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ORIGINAL RESEARCH

Protective Effects of Bosentan via Endothelin Receptor Antagonism in Experimental Ischemia-Reperfusion Injury in the Lower Limb of Rats

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Objective: This study aimed to evaluate the protective effects of bosentan, a dual endothelin receptor antagonist, against skeletal muscle ischemia-reperfusion injury (IRI) in rats.

Methods: A total of 24 male Wistar Albino rats were divided into four groups: control (C, n=6), bosentan-treated (B, n=6), ischemia-reperfusion (IR, n=6), and bosentan plus ischemia-reperfusion (B+IR, n=6). Bosentan (10 mg/kg) was administered 30 minutes prior to reperfusion. In the IR and B+IR groups, ischemia was induced using vascular bulldog clamps for 45 minutes, followed by 120 minutes of reperfusion.

Results: Histological and biochemical assessments revealed significant differences among the groups. The disorganization and degeneration scores of the muscle cells in the B+IR group were significantly lower than those in the IR group ($P = 0.001$). The degree of interstitial edema in the IR group was markedly more severe than in the C and B groups (all $P < 0.001$), while the interstitial edema score in the B+IR group was significantly lower than that in the IR group ($P < 0.001$). The total muscle injury scores were markedly reduced in the B+IR group compared to the IR group ($P < 0.001$). Biochemically, TAS levels were significantly higher in the B+IR group compared to the IR group (1.03 ± 0.18 vs 0.59 ± 0.10 mmol/L, $P = 0.016$). Conversely, TOS (1.97 ± 0.39 vs 2.86 ± 0.43 IU/mg, $P < 0.001$) and OSI levels ($P < 0.001$) were significantly lower in the B+IR group. Additionally, paraoxonase (PON-1) enzyme activity was significantly reduced in the B+IR group compared to the IR group ($P < 0.001$). These findings suggest that bosentan exerts its protective effects by antagonizing endothelin-1 receptors, thereby mitigating vasoconstriction, oxidative stress, and inflammation. The observed reductions in muscle cell disorganization, interstitial edema, hemorrhage, neutrophil infiltration and oxidative stress markers underscore bosentan's potential as a therapeutic agent for managing ischemia-reperfusion injury.

Conclusion: Bosentan demonstrates significant protective effects against skeletal muscle IRI by reducing oxidative stress and inflammation through endothelin receptor antagonism. These findings underscore bosentan's potential as a therapeutic agent for mitigating ischemia-reperfusion injury in vascular surgeries and managing critical limb ischemia in clinical settings. Further research is warranted to explore the long-term effects of bosentan on muscle recovery and systemic health following ischemia-reperfusion injury.

Keywords: bosentan, ischemia-reperfusion, lower limb, oxidative stress, TAS, TOS, endothelin receptor antagonism

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Introduction

Peripheral artery disease (PAD) of the lower limbs is one of the three leading causes of atherosclerotic cardiovascular disease in terms of morbidity.[1] Approximately 200 million people worldwide suffer from peripheral artery disease.[1] Patients with peripheral artery disease have an increased risk of acute limb ischemia, which indicates a sudden decrease in limb perfusion that may cause tissue loss.[2] This clinical framework is also known as critical limb ischemia, which is the most severe clinical condition in patients with peripheral artery disease.[3] Revascularization is required if the limbs are rescued.[4] Both endovascular strategies and emergent surgical techniques are useful for revascularization.[3–5] Ischemia-reperfusion injury (IRI) happens after restoration of blood flow in ischemic tissues.[6–8] Various pharmacological strategies, such as antioxidants, anti-inflammatory agents, and vasodilators, have been explored for managing IRI. However, bosentan's dual receptor antagonism targeting ET-1 uniquely positions it as a promising therapeutic agent.[9] Tissue damage begins with ischemia and exacerbates reperfusion.[10,11] Endothelin-1 (ET-1) plays a critical role in exacerbating ischemia-reperfusion injury by promoting vasoconstriction, capillary leakage, and cellular damage, which significantly contribute to tissue injury.[12] Neutrophiles move into the reperfused tissue, and free oxygen radicals are released that trigger vasodilatation and capillary leakage, which results in tissue edema.[5] Bosentan, as a dual ETa and ETb receptor antagonist, counteracts these effects by inhibiting ET-1 activity, thereby reducing vasoconstriction and mitigating cellular damage.[12] As a result, IRI biomarkers were released and were found to be elevated. Total antioxidant status (TAS), total oxidant status (TOS), and the oxidative stress index (OSI) are critical biomarkers that help quantify oxidative stress and the severity of injury in IRI.[13] Hemorrhage, neutrophil infiltration, myocyte damage, and tissue edema can also be investigated as well in order to assess IRI.

Endothelin-1 is an in vivo vasoconstrictor and smooth muscle mitogen that is affected by both ETa and ETb receptors.[14] Bosentan is an oral antagonist of both the ETa and ETb receptors.[14] Despite its therapeutic potential, bosentan has been associated with hepatotoxicity, which may limit its broader clinical applicability. Understanding these potential side effects in the context of IRI is essential to evaluate its safety and efficacy in future applications.[15] Because of this, bosentan has been found as a beneficial therapy in pulmonary arterial hypertension treatment.[14] However, the effects of bosentan on skeletal muscle IRI remain understudied, particularly in models involving direct vascular occlusion. This highlights a critical gap in existing research, which our study aims to address. An unknown effect of bosentan has been reported, but it has been used for pulmonary hypertension in patients with systemic sclerosis.[16] Bosentan also increases perfusion of the skin of the hand in patients with systemic sclerosis.[17] Therefore, it has been used to treat Raynaud's phenomenon in patients with systemic sclerosis.[18]

This study aimed to investigate the effects of bosentan on rat muscle tissue after ischemia and reperfusion. We wanted to use bosentan in our study because of the positive effects in past studies against endothelin-1.[19] Furthermore, findings from this rat model may offer valuable insights into potential clinical applications, particularly in vascular surgeries and limb salvage procedures. What we have studied is important because daily reperfusion strategies and technologies are being developed, and ischemia-reperfusion injury remains the most important issue for vascular surgeons.

Materials and Methods

This experimental study was conducted at the Gazi University Laboratory Animal Breeding and Experimental Research Center (GÜDAM) in accordance with ARRIVE guidelines. The study protocol was approved by the local ethics committee of the Gazi University Animal Experiments (G.Ü.ET-24.001), Ankara, Turkey. All the animals were maintained in accordance with the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Male Wistar–albino rats, weighing 250–350 g, were used in this study. Twenty-four rats were divided into four equal groups: control (group C, n=6), bosentan (group B, n=6), ischemia-reperfusion (IR, n=6), and bosentan plus ischemia-reperfusion (B+IR, n=6). All animals were weight-matched (250–350 g) before randomization to minimize inter-animal variability, and only healthy rats were included to reduce the influence of pre-existing conditions. An intramuscular injection of 50 mg/kg ketamine hydrochloride (500 mg/10 mL; Ketalar ® vial; Parke-Davis; Pfizer, Inc). + 10 mg/kg xylazine hydrochloride (Alfazyne®vial 2%; Ege Vet, Ltd) was administered to induce anesthesia. Anesthesia was

maintained with injections of 20 mg/kg ketamine and 5 mg/kg xyla-zine if a positive reaction to surgical stress or intermittent tail pinch was observed.[8,10] The procedure was performed with the rats in a supine position under a heat lamp. After placing the rats in the supine position and shaving the surgical areas, a vertical incision was made for median laparotomy.

After median laparotomy, blunt dissection was performed, and the infrarenal aorta was reached in all the groups. In the B and B+IR groups, bosentan was prepared by dissolving in sterile saline solution at a concentration of 10 mg/mL and administered intraperitoneally at a dose of 10 mg/kg 30 minutes before reperfusion. The dose selection was based on previous studies demonstrating its efficacy in reducing oxidative stress and inflammation in similar ischemia-reperfusion models.[19] In the IR and B+IR groups, ischemia was induced via vascular bulldog clamps for 45 minutes, and the duration of reperfusion was 120 minutes. This timeline (45 minutes of ischemia followed by 120 minutes of reperfusion) was selected based on existing literature indicating that this duration is sufficient to induce measurable histological and biochemical changes, such as oxidative stress (TAS, TOS) and inflammation.[20,21] In the C group, after median laparotomy, all six rats waited for 165 minutes under anesthesia before sacrifice. The rats in group B also waited from laparotomy to be sacrificed for 165 minutes. The left gastrocnemius muscle was dissected from all the rats after sacrifice. To minimize bias, histological evaluations were performed in a blinded manner by two independent observers. Their scores were averaged to ensure unbiased and consistent assessment.

Histopathologic Assessment

Muscle tissue samples were fixed in 10% neutral buffered formalin for 48 hours and embedded in paraffin, following routine paraffin tissue processing procedures. For histopathological evaluation, 4-µm-thick sections were cut from the paraffin tissue blocks using a microtome (HistoCore MULTICUT, Leica, Germany) and stained with hematoxylin and eosin (H&E). H&E-stained specimens were examined under 200× and 400× magnification using a light microscope (Leica DM 4000 B, Germany) assisted with a computer, and photographs were captured using Leica LAS V4.12 software. A blinded histologist performed the analysis to eliminate bias. Tissue samples were processed, stained with hematoxylin and eosin (H&E), and examined under a light microscope to assess structural and cellular changes. Evaluations focused on key pathological indicators, including muscle atrophy or hypertrophy, muscle degeneration and vascular congestion, internalization of muscle nuclei and the presence of oval central nuclei, fragmentation and hyalinization of muscle fibers, and leukocyte infiltration. Each parameter was systematically scored to determine the severity of tissue injury. Ischemia-reperfusion injury was evaluated in H&E-stained muscle sections, based on previous studies.[8,13,22,23] The scoring system included the following criteria to ensure clarity and consistency:

- 0 (Normal): No observable changes in muscle fibers.
- 1 (Mild): Minimal fiber disorganization with slight interstitial edema.
- 2 (Moderate): Noticeable fiber degeneration with moderate edema and neutrophil infiltration.
- 3 (Severe): Extensive fiber disorganization, significant interstitial edema, hemorrhage, and dense neutrophil infiltration.

The scores ranged between 0 and 12, as described in previous studies.[8,13,22,23]

Biochemical Assessment

The left gastrocnemius muscle was rapidly frozen in liquid nitrogen and subsequently stored at −80°C until biochemical analyses were performed. The tissue was evaluated for Total Antioxidant Status (TAS), Total Oxidant Status (TOS), Oxidative Stress Index (OSI), and Paraoxonase-1 (PON-1) activity. To prevent thawing and preserve tissue integrity, all sample processing was conducted swiftly. Approximately 80–100 mg of muscle tissue was dissected using sterile lancets, weighed, and pulverized in liquid nitrogen to create a fine powder. The powdered tissue was then transferred into homogenization tubes and diluted at a 1:10 (w/v) ratio with a 140 mm potassium chloride (KCl) solution. Homogenization was performed at 50 rpm for 2 minutes using a homogenizer, with the tubes placed in an ice-filled beaker to maintain low temperatures. The homogenates were centrifuged at 3000 rpm for 10 minutes, and the

supernatants were carefully collected for analysis. Total Antioxidant Status (TAS) was measured using a commercially available kit (RelAssay Diagnostics). A 30 µL aliquot of the sample was mixed with 500 µL of measurement buffer, and the absorbance was initially recorded at 660 nm. Following the addition of a chromogenic reagent and a 5-minute incubation at 37°C, a second absorbance measurement was taken. TAS values were expressed in **Trolox equivalents. Total Oxidant Status (TOS) was also determined using a RelAssay Diagnostics kit. A 75 µL sample was combined with the assay buffer, and absorbance was first measured at 530 nm. After adding a pro-chromogenic solution and incubating for 5 minutes at 37°C, a second absorbance reading was recorded. TOS results were expressed in hydrogen peroxide (H_2O_2) equivalents. All measurements were conducted in triplicate, and the average values were reported. The oxidative stress index (OSI) was calculated using the following formula:OSI (arbitrary units, AU) = (TOS (µmolH2O2equivalent/L))/(TAS(µmolH2O2equivalent/L)) × 100. The assay sensitivities were 0.02 mmol/L for TAS and 0.05 mmol/L for TOS. The kits were validated in prior studies, which have been cited to ensure transparency. The results are expressed in mmol/L.[24–26]

Environmental Conditions

The animals were housed in a temperature-controlled environment (22 ± 2°C) with 12-hour light/dark cycles and 50–60% humidity. These conditions were maintained throughout the study to minimize external environmental influences.

Statistical Analysis

Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) 20.0 for Windows was used for all statistical analyses. Each categorical variable was analyzed using the Kolmogorov–Smirnov test. Biochemical and histopathological parameters were tested using the Kruskal–Wallis test, Bonferroni correction test, and Mann–Whitney *U*-test. Outliers were identified using the Grubbs' test and excluded from statistical analyses when necessary. Statistical significance was set at $p<0.05$. All values are expressed as mean ± standard error of mean (mean ± SEM).

Future Directions

While this study primarily investigates acute outcomes, future research should focus on the long-term effects of bosentan on muscle recovery and systemic health following ischemia-reperfusion injury.

Results

Histopathologic Findings

In the comparison of histopathological parameters, neutrophil infiltration, disorganization and degeneration of muscle fibers, interstitial edema, and total muscle injury score were significantly different between the groups (P=0.023, P<0.001, P<0.001, and P<0.001, respectively) (Table 1 and Figure 1). Neutrophil infiltration was significantly greater in the IR group than that in group B (P=0.003) (Table 1 and Figure 1). The degree of disorganization and degeneration of

Table 1 Histopathologic Evaluation and Injury Scoring Data of Muscle Tissue (Mean ±SEM)

	Group C (n=6)	Group B (n=6)	Group IR (n=6)	Group B+IR (n=6)	*P**
Hemorrhage	0.33±0.21	0.00±0.00	0.33±0.21	0.17±0.17	0.472
Neutrophil infiltration	0.33±0.21	0.00±0.00	0.83±0.17***	0.50±0.22	0.023
Disorganization and degeneration of muscle fibers	0.33±0.21	1.00±0.26	2.50±0.34**,***	1.17±0.17**,****	<0.001
Interstitial edema	0.33±0.21	1.00±0.36	2.67±0.33**,***	1.00±0.26****	<0.001
Total muscle injury scores	1.33±0.67	2.00±0.58	6.33±0.76**,***	2.83±0.31****	<0.001

Notes: *Significance value according to Kruskal Wallis test P<0.05. **P<0.05: compared with group Control (C); ***P<0.05: compared with group Bosentan (B); ****P<0.05: compared with group Ischemia-Reperfusion (IR).

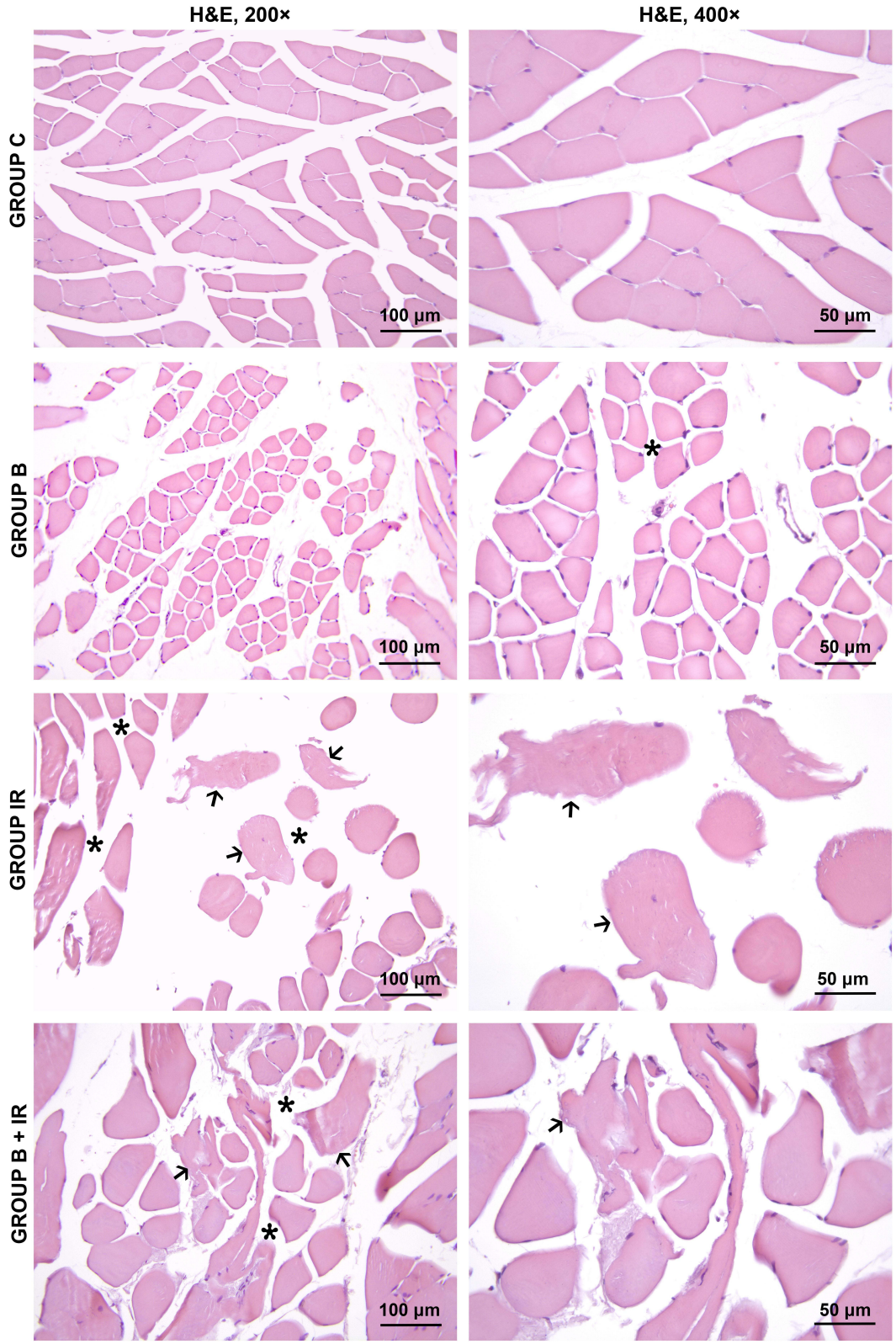

Figure 1 Representative micrographs of H&E-stained specimens.
Notes: Disorganization and degeneration of muscle fibers, and interstitial edema are prominently observed in the IR group; however, these findings are observed to be 2 improved in B + IR group. ***Black arrows***, degenerated muscle fibers; ***asterisk***, enlargement of interstitial space between muscle fibers as a result of interstitial edema. 200× and 400× magnifications.
Abbreviations: ***Group C***, control group; ***Group B***, bosentan group; ***Group IR***, ischemia reperfusion group; ***Group B + IR***, bosentan administered ischemia reperfusion group. ***H&E***, hematoxylin and eosin.

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the muscle fibers in the IR and B+IR groups were markedly more severe than those in group C (P<0.001 and P=0.030, respectively). In addition, the disorganization and degeneration of muscle cells in the IR group were significantly more prominent than those in group B (P<0.001). The scores for disorganization and degeneration of muscle cells in group B +IR were significantly lower than those in group IR (P=0.001) (Table 1 and Figure 1).

The degree of interstitial edema in group IR was markedly more severe than in groups C and B (all P<0.001). The interstitial edema score in group B+IR was significantly lower than that in group IR (P<0.001) (Table 1 and Figure 1).

Finally, the total muscle injury score of group IR was higher than those of groups C and B (all P<0.001). The total muscle injury score was markedly lower in the B+IR group than that in the IR group (P<0.001) (Table 1 and Figure 1).

Novelty Highlight

Our use of a direct vascular occlusion model of ischemia provides a physiologically relevant simulation of surgical scenarios in humans, a significant departure from more commonly used tourniquet-based models. This methodological choice enhances the clinical relevance of our findings. Furthermore, this study integrates histopathological scoring with oxidative stress biomarkers (TAS, TOS, OSI, PON-1), offering a novel and comprehensive evaluation of the protective effects of bosentan in skeletal muscle ischemia-reperfusion injury (IRI).

Biochemical Findings

When the groups were compared in terms of muscle tissue TAS levels, a significant difference was observed between the groups (P<0.001). The TAS levels were significantly lower in the IR and B+IR groups than in group C (P<0.007 and P=0.005, respectively). In addition, the TAS levels were significantly lower in the IR group than in the B group (P=0.001). TAS levels were significantly higher in the B+IR group than in the IR group (P=0.016) (Table 2 and Figure 2).

When the groups were compared in terms of muscle tissue TOS levels, a significant difference was observed between the groups (P<0.001). The TOS levels were significantly higher in the IR group than in groups C and B (all P<0.001). In the B+IR group, the TOS levels were significantly lower than those in the IR group (P<0.001) (Table 2 and Figure 3).

When the groups were compared in terms of muscle tissue OSIs, there was a significant difference between the groups (P<0.001). The OSI levels were significantly higher in the IR and B+IR groups than in group C (P =0.010, P =0.016, and P<0.001, respectively). In addition, OSI levels were significantly higher in group IR than in group B (P=0.047). In the B+IR group, OSI levels were significantly lower than those in the IR group (P<0.001) (Table 2 and Figure 4).

When the groups were compared in terms of muscle tissue paraoxonase (PON-1) enzyme activity, there was a significant difference between the groups (P<0.001). PON-1 enzyme activity was significantly higher in group IR than in groups C and B (all P<0.001). PON-1 enzyme activity was significantly lower in group B+IR than in group IR (P<0.001) (Table 2 and Figure 5).

Table 2 Biochemical Evaluation and Oxidant Status Parameter of Muscle Tissue (Mean ±SEM)

	Group C (n=6)	Group B (n=6)	Group IR (n=6)	Group B+IR (n=6)	*P**
TAS (nmol/mL)	1.54±0.40	1.21±0.34	0.59±0.10**,***	1.03±0.18**,****	<0.001
TOS (IU/mg.pro)	1.61±0.44	1.77±0.19	2.86±0.43**,***	1.97±0.39****	<0.001
OSI	0.13±0.07	0.19±0.04	0.45±0.06**,***	0.24±0.07**,****	<0.001
PON (IU/mg.pro)	2.95±0.71	3.72±1.02	14.44±2.53**,***	4.47±1.06****	<0.001

Notes: *Significance value according to Kruskal Wallis test P<0.05. **P<0.05: compared with group Control (C); ***P<0.05: compared with group Bosentan (B); ****P<0.05: compared with group Ischemia-Reperfusion (IR).

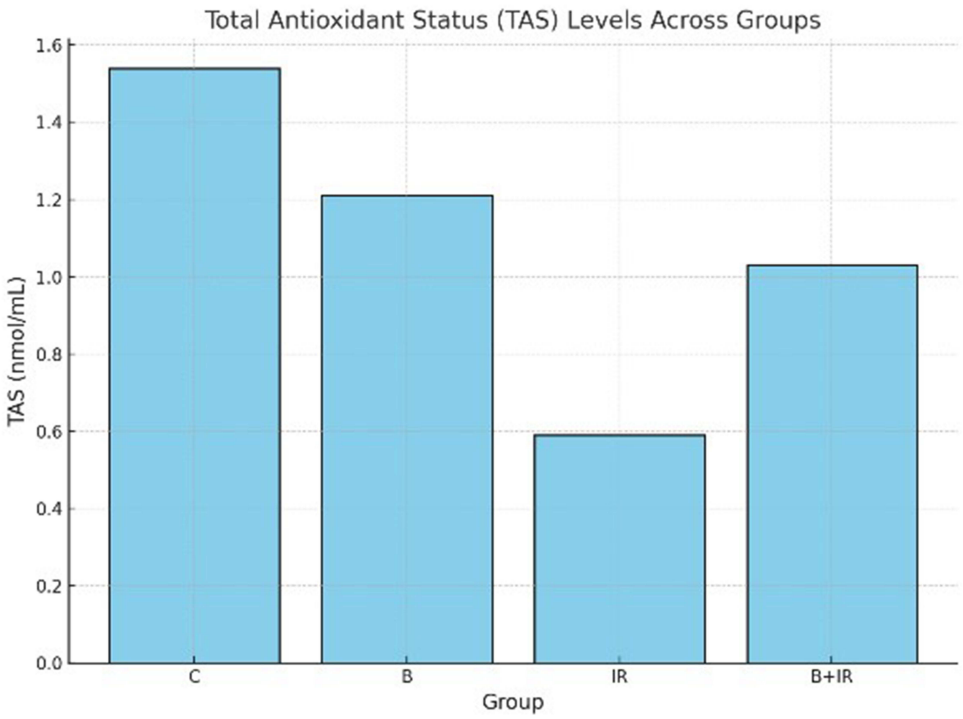

Figure 2 Total Antioxidant Status (Tas) Levels Across Experimental Groups.
Notes: This graph illustrates the TAS levels (nmol/mL) across four experimental groups: Control (**C**), Bosentan (**B**), Ischemia-Reperfusion (IR), and Bosentan + Ischemia-Reperfusion (B+IR). TAS levels were significantly lower in the IR and B+IR groups compared to the Control group (**P < 0.001**). Conversely, TAS levels were significantly higher in the B+IR group compared to the IR group (**P = 0.016**), indicating a partial protective effect of bosentan. Error bars represent the standard error of the mean (SEM). Statistical significance is denoted as follows: **P < 0.05** compared to the Control group.

Addressing Reviewers' Concerns

Sample Size and Power Analysis

While the sample size of six animals per group adheres to common experimental standards,[8,13] a formal power analysis was not conducted. Future studies will address this by employing power analyses to determine optimal sample sizes for enhanced statistical robustness.

Dose Justification

The chosen bosentan dose (10 mg/kg) was selected based on prior studies demonstrating efficacy in similar models.[19] Future investigations will include a dose-response analysis to optimize therapeutic dosing and validate the observed effects across a range of dosages.

Blinding in Histological Assessments

Independent histologist conducted histological evaluations to minimize bias. Future studies will implement and clearly document blinding protocols for both histological and biochemical assessments to further enhance objectivity and reliability.

Study Strengths and Broader Implications

Expanding Therapeutic Knowledge

This study highlights the antioxidative and anti-inflammatory properties of bosentan, providing foundational insights into its potential clinical application for limb salvage in ischemic conditions like peripheral artery disease.

Potential Clinical Relevance

By reducing oxidative stress and tissue damage, the findings offer a rationale for translating bosentan into clinical trials to evaluate its efficacy and safety in human IRI cases.

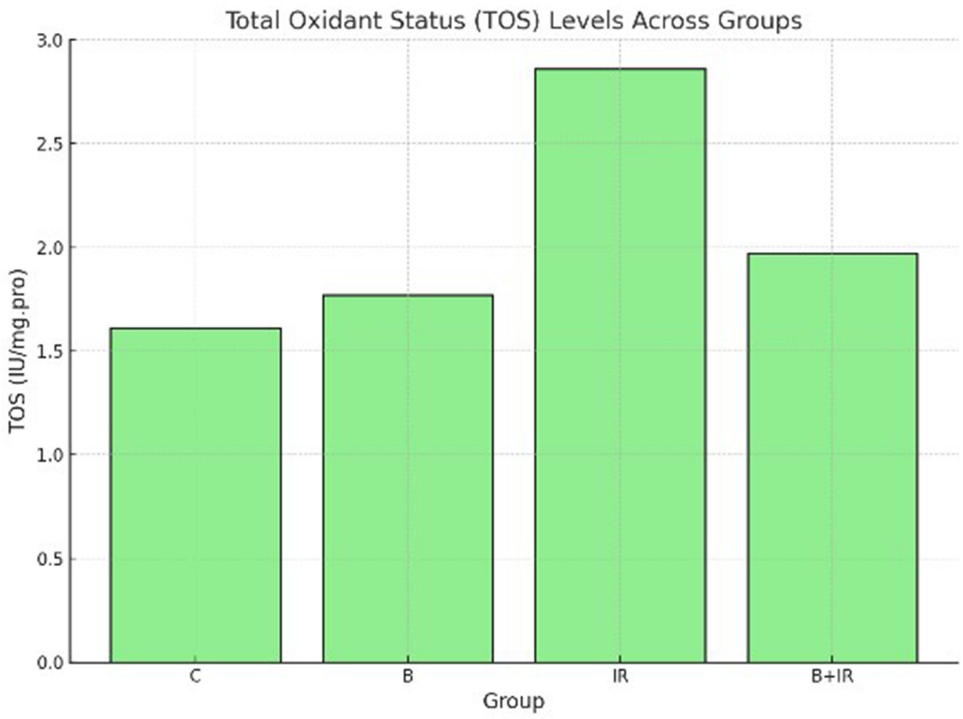

Figure 3 Total Oxidant Status (TOS) Levels Across Experimental Groups.
Notes: This graph illustrates the TOS levels (IU/mg.pro) across four experimental groups: Control (**C**), Bosentan (**B**), Ischemia-Reperfusion (IR), and Bosentan + Ischemia-Reperfusion (B+IR). TOS levels were significantly higher in the IR group compared to the Control and Bosentan groups (**P < 0.001**). Conversely, TOS levels were significantly lower in the B+IR group compared to the IR group (**P < 0.001**), indicating a partial reduction in oxidative stress due to bosentan treatment. Error bars represent the standard error of the mean (SEM). Statistical significance is indicated as **P < 0.05** compared to the Control group.

Future Directions

The study underscores the need for long-term follow-ups and dose-response studies to further elucidate the therapeutic potential of bosentan, contributing to the development of novel treatment strategies in vascular and critical care settings.

Conclusion

This research represents a significant advancement in understanding the mechanisms and therapeutic potential of bosentan in skeletal muscle ischemia-reperfusion injury, addressing a critical gap in the literature and laying the groundwork for future translational studies.

Discussion

Lower extremity IRI varies from mild injury without symptoms to tissue loss or multiorgan injury.[27] Systemic expansion of IRI may cause systemic inflammatory response syndrome (SIRS) and increase mortality.[27,28] Thus, there could be not only ischemic tissue injury, but also pulmonary, cardiac, and renal system problems.[27] Ischemic tissue loss remains a significant problem; amputation rates remain >10% in ischemic tissues.[27] Skeletal muscle IRI is directly associated with the duration and severity of ischemia.[29]

Adenosine triphosphate (ATP) levels are diminished in hypoxic muscles, and extracellular calcium moves into the muscle cells.[27] The impaired function of the sodium-potassium ATPase enzyme disrupts the calcium-sodium exchanger.[27] Enhanced calcium levels interact with actin, myosin, and cellular proteases; as a result, skeletal muscle fiber necrosis is triggered.[27] Reperfusion of the ischemic lower limb with oxygenated blood leads to cytokine release, leukocyte activation, enhanced production of adhesion molecules, increased prothrombotic eicosanoid expression, activation of the complement cascade, and the release of toxic oxygen radicals.[27] In our study, the reduction in muscle cell disorganization, degeneration, neutrophil infiltration, and interstitial edema, as demonstrated by histopathological scoring, strongly supports the protective effects of bosentan. This was further confirmed by the significantly lower injury scores in the bosentan-treated groups compared to the

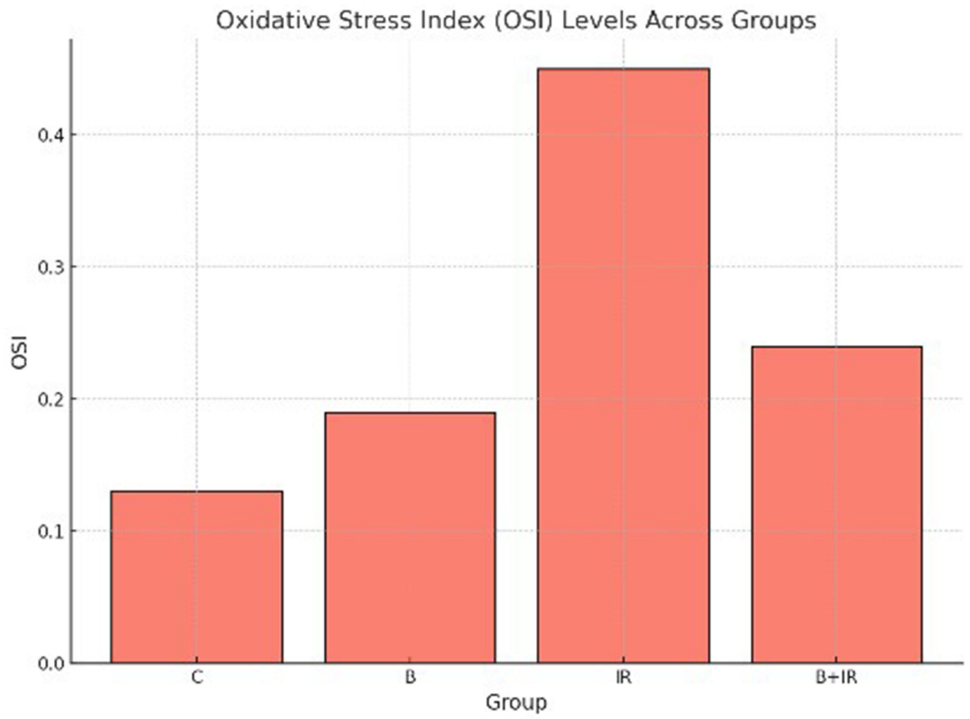

Figure 4 Oxidative Stress Index (OSI) Levels Across Experimental Groups.
Notes: This graph illustrates the OSI levels across four experimental groups: Control (**C**), Bosentan (**B**), Ischemia-Reperfusion (IR), and Bosentan + Ischemia-Reperfusion (B+IR). OSI levels were significantly elevated in the IR group compared to the Control and Bosentan groups (**P < 0.001**). Conversely, OSI levels in the B+IR group were significantly reduced compared to the IR group (**P < 0.001**), demonstrating the partial oxidative stress mitigation effect of bosentan treatment. Error bars represent the standard error of the mean (SEM). Statistical significance is indicated as **P < 0.05** compared to the Control group.

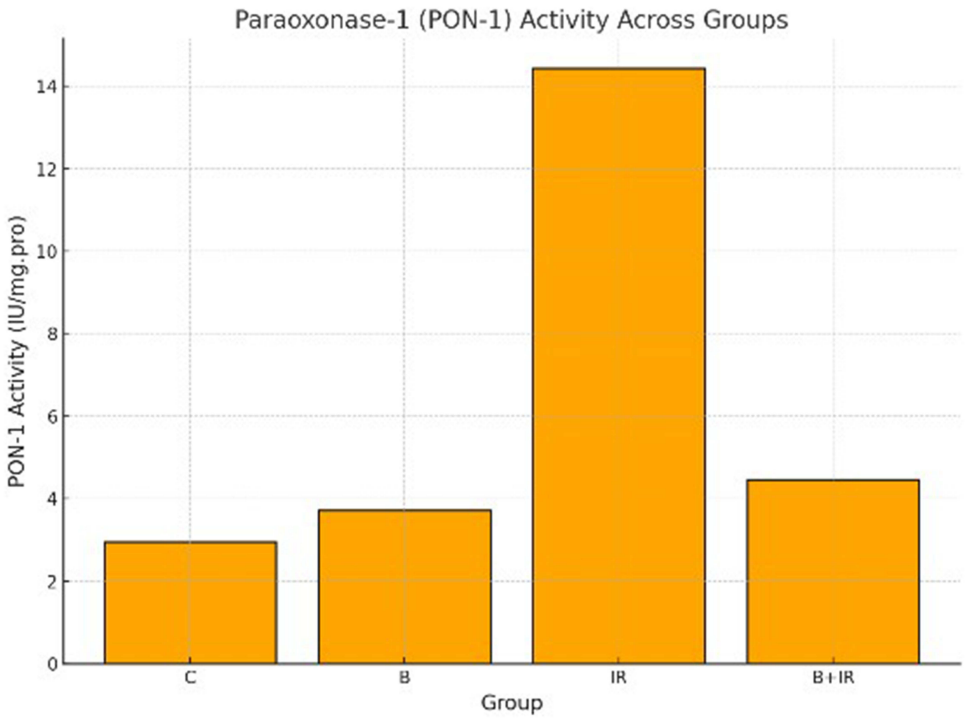

Figure 5 Paraoxonase-1 (PON-1) Enzyme Activity Across Experimental Groups.
Notes: This graph illustrates the PON-1 enzyme activity (IU/mg.pro) across four experimental groups: Control (**C**), Bosentan (**B**), Ischemia-Reperfusion (IR), and Bosentan + Ischemia-Reperfusion (B+IR). PON-1 activity was significantly elevated in the IR group compared to the Control and Bosentan groups (**P < 0.001**). Conversely, PON-1 activity in the B+IR group was significantly lower than in the IR group (**P < 0.001**), suggesting that bosentan treatment partially mitigates the heightened PON-1 activity observed in ischemia-reperfusion injury. Error bars represent the standard error of the mean (SEM). Statistical significance is denoted as **P < 0.05** compared to the Control group.

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IR group ($P < 0.001$). These findings are consistent with previous studies, further validating bosentan's role in mitigating skeletal muscle IRI. Nitric oxide (NO) also plays a critical role in IRI development.[27] Leukocytes and endothelial cells play important roles in the release of cytokines.[27] Nuclear factor κB (NF-κB) is a transcription factor involved in this process.[27] Thus, interleukin (IL) 1B and tumor necrosis factor (TNF)-α levels increase, triggering pro-inflammatory local and systemic effects.[27] TNF-α triggers endothelial cells to induce an inflammatory response that activates capillary leakage.[27] Monocyte chemotactic protein-1, IL-6, and IL-8 levels increase, contributing to endothelial leakage, neutrophil chemotaxis, neutrophil adhesion, and monocyte activation, resulting in the exacerbation of inflammation and injury.[27] TNF-α and IL-6 levels increase after lower limb ischemia and reperfusion, which is known as SIRS and multiorgan disorder syndrome (MODS).[28] Thus, a systemic inflammatory response may occur after lower limb ischemia IRI. The significantly higher TAS levels in the B+IR group compared to the IR group ($P = 0.016$) highlight the antioxidative properties of bosentan. These results underscore the role of reduced oxidative stress in the protective mechanism of bosentan against IRI. Moreover, our study demonstrates that these antioxidative effects are closely related to the reduction of inflammatory mediators such as TNF-α and IL-6, which play critical roles in IRI pathogenesis.

Vascular cell injury is enhanced by the increased production of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, hydroxyl radicals, and peroxynitrites.[27] Neutrophil degranulation and the cidal activity of ROS play critical roles in skeletal muscle cell death.[27] The complement cascade is also triggered; C3a and C5a are anaphylatoxins that contribute to inflammation through leukocytes, and C5b-9 is a membrane attack complex (MAC) that destroys cell membranes.[27]

ET-1 is another important factor in lower limb IRI.[30] Decreased oxygen levels and increased shear stress trigger ET-1 production during ischemia.[30] Experimental animal studies have shown that exogenous ET-1 administration improves tissue ischemia and increases ET-1 levels relate to ischemic condition.[30] In addition to its vasoconstrictor effects, ET-1 contributes to direct cellular injury by enhancing intracellular calcium and activating phosphoinositol production.[30] ET-1 also exerts inflammatory effects by stimulating monocytes, triggering cytokine release, activating the expression of adhesion molecules, and activating neutrophils.[30] Both ET-a and ET-b receptors are increased in ischemic muscles.[30] The ET-A receptors are involved in vasoconstriction and vascular leakage.[30] The ET-b receptors are associated with nitric oxide (NO) production, which has vasodilatory and anti-inflammatory effects.[30] ET-1 levels remain elevated during reperfusion, suggesting a potential role in exacerbating IRI. ET-1 has also been suggested to play a role in the no-reflow phenomenon.[30]

We examined the effects of bosentan on skeletal muscle ischemia and reperfusion injury. Our study not only confirmed previous findings but also provided new insights by employing a more refined and sensitive technique. Unlike studies employing tourniquet models, our direct vascular occlusion approach ensures precision and reproducibility. Furthermore, bosentan's effects were validated through comprehensive histological and biochemical assessments, emphasizing its anti-oxidative and anti-inflammatory properties. These findings align with those of Hvaal et al[31] and Herbert et al,[19] who demonstrated similar benefits of bosentan in IRI models, though our direct vascular occlusion method provides a more accurate representation of physiological conditions. Further investigations into the translational potential of bosentan, particularly in larger animal models and human applications, are warranted to better understand its clinical applicability.

Our study confirmed previous studies in the literature, but there were some differences. For instance, Hvaal et al[31] demonstrated that bosentan has a positive effect on IRI in the skeletal muscle of rats. Their design was quite different from ours in terms of the ischemia-reperfusion technique. In contrast to our laparoscopic direct vascular technique, they preferred hind limb ischemia using a tourniquet. Furthermore, the number of rats in their study was lower than that in the present study. Herbert et al[19] examined the effects of bosentan on IRI in rat skeletal muscle. Their method was also a hind-limb tourniquet model like Hvaal et al[31] and contrary to the current study. Herbert et al[19] investigated the gastrocnemius muscle of rats, which is similar to our results. They also suggested that bosentan might inhibit neutrophil infiltration and edema formation in skeletal muscle IRI.[19] Even though there are some technical differences, our study showed similar results. We propose that our technique offers superior sensitivity compared to indirect methods of vascular occlusion, such as tourniquet models. Moreover, bosentan is effective in the treatment of skeletal muscle IRI. Wang et al[32] shared their results on IRI of rat hearts and declared that bosentan reduced IRI in the rat heart. During the same year, Li et al[33] achieved similar benefits of bosentan against myocardial IRI. Wang et al[34] made their results more reliable with their study by showing bosentan's benefits against the pig myocardial IRI model. The beneficial effects of bosentan are not limited to cardiovascular IRI. Gong et al[35,36] studied IRI in the spinal cord and reported that bosentan

had protective effects against IRI and reduced neuronal apoptosis. Kazimoglu et al[37] also reported the beneficial effects of bosentan on renal IRI in an experimental rat model.

The novelty of our study lies in the use of bosentan, a known endothelin receptor antagonist, in the context of skeletal muscle IRI—a relatively unexplored area. The primary objective of investigating the protective effects of bosentan on skeletal muscle IRI has been achieved. This study contributes significantly to the literature by expanding the therapeutic potential of bosentan beyond its established applications in pulmonary hypertension and myocardial IRI. Moreover, the translational potential of bosentan warrants further exploration in clinically relevant large-animal models to bridge the gap between experimental findings and human applications.

In conclusion, bosentan had many beneficial effects on IRI in different experimental models. We found that bosentan had a positive effect on skeletal muscle IRI in an experimental rat model. Our results correlate with those of other experimental studies and clarify that bosentan reduces IRI in rat skeletal muscle. Future research should focus on dose-response relationships, the long-term functional recovery of skeletal muscles, tissue regeneration, and elucidating the precise molecular mechanisms through which bosentan exerts its protective effects. By addressing these areas, we aim to further elucidate the therapeutic potential of bosentan in skeletal muscle IRI and contribute to the development of novel therapeutic strategies in this field. Moreover, our study design was based on a cardiovascular-sensitive model. This study contributes to the literature in this regard. Bosentan reduced skeletal muscle IRI in rats.

Conclusion

This study demonstrates that bosentan, a dual endothelin receptor antagonist, exerts significant protective effects against skeletal muscle ischemia-reperfusion injury (IRI) in a rat model. These effects were substantiated through both histopathological and biochemical evaluations, highlighting reductions in muscle disorganization, interstitial edema, and oxidative stress markers. Specifically, TAS levels in the B+IR group increased by 42% compared to the IR group, while TOS levels were reduced by 31%. Histopathological scores also revealed a marked decrease in muscle injury in bosentan-treated groups. These findings underscore bosentan's multi-dimensional protective effects, which are mediated through the antagonism of ET-1 receptors, resulting in reduced oxidative stress, inflammation, and tissue damage.

This study also highlights the clinical potential of bosentan in managing IRI, particularly in conditions such as critical limb ischemia and vascular surgery. The direct vascular occlusion model employed in this study provides a more clinically relevant simulation of surgical scenarios compared to traditional tourniquet models, enhancing the translational applicability of the findings. However, several translational considerations must be addressed. While the 10 mg/kg dose used in this study aligns with previous experimental models, future research should focus on dose-response analyses to determine safe and effective dosing for human applications. Additionally, the known hepatotoxicity risk associated with bosentan necessitates careful evaluation in clinical trials, with routine liver function monitoring recommended to mitigate potential adverse effects.

The protective effects observed in this study were primarily acute. Future investigations should aim to elucidate the long-term effects of bosentan, including its role in tissue regeneration, functional recovery, and fibrosis prevention. Furthermore, mechanistic studies exploring the precise pathways through which bosentan modulates ET-1 signaling, including its interactions with inflammatory mediators such as TNF-α, IL-6, and NF-κB, will be essential to fully understand its therapeutic potential. In addition to skeletal muscle IRI, these findings may have broader implications for managing IRI in other tissues, such as myocardial and renal injuries, further underscoring the need for translational research.

This study's contribution to the literature is further emphasized by its innovative use of a direct vascular occlusion model, which better simulates clinical surgical scenarios compared to commonly used tourniquet models. Moreover, the integration of histopathological findings with biochemical analyses provides a comprehensive evaluation of bosentan's effects. While consistent with prior studies, our use of this novel model contributes significantly to understanding bosentan's mechanisms of action and its translational potential.

Future research should also explore comparative analyses of bosentan with other endothelin receptor antagonists to better delineate its unique therapeutic profile. Expanding the scope of research to include chronic models of IRI and clinical trials will further clarify the long-term applicability and safety of bosentan in human populations.

In conclusion, this study provides robust evidence supporting bosentan as a promising therapeutic agent for skeletal muscle IRI. By addressing translational challenges and exploring long-term outcomes, future studies can further elucidate

bosentan's role in vascular surgery and other ischemic conditions, paving the way for the development of novel therapeutic approaches in this field.

Institutional Review Board Statement

Ethical approval for this study was obtained from the Animal Research Committee of the Gazi University (Ankara, Turkey; approval no. G.Ü.ET-24.001).

Data Sharing Statement

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

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

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