinforce the histopathological improvements observed in our

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study, supporting BPC 157's capacity to attenuate structural injury and preserve tissue integrity in skeletal muscle I/R.

Regarding human use, available clinical data remain limited. Early clinical studies of PL 14736/BPC 157 in inflammatory bowel disease and healthy volunteers have reported favorable tolerability and no major safety concerns.[44,45] However, robust evidence of efficacy in ischemia–reperfusion injury in humans is currently lacking. Furthermore, although experimental studies have not consistently identified significant adverse effects, the overall safety profile, optimal dosing strategies, long-term safety, and potential drug–drug interactions of BPC 157 in humans remain insufficiently characterized. Therefore, despite its promising experimental profile, clinical translation will require well-designed human studies, including pharmacokinetic, dose–response, safety, and drug interaction evaluations.

LIMITATIONS

This study has several limitations that should be acknowledged. The rat model does not fully replicate the complexity of human PAD, thereby limiting clinical translation. The relatively small sample size and short follow-up period may reduce statistical power and generalizability. Although a sample size of six animals per group aligns with established experimental practice, it does not substitute for a formal a priori power calculation and should therefore be considered a methodological limitation. In addition, only a single administration protocol was tested, and the use of a single dose of BPC 157 together with a single reperfusion time point (2 hours) does not allow evaluation of dose–response relationships, optimal therapeutic window, or long-term durability of the observed effects. The selected experimental design reflects an acute-phase model of ischemia–reperfusion injury and represents a controlled snapshot that may not fully capture the complexity of clinical conditions. Long-term outcomes such as functional recovery, limb salvage, and systemic side effects were not assessed.

Our analysis focused mainly on oxidative stress and inflammation, without exploring other potentially relevant mechanisms such as mitochondrial dynamics, autophagy, endothelial–immune interactions, or systemic organ cross-talk. Furthermore, the lack of protein-level assessment of key apoptotic markers such as Bax and Bcl-2, as well as the absence of Bax/Bcl-2 ratio analysis, represent additional limitations that restrict mechanistic interpretation of apoptotic regulation.

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Another limitation of this study is that oxidative stress markers (MDA, SOD, TAS, and TOS) were measured only in serum and not directly in skeletal muscle tissue. Although serum measurements provide valuable information regarding systemic oxidative stress, they may not fully reflect the local redox environment within the affected muscle tissue. This compartmental discrepancy may limit the direct correlation between biochemical, molecular, and histopathological findings. Tissue-specific biochemical assessment could provide more precise insights into local oxidative alterations, and future studies incorporating both systemic and tissue-level analyses are warranted.

In addition, discrepancies between mRNA and protein expression levels, particularly for IL-6, were observed. The absence of quantitative protein validation methods such as Western blot analysis represents a limitation of the study and may affect the interpretation of the anti-inflammatory effects. Comparisons with standard or adjunctive therapies were not included, limiting contextualization of efficacy.

The observed increase in oxidative stress markers in the BPC 157-only group raises questions regarding its baseline effects on redox homeostasis, which were not fully explored in this study. In addition, histopathological scoring was performed by a single blinded evaluator, and inter-observer variability or kappa statistics were not assessed, which may limit the reproducibility of the scoring system.

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Therefore, this work should be considered as preliminary evidence of the protective effects of BPC 157 in skeletal muscle ischemia–reperfusion injury. Further studies with larger cohorts, multiple dosing regimens, extended reperfusion periods, longer follow-up, and more comprehensive mechanistic investigations are required to validate and extend these findings.

CONCLUSION

Ischemia–reperfusion injury remains a major contributor to local and systemic complications in peripheral arterial disease, largely driven by oxidative stress and inflammation. The present findings suggest that BPC 157 may exert cytoprotective effects by reducing oxidative damage, modulating inflammatory responses, and supporting tissue repair. While these results highlight its potential therapeutic relevance, translation into clinical practice will require further validation in larger cohorts, dose–response studies, long-term follow-up, and well-designed clinical trials. BPC 157 may therefore represent a promising candidate for expanding treatment strategies in I/R injury, although its clinical utility remains to be established.

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Author Contributions: Conceptualization, A.Ö.; methodology, H.D., A.Ö., A.K.Y., 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., 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., and M.A.; project administration, A.Ö. All the authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical approval for the study was obtained from the Gazi University Animal Experiments Local Ethics Committee (Ankara, Turkey; Approval number: G.Ü.ET-24.049, date: 27.05.2024). All methods were performed in accordance with relevant institutional and international guidelines and regulations, including the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1986) and the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020). The study was conducted and reported in accordance with the ARRIVE guidelines.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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