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Protective role of bromelain's antioxidant and anti-inflammatory effects in experimental lower limb ischemia-reperfusion injury

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Ischemia-reperfusion (IR) injury is a multifaceted pathological process characterized by excessive oxidative stress and inflammatory responses upon restoration of blood flow. Bromelain, a proteolytic enzyme complex derived from pineapple, exhibits robust antioxidant and anti-inflammatory activities. This study aimed to evaluate the protective effects and underlying mechanisms of bromelain on oxidative stress and inflammation in an experimental rat model of lower limb ischemia-reperfusion injury. Twenty-four male Wistar Albino rats were randomly allocated into four groups: Sham-operated control (SHAM), Bromelain-only (BR), Ischemia-Reperfusion (IR), and Ischemia-Reperfusion with Bromelain treatment (IR + BR). Bromelain (40 mg/kg) was administered intraperitoneally before ischemia induction. The IR model involved 45 min of infrarenal abdominal aorta occlusion followed by 120 min of reperfusion. Oxidative biomarkers (total antioxidant status [TAS], total oxidant status [TOS], oxidative stress index [OSI]) and histopathological parameters (muscle atrophy, degeneration, leukocyte infiltration, internalization of nuclei, fragmentation, and hyalinization) were analyzed. Significant increases in muscle degeneration, leukocyte infiltration, nuclear internalization, fragmentation, and elevated oxidative stress biomarkers (increased TOS and OSI, decreased TAS) were observed in the IR group compared to controls. Bromelain treatment (IR + BR) significantly ameliorated these effects, reducing muscle tissue damage, inflammation, and oxidative imbalance compared to the untreated IR group. Bromelain effectively mitigates lower limb ischemia-reperfusion injury by reducing oxidative stress, restoring antioxidant capacity, and suppressing inflammatory responses. These protective effects suggest that bromelain holds potential as a therapeutic agent for managing oxidative and inflammatory damages associated with IR conditions, warranting further clinical investigation.

Keywords Bromelain, Ischemia-reperfusion, Oxidative stress, Rat model

Ischemia-reperfusion (IR) injury represents a complex, multifactorial pathophysiological process triggered by temporary cessation (ischemia) and subsequent restoration (reperfusion) of blood flow[1,2]. Although timely reperfusion is critical to prevent permanent tissue necrosis, the sudden reintroduction of oxygenated blood

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paradoxically exacerbates the initial ischemic damage through several interconnected biochemical and cellular disturbances[3–7].

During the ischemic phase, oxygen and nutrient deprivation induces cells to transition from aerobic metabolism to anaerobic glycolysis, leading to rapid depletion of adenosine triphosphate (ATP), intracellular acidosis, and lactic acid accumulation[4,5]. This ATP shortage severely impairs ATP-dependent ion pumps, notably Na^+/K^+-ATPase and Ca^{2+}-ATPase, resulting in cellular sodium accumulation, calcium overload, and membrane depolarization. Elevated intracellular calcium activates calcium-dependent proteases, such as calpains and phospholipases, exacerbating structural damage and initiating apoptotic cascades[3,5].

Upon reperfusion, the sudden influx of oxygen leads to an overwhelming production of reactive oxygen species (ROS), primarily through enzymatic systems including xanthine oxidase, NADPH oxidase, and the mitochondrial electron transport chain[3,5]. This excessive oxidative stress surpasses endogenous antioxidant defenses, causing lipid peroxidation, protein oxidation, mitochondrial membrane disruption, and DNA fragmentation. Particularly critical is the opening of mitochondrial permeability transition pores (mPTP), which results in the release of apoptogenic factors such as cytochrome c, driving further cell death via apoptosis and necrosis pathways[3,8,9]. These disturbances manifest as elevated biochemical markers, including malondialdehyde (MDA)—a lipid peroxidation byproduct—as well as changes in total oxidant status (TOS), total antioxidant status (TAS), and oxidative stress index (OSI), clearly reflecting oxidative imbalance[4–9].

Inflammation significantly contributes to the pathology of IR injury. Reperfusion activates resident macrophages and endothelial cells, leading to upregulation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1[3–9]. These cells also secrete potent pro-inflammatory cytokines, notably tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), which recruit and activate neutrophils[8,9]. Activated neutrophils exacerbate tissue damage through additional ROS production and the release of degradative enzymes such as elastase and myeloperoxidase, further promoting endothelial dysfunction and increased microvascular permeability. This inflammatory response significantly contributes to the "no-reflow" phenomenon, characterized by impaired capillary perfusion, interstitial edema, and exacerbated tissue necrosis[8,9].

Collectively, these interconnected mechanisms of oxidative stress, mitochondrial dysfunction, and inflammation establish a vicious cycle that significantly enhances tissue injury and may precipitate systemic organ dysfunction[3]. Given these severe consequences, effective therapeutic strategies targeting these critical pathways are essential. Recently, natural products exhibiting antioxidant and anti-inflammatory properties have garnered significant attention[10–15].

Bromelain is a complex mixture of proteolytic enzymes primarily extracted from pineapple stems and fruit (Ananas comosus), renowned for its potent antioxidant and anti-inflammatory properties. Bromelain exhibits antioxidant activities by scavenging reactive oxygen species (ROS), thus mitigating oxidative stress, which significantly contributes to tissue injury in various pathological conditions, including ischemia-reperfusion injury, cancer, and chronic inflammatory diseases[16]. Its anti-inflammatory mechanisms involve modulation of key inflammatory pathways, including suppression of nuclear factor-kappa B (NF-κB) activation, inhibition of cyclooxygenase-2 (COX-2) enzyme expression, and reduction of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6[17–22]. Bromelain also reduces inflammatory edema by enhancing fibrinolytic activity, degrading fibrin, and down-regulating plasma kininogen, thereby attenuating vasodilation and vascular permeability[23–25]. These multifaceted biological activities position bromelain as a promising therapeutic agent for conditions associated with excessive inflammation and oxidative stress, including arthritis, inflammatory bowel disease, certain cancers, sinusitis, rhinosinusitis, respiratory disorders, cardiovascular diseases, and skin wounds, as demonstrated by preclinical studies using experimental models such as rodents and cell cultures, which have reported protective effects on various organs, notably the liver, lungs, and gastrointestinal tract[17–39]. However, despite extensive preclinical evidence supporting these beneficial effects, detailed mechanistic insights into its specific roles in lower limb ischemia-reperfusion injury remain limited.

The aim of this study is to investigate the protective effects of bromelain on oxidative stress and inflammation in an experimental model of lower limb ischemia-reperfusion injury. Specifically, this research aims to clarify the potential mechanisms underlying bromelain's therapeutic actions by assessing its ability to reduce oxidative biomarkers (TOS, TAS, OSI), modulate inflammatory cytokine levels, and evaluate histopathological changes, including muscle degeneration, atrophy, central nuclear internalization, and leukocyte infiltration[17]. Ultimately, this study seeks to provide valuable insights into the potential clinical applications of bromelain in ischemia-reperfusion injury management. To our knowledge, this is the first experimental study to demonstrate bromelain's combined antioxidant and anti-inflammatory protective effects in a rat model of lower-limb ischemia-reperfusion injury, providing a novel mechanistic link between systemic oxidative balance and local tissue protection.

Materials and methods

Animals

This study was conducted using twenty-four male Wistar Albino rats aged 10–12 weeks, with body weights between 250 and 350 g. All rats were housed in polycarbonate cages (three per cage) under controlled environmental conditions (temperature 22 ± 2 °C, relative humidity 50 ± 10%, 12-h light/dark cycle) with free access to standard pellet diet and tap water. Animals were age-matched (10–12 weeks) adult male Wistar rats, and the reported weight range reflects normal variation within this age group. The animals were sourced from and maintained at the Gazi University Life Sciences Application and Research Center, Ankara, Türkiye, a certified and dedicated facility for experimental animal research. Ethical approval for all experimental procedures was granted by the Gazi University Animal Experiments Local Ethics Committee (Approval number: G.Ü.ET-25.025, date: 03.03.2025), ensuring adherence to international guidelines for the humane care and use of laboratory animals. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1986). The study was conducted and reported in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments, https://arriveguidelines.org) guidelines.

Drug

Bromelain, a proteolytic enzyme complex derived from pineapple (Ananas comosus), was used as the therapeutic agent in this study. Commercially available powdered bromelain (Sigma-Aldrich, USA) was aseptically dissolved in phosphate-buffered saline (PBS), serving as the solvent vehicle to optimize solubility and bioavailability. The bromelain solution was freshly prepared under sterile laboratory conditions immediately prior to administration. The final administered solution was carefully adjusted to a concentration corresponding to a dosage of 40 mg/kg body weight, consistent with previously validated dosage guidelines from earlier experimental models[27,28]. The selected dose was chosen based on previously published experimental studies demonstrating its antioxidant and anti-inflammatory efficacy and safety in rats[27,28]. The present study aimed to evaluate the protective effects of a literature-validated therapeutic dose rather than to establish a dose-response curve. Intraperitoneal (i.p.) injection was selected as the route of administration, in line with established experimental protocols for assessing the antioxidant and anti-inflammatory effects of bromelain.

Experimental protocol

A total of 24 male Wistar Albino rats were randomly assigned into four groups (n = 6 per group): Sham-operated control (SHAM), Bromelain only (BR), Ischemia-Reperfusion (IR), and Ischemia-Reperfusion with Bromelain treatment (IR + BR). The ischemia-reperfusion procedure (45 min ischemia + 120 min reperfusion) and anesthesia protocol were adapted from previously validated models used in our earlier experimental studies and comparable literature[10,12,14]. The sample size determination was based on previous similar experimental studies investigating ischemia-reperfusion injury, ensuring sufficient statistical power to detect intergroup differences in both biochemical and histological evaluations. General anesthesia was induced intramuscularly using ketamine (50 mg/kg) combined with xylazine (5 mg/kg), and anesthetic depth was periodically monitored by evaluating the loss of the ear pinch reflex. Supplemental doses of ketamine (20 mg/kg) and xylazine (5 mg/kg) were provided at 90-minute intervals as necessary. After a 30-minute stabilization period, surgical interventions were performed in a supine position with animals placed on a heating pad, maintaining body temperature at 37 ± 0.5 °C to prevent hypothermia. In the SHAM and BR groups, a midline laparotomy was performed without induction of ischemia, followed by intraperitoneal administration of either isotonic saline (0.3 mL) or bromelain (40 mg/kg), respectively. For the IR and IR + BR groups, ischemia was established by occluding the infrarenal abdominal aorta with an atraumatic bulldog clamp for 45 min, followed by reperfusion for 120 min after clamp removal. In the IR + BR group, bromelain (40 mg/kg) was administered intraperitoneally immediately prior to ischemia induction. At the conclusion of the reperfusion period, rats were euthanized under deep anesthesia via an overdose of ketamine (100 mg/kg) and xylazine (10 mg/kg), and euthanasia was confirmed by exsanguination from the inferior vena cava. Bilateral gastrocnemius muscle tissues were immediately harvested for subsequent histopathological (left side) and biochemical (right side) analyses.

Histopathological evaluation

Following euthanasia, the left gastrocnemius muscle was carefully dissected from each rat and immediately fixed in 10% neutral buffered formalin to preserve tissue morphology for subsequent histopathological analysis. After standard histological processing, the tissue samples were embedded in paraffin blocks. Serial cross-sectional slices measuring approximately 4 µm in thickness were prepared from each block and stained with hematoxylin and eosin (H&E) to allow detailed microscopic examination. Histological images were captured under ×400 magnification, and scale bars (50 µm) were inserted for all figures. Tissue injury was assessed semi-quantitatively using a 0–3 grading system (0 = none, 1 = mild, 2 = moderate, 3 = severe) for parameters including muscle-fiber degeneration, edema, vascular congestion, and inflammatory infiltration in ten randomly selected fields per sample. Mean scores were calculated for statistical comparison. The scoring criteria were adapted from previously validated ischemia-reperfusion injury models described by Eren et al. and Ergene et al.[21,22]. All slides were examined by a blinded histopathologist under standardized conditions (×400 magnification, identical illumination and calibration), and ten random microscopic fields per sample were evaluated to ensure objectivity and reproducibility. The histological assessment was performed under a light microscope at magnifications of 100× and 400× by an experienced pathologist who was blinded to the experimental groups to eliminate observer-related biases. Histopathological evaluation specifically targeted crucial morphological indicators of skeletal muscle injury associated with ischemia-reperfusion. The assessed parameters included muscular fiber atrophy or hypertrophy, myocyte degeneration, vascular congestion, internalized nuclei, centrally located oval nuclei, fragmentation and hyalinization of myofibers, and inflammatory cell infiltration. Each of these parameters was systematically graded using a semi-quantitative scoring scale to provide a comprehensive evaluation of muscle tissue integrity and to precisely quantify the extent of ischemia-reperfusion injury among the experimental groups. The histopathological assessment and scoring approach were performed in accordance with validated methods previously described in similar ischemia-reperfusion studies[10,14].

Oxidative stress parameters

Immediately following collection, the right gastrocnemius muscle samples were rapidly snap-frozen in liquid nitrogen and stored at − 80 °C to maintain the biochemical integrity and stability of oxidative stress biomarkers. Quantitative analyses included the measurement of TAS and TOS. Additionally, the OSI was calculated as the ratio of TOS to TAS, serving as a reliable indicator of the systemic oxidative balance and extent of oxidative injury within the tissue. Quantitative analyses were performed following standardized, validated protocols described in

	SHAM Group (S) (*n* = 6)	Bromelain Group (BR) (*n* = 6)	Ischemia reperfusion group (IR) (*n* = 6)	IR + Bromelain Group (IR + BR) (*n* = 6)	*p*-value
Muscle atrophy and hypertrophy	0.33 ± 0.21	0.33 ± 0.21	1.33 ± 0.21*, **	0.50 ± 0.22***	0.010
Muscle degeneration and congestion	0.33 ± 0.21	0.17 ± 0.17	1.50 ± 0.22*, **	0.67 ± 0.33***	0.004
Internalization of muscle nuclei (oval-central nuclei)	0.33 ± 0.21	0.33 ± 0.21	1.33 ± 0.33*, **	0.67 ± 0.21	0.030
Fragmentation and hyalinization	0.33 ± 0.21	0.33 ± 0.21	1.50 ± 0.22*, **	0.67 ± 0.33***	0.011
Leukocyte cell infiltration	0.33 ± 0.21	0.33 ± 0.21	1.50 ± 0.22*, **	0.50 ± 0.22***	0.003

Table 1. Histopathological analysis scores of the gastrocnemius muscle tissue. Data are presented as Mean ± Standard Error of the Mean (SEM); ANOVA test was used for statistical analysis; significance level was set at $p < 0.05$; *$p < 0.05$: significant difference compared to the SHAM Group (S); **$p < 0.05$: significant difference compared to the Bromelain Group (BR); ***$p < 0.05$: significant difference compared to the Ischemia reperfusion group (IR).

	SHAM Group (S) (*n* = 6)	Bromelain Group (BR) (*n* = 6)	Ischemia reperfusion group (IR) (*n* = 6)	IR + Bromelain Group (IR + BR) (*n* = 6)	*p*-value
Total antioxidant status (TAS) (mmol/L)	0.97 ± 0.15	0.99 ± 0.22	0.68 ± 0.08*, **	0.90 ± 0.09***	0.006
Total oxidant status (TOS) (μmol/L)	4.18 ± 0.90	4.46 ± 1.69	9.94 ± 1.32*, **	5.48 ± 1.02***	< 0.001
Oxidative stress index (OSI)	0.44 ± 0.11	0.45 ± 0.16	1.49 ± 0.21*, **	0.61 ± 0.10***	< 0.001

Table 2. Oxidative status parameters of the gastrocnemius muscle tissue. Data are presented as Mean ± Standard Error of the Mean (SEM); ANOVA test was used for statistical analysis; significance level was set at $p < 0.05$; *$p < 0.05$: significant difference compared to the SHAM Group (S); **$p < 0.05$: significant difference compared to the Bromelain Group (BR); ***$p < 0.05$: significant difference compared to the Ischemia reperfusion group (IR).

prior ischemia-reperfusion models[10,14]. All biochemical procedures and calculation methods adhered strictly to validated, standardized protocols previously established and applied in earlier experimental studies conducted by our research team[10,14]. All biochemical analyses were performed under blinded conditions. Sample tubes were coded by an independent researcher, and the investigator conducting the TAS, TOS, and OSI assays was blinded to the experimental groups until all measurements were completed. All reagents were purchased from validated commercial suppliers (Sigma-Aldrich, USA; Rel Assay Diagnostics, Türkiye). RRID numbers were not available for these specific products.

Statistical analysis

The primary outcomes consisted of biochemical markers (TAS, TOS, and OSI) and histopathological scoring obtained through the semiquantitative evaluation of muscle tissue injury. Both biochemical results and histopathological scores, numerically assessed by a pathologist blinded to experimental conditions, were considered continuous variables for statistical evaluation. Statistical analyses were conducted using IBM SPSS Statistics software version 26.0 (IBM Corp., Armonk, NY, USA). Normality of data distribution was verified through the Shapiro–Wilk test. Comparisons across experimental groups for biochemical parameters and histopathological data were performed using one-way analysis of variance (ANOVA). When statistically significant differences were identified, Bonferroni-adjusted post hoc tests were utilized to pinpoint specific intergroup variations. Statistical significance was set at a threshold of $p < 0.05$. Results were presented as mean ± standard error of the mean (SEM), reflecting the overall distribution characteristics of the dataset. The normality of data distribution was assessed using the Kolmogorov–Smirnov test. Although histopathological scores were approximately normally distributed and analyzed by one-way ANOVA, a non-parametric Kruskal–Wallis test was also performed to verify robustness. The Kruskal–Wallis results were consistent with the ANOVA findings, demonstrating identical patterns of statistical significance across groups. Data distribution and variance homogeneity were tested using the Kolmogorov–Smirnov and Levene tests. One-way ANOVA followed by Bonferroni post-hoc correction was applied to minimize Type I error in multiple comparisons. A non-parametric Kruskal–Wallis analysis was also performed to verify robustness, yielding consistent results. A post-hoc power analysis indicated adequate statistical power (> 0.80) for the main biochemical and histopathological variables.

Results

Histopathological evaluation

Histopathological analysis using light microscopy demonstrated statistically significant differences among groups regarding muscle atrophy and hypertrophy ($p = 0.010$). Notably, the ischemia-reperfusion (IR) group exhibited significantly increased muscle atrophy and hypertrophy compared to both the sham (S) and bromelain (BR) groups ($p = 0.004$, each). Furthermore, muscle atrophy and hypertrophy were significantly reduced in the IR + BR group relative to the IR group ($p = 0.012$) (Table 1 ,Figs. 1, 2, 3, 4, 5, 6, 7 and 8).

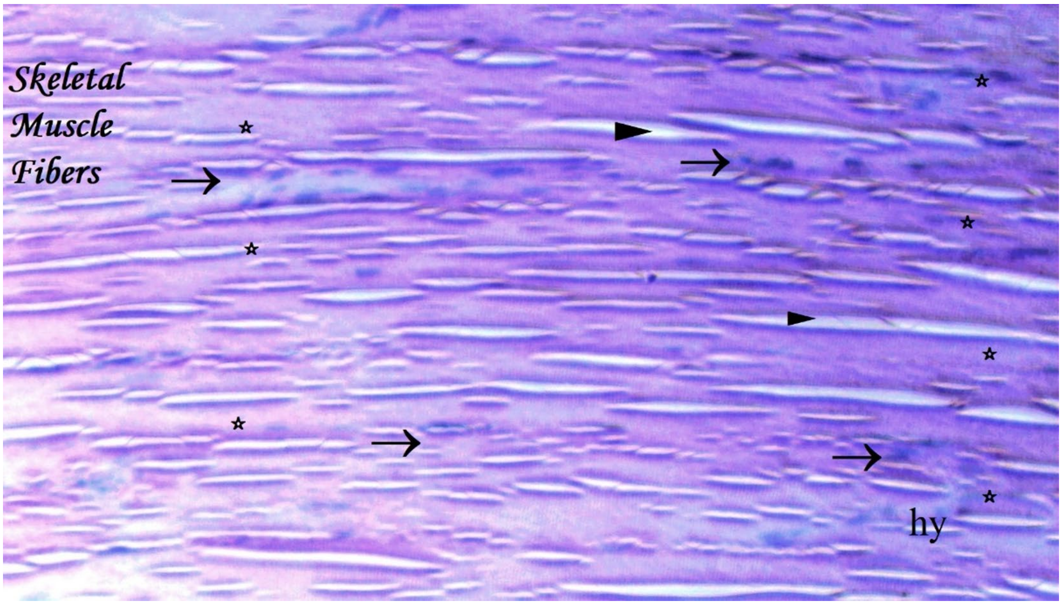

Fig. 1. Representative histological evaluation of gastrocnemius muscle tissue from the SHAM group. Examination demonstrated characteristic peripheral positioning of myocyte nuclei (indicated by →), distinct intercellular spaces (►), and clearly identifiable muscle fibers (*). The tissue sections were processed with hematoxylin and eosin (H&E) staining and visualized at 100× magnification.

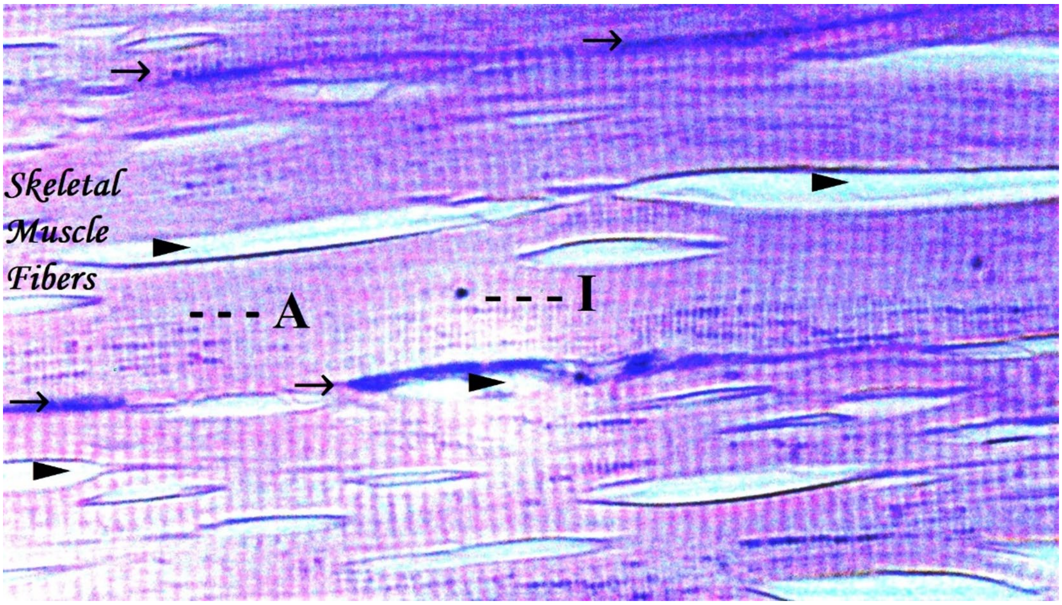

Fig. 2. Histological examination of gastrocnemius muscle tissue in the SHAM group demonstrated peripheral localization of muscle cell nuclei (indicated by →), clearly visible intercellular spaces (►), and intact muscle fibers (*), (---A) A band and (---I), I band are distinctly observable, indicating normal muscle fiber architecture. Tissue sections were stained using hematoxylin and eosin (H&E) and visualized at 400× magnification.

Similarly, muscle degeneration and congestion differed significantly among the groups ($p = 0.004$). The IR group showed markedly higher levels of muscle degeneration and congestion compared to both the S and BR groups ($p = 0.003$ and $p < 0.001$, respectively). Importantly, muscle degeneration and congestion were significantly decreased in the IR + BR group compared to the IR group ($p = 0.024$) (Table-1; Figs. 1, 2, 3, 4, 5, 6, 7 and 8).

Internalization of muscle nuclei, characterized by oval-central nuclei, revealed significant intergroup variations ($p = 0.030$). The IR group demonstrated significantly higher internalization of muscle nuclei compared to both the S and BR groups ($p = 0.010$, each). Although a reduction in the internalization of muscle nuclei was

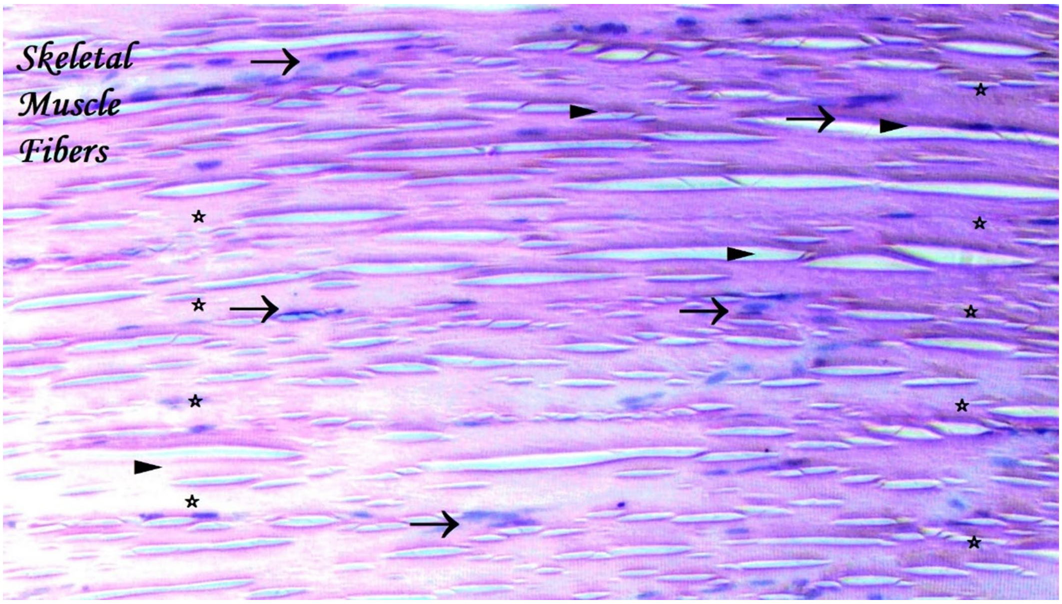

Fig. 3. Histological examination of gastrocnemius muscle tissue from the Bromelain (BR) group revealed predominantly preserved muscle morphology. Peripheral localization of muscle cell nuclei (→), intact muscle fibers (*), and mild intercellular spacing (►) were observed. Tissue samples were stained with hematoxylin and eosin (H&E) and analyzed at 100× magnification.

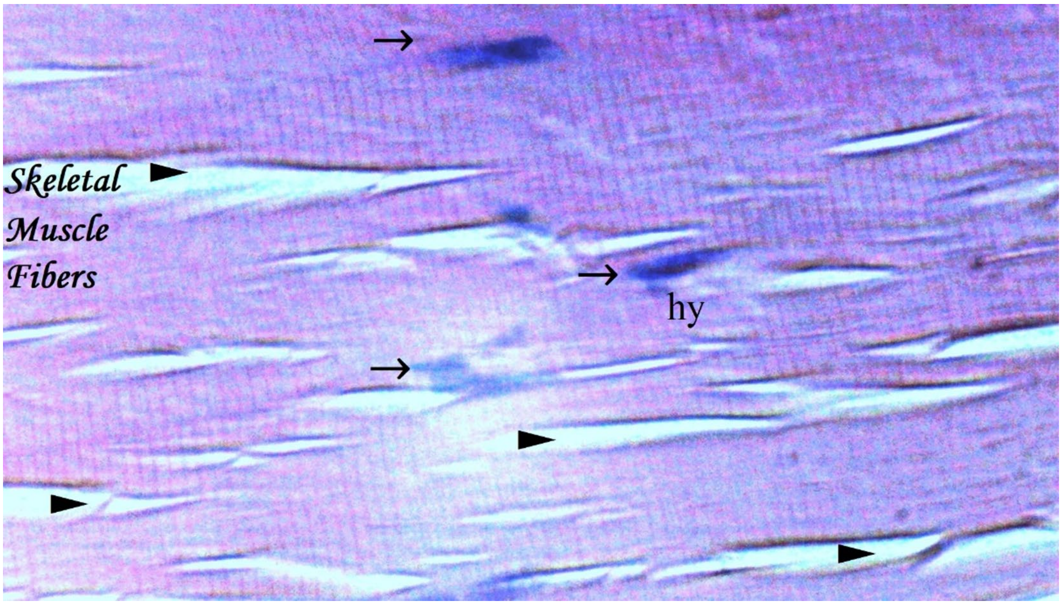

Fig. 4. Microscopic evaluation of the gastrocnemius muscle in the Bromelain (BR) group demonstrated largely preserved muscle tissue architecture. Muscle cell nuclei exhibited peripheral localization (→), muscle fibers (*) appeared intact, and intercellular spacing (►) was minimally present. Notably, minimal sign of muscle fiber hypertrophy (hy) was also observed. Tissue sections were stained using hematoxylin and eosin (H&E) and analyzed at 400× magnification.

observed in the IR + BR group compared to the IR group, this difference did not reach statistical significance ($p = 0.071$) (Table-1; Figs. 1, 2, 3, 4, 5, 6, 7 and 8).

Histopathological evaluation also identified significant differences in fragmentation and hyalinization among groups ($p = 0.011$). The IR group presented significantly elevated fragmentation and hyalinization compared to both the S and BR groups ($p = 0.004$, each). Conversely, the IR + BR group exhibited significantly reduced fragmentation and hyalinization relative to the IR group ($p = 0.029$) (Table-1; Figs. 1, 2, 3, 4, 5, 6, 7 and 8).

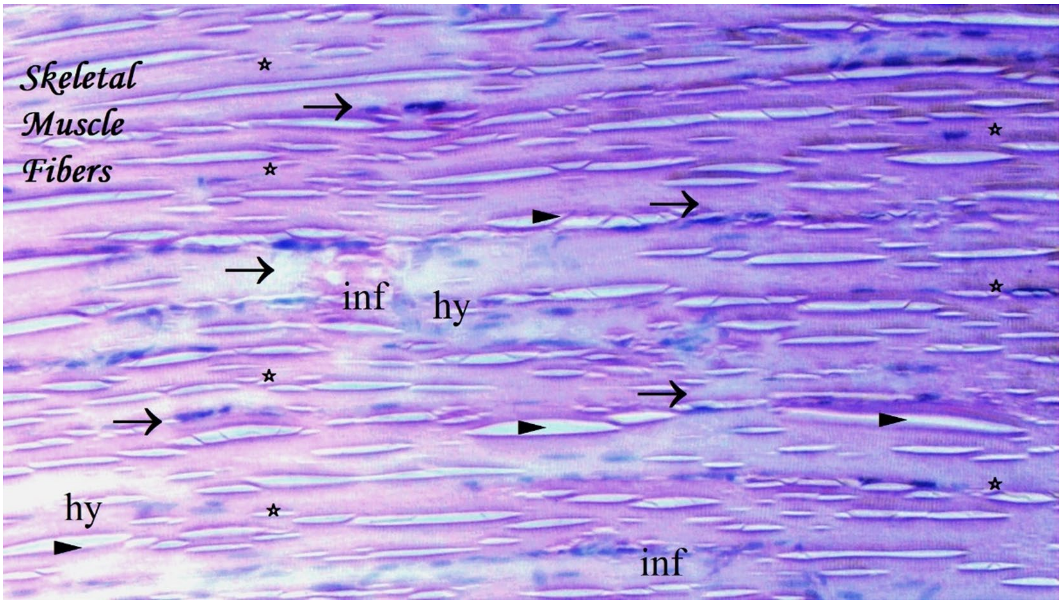

Fig. 5. Histological examination of gastrocnemius muscle tissue from the ischemia-reperfusion (IR) group revealed significant pathological alterations. Peripheral nuclei (→) and muscle fibers (*) were visible; however, notably widened intercellular spaces (►) indicated tissue disruption. Observed pathological changes included the presence of nuclear displacement, muscle fiber fragmentation (fr), degenerative alterations, inflammatory cell infiltration (inf), and muscle fiber hypertrophy (hy). Tissue sections were stained with hematoxylin and eosin (H&E) and analyzed under 100× magnification.

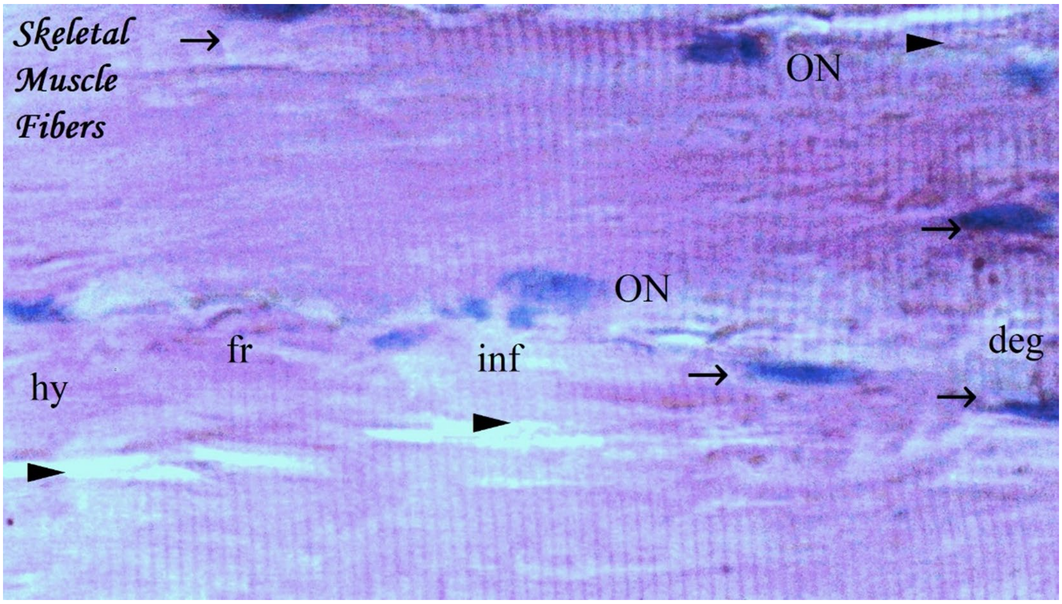

Fig. 6. Histological examination of gastrocnemius muscle tissue from the ischemia-reperfusion (IR) group revealed significant pathological alterations. Peripheral nuclei (→) were visible; however, notably widened intercellular spaces (►) indicated tissue disruption. Observed pathological changes included oval nuclei (ON), suggestive of nuclear displacement, muscle fiber fragmentation (fr), muscle fiber degeneration (deg), inflammatory cell infiltration (inf), and muscle fiber hypertrophy (hy). Tissue sections were stained with hematoxylin and eosin (H&E) and analyzed at 400× magnification.

Moreover, leukocyte infiltration differed significantly among the groups ($p = 0.003$). The IR group exhibited significantly increased leukocyte infiltration compared to the S and BR groups ($p = 0.001$, each). Notably, leukocyte infiltration was significantly decreased in the IR + BR group compared to the IR group ($p = 0.004$) (Table-1; Figs. 1, 2, 3, 4, 5, 6, 7 and 8).

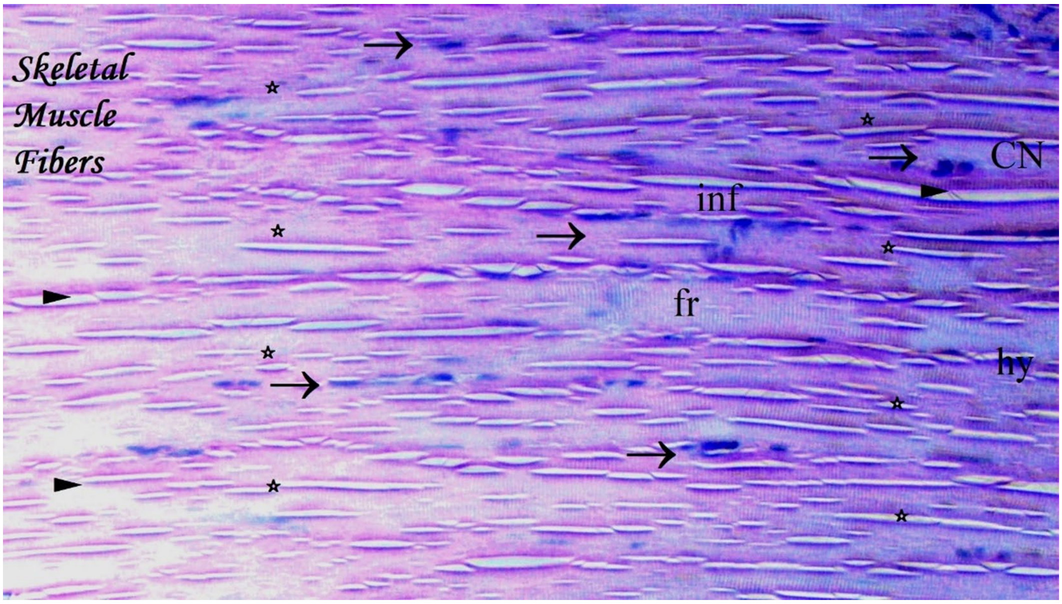

Fig. 7. Histopathological examination of gastrocnemius muscle tissue from the Ischemia-Reperfusion + Bromelain (IR + BR) group revealed a partially preserved muscle architecture. Peripheral localization of nuclei (→) and intact muscle fibers (*) were observed, along with a limited presence of central nuclei (CN) and oval nuclei (ON). Mild muscle fiber fragmentation (fr) were also noted; however, these changes appeared less severe compared to the IR group. Tissue sections were stained using hematoxylin and eosin (H&E) and analyzed at 100× magnification.

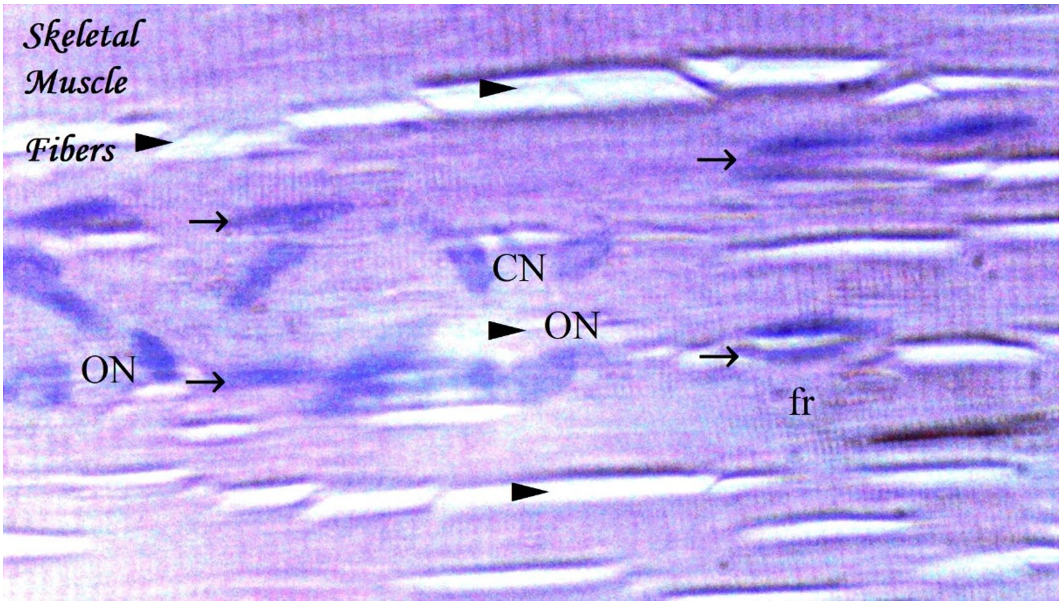

Fig. 8. Histopathological assessment of gastrocnemius muscle tissue from the Ischemia-Reperfusion + Bromelain (IR + BR) group demonstrated partially preserved muscle morphology. Peripheral nuclei (→) and mild intercellular spaces (►) were observed, accompanied by occasional central nuclei (CN) and oval nuclei (ON). Additionally, mild muscle fiber fragmentation (fr) was present, although these alterations appeared markedly reduced compared to the IR group. Tissue sections were stained with hematoxylin and eosin (H&E) and examined under 400× magnification.

Oxidative stress biomarkers

Analysis of oxidative stress parameters in muscle tissue revealed significant differences among experimental groups. TAS levels varied significantly across groups ($p = 0.006$). Specifically, TAS levels in the ischemia-reperfusion (IR) group were markedly lower compared to the sham (S) and bromelain (BR) groups ($p = 0.002$, each). Conversely, treatment with bromelain (IR + BR group) significantly increased TAS levels compared to the IR group ($p = 0.015$) (Table-2).

TOS also exhibited significant variability among groups ($p < 0.001$). Notably, the IR group presented significantly elevated TOS levels compared to both the S and BR groups ($p < 0.001$, each). Importantly, bromelain treatment significantly reduced TOS levels in the IR + BR group compared to the IR group ($p < 0.001$), indicating effective mitigation of oxidative stress by bromelain (Table-2).

Furthermore, the OSI, reflecting the balance between oxidative and antioxidative processes, displayed significant intergroup differences ($p < 0.001$). OSI values were significantly higher in the IR group than in both the S and BR groups ($p < 0.001$, each). However, OSI levels in the IR + BR group were significantly lower compared to the IR group ($p < 0.001$), demonstrating the beneficial impact of bromelain in reducing oxidative stress (Table-2).

Discussion

Oxidative stress plays a pivotal role in IR injury through mechanisms involving the generation of ROS, mitochondrial dysfunction, and disruption of cellular homeostasis. Initially, ischemia causes ATP depletion, anaerobic metabolism, and intracellular acidosis, resulting in impaired ion pumps and calcium overload[1–3]. Upon reperfusion, the sudden influx of oxygen triggers enzymatic systems such as xanthine oxidase, NADPH oxidase, and mitochondrial electron transport chains, significantly enhancing ROS production[3]. Excessive ROS surpass the endogenous antioxidant defenses, leading to increased TOS, OSI, and malondialdehyde (MDA), along with decreased TAS[8,9]. ROS also activate several signaling cascades including nuclear factor-kappa B (NF-κB), Nrf-1, Nrf-2, cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE-2), and mitogen-activated protein kinase (MAPK), further exacerbating inflammation and cellular injury[3,8,9]. Although the present study did not directly measure the activity or expression of these signaling molecules, these pathways were discussed based on previously published mechanistic studies demonstrating bromelain's regulatory effects on NF-κB, MAPK, MLCK, Nrf2, and Akt/FOXO signaling in various experimental models. Therefore, our discussion should be interpreted as a mechanistic inference supported by prior literature rather than a direct molecular confirmation[18,21,29,31,32]. Inflammation is another crucial contributor to IR injury. Reperfusion-induced inflammation is characterized by elevated levels of cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10). These cytokines facilitate leukocyte adhesion to endothelial cells and subsequent infiltration into affected tissues, aggravating histopathological changes such as muscle fiber degeneration, congestion, leukocyte infiltration, hyalinization, and internalization of muscle nuclei[8,9]. Although cytokines such as IL-1β, IL-6, and TNF-α are repeatedly discussed in the context of ischemia-reperfusion injury and bromelain's anti-inflammatory effects, these mediators were not directly quantified in the present study. Their inclusion serves solely to frame our biochemical and histopathological findings within the inflammatory mechanisms previously established in the literature. Nevertheless, the significant improvements observed in oxidative stress parameters (decreased TOS and OSI, increased TAS) and the marked histopathological recovery (reduced leukocyte infiltration, muscle degeneration, and edema) provide indirect but consistent evidence of bromelain's anti-inflammatory efficacy, which aligns with previous cytokine-based studies in the literatüre[18,31,32]. Collectively, these intertwined oxidative and inflammatory pathways significantly amplify the overall tissue damage during ischemia-reperfusion injury, underscoring the necessity of targeted therapeutic interventions aimed at mitigating these detrimental processes. In this context, the present study demonstrated that bromelain significantly ameliorated oxidative stress and histopathological damage induced by lower limb IR injury. Specifically, bromelain treatment resulted in reductions of TOS and OSI, restoration of TAS, and notable improvements in muscle architecture, as evidenced by decreased muscle atrophy, degeneration, leukocyte infiltration, and structural disruption of myocytes. These beneficial effects align closely with previous comprehensive mechanistic and translational studies, reinforcing bromelain's therapeutic potential as an effective intervention in oxidative and inflammatory conditions associated with ischemia-reperfusion injury.

The protective effects of bromelain observed in our study align with various reports from different organ systems and animal models. In an ocular toxicity model induced by cisplatin, bromelain effectively mitigated oxidative stress by significantly enhancing antioxidant capacity—reflected by increases in total antioxidant capacity (TAC) and SOD—and reducing oxidative markers such as TOS, ROS, and MDA[28]. Similar antioxidant mechanisms were observed in our ischemia-reperfusion (IR) injury model, where bromelain restored oxidative balance through reductions in TOS and OSI and by increasing TAS. Furthermore, bromelain exhibited notable anti-inflammatory effects by suppressing NF-κB-mediated pro-inflammatory cytokines (IL-1β, TNF-α) and promoting anti-inflammatory cytokine (IL-10) production, mechanisms likely contributing to reduced leukocyte infiltration and decreased tissue damage in our IR model[28]. In addition to oxidative stress management, bromelain's anti-inflammatory properties have also been reported in inflammatory bowel disease (IBD) research. Bromelain treatment significantly reduced secretion of pro-inflammatory cytokines and chemokines such as granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), and TNF-α from inflamed colon biopsies in IBD patients[26]. The authors suggested that bromelain's proteolytic activity alters cell surface molecules, thereby inhibiting leukocyte activation and adhesion. Such findings closely parallel our results, where bromelain similarly attenuated inflammatory cell infiltration and muscle tissue damage related to IR injury. Bromelain-mediated reduction of cytokines, particularly TNF-α and IFN-γ, is critical for limiting leukocyte recruitment and subsequent inflammatory responses, reinforcing its therapeutic potential in inflammatory conditions[26].

Bromelain has demonstrated significant anti-inflammatory effects through multiple mechanisms. It effectively suppresses pro-inflammatory mediators, such as nitric oxide (NO), IL-6, TNF-α, IL-1β, and IL-8, as reported in macrophages stimulated by lipopolysaccharides (LPS) and human dental pulp cells exposed to inflammatory stimuli. These effects were attributed to the downregulation of critical inflammatory signaling pathways, including nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), along with inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression[32,33]. Additionally, bromelain significantly reduced inflammation markers such as TNF-α, IL-6, and prostaglandin E2 (PGE2) in osteoarthritis models, highlighting its broad anti-inflammatory capabilities[34]. Furthermore, bromelain exhibited analgesic and anxiolytic effects in neuropathic pain models, characterized by reduced production of inflammatory mediators (IL-1β, IL-6, TNF-α, and PGE2), decreased nitric oxide production, and suppressed NF-κB activation[38]. Similarly, comprehensive discussions by Kansakar et al. underscored bromelain's diverse therapeutic applications, emphasizing its antioxidant, anti-inflammatory, analgesic, and antiangiogenic activities, largely mediated by NF-κB inhibition, cytokine modulation, and regulation of nitric oxide pathways[17]. Recent studies further support bromelain's anti-inflammatory efficacy in various disease contexts. Lu et al. reported that bromelain inhibited inflammation and cellular senescence in human gingival fibroblasts exposed to advanced glycation end-products (AGEs), primarily through suppression of pro-inflammatory cytokines (IL-6, IL-8) and the NF-κB and MAPK/ERK pathways[31]. Moreover, Zhou et al. demonstrated significant reduction in inflammation and improved epithelial barrier function in a TNBS-induced colitis model, mediated by downregulation of TNF-α receptors (TNFR1, TNFR2), NF-κB, and MLCK signaling pathways, which are critical in inflammatory responses and barrier integrity[18]. Collectively, these findings strongly align with our study, in which bromelain administration substantially reduced oxidative stress biomarkers (TOS, OSI), restored antioxidant balance (TAS), and attenuated inflammatory cell infiltration and muscle tissue injury following ischemia-reperfusion.

The antioxidant potential of bromelain has also been demonstrated in multiple experimental models. In a study evaluating bromelain's radiosensitizing and radioprotective effects, bromelain significantly reduced lipid peroxidation (MDA), ROS, restored paraoxonase-1 (PON1) activity, and normalized liver function markers, involving the downregulation of NF-κB and PARP1 expression, and upregulation of PPAR-α[20]. Similar protective effects were observed against high-fat diet-induced non-alcoholic fatty liver disease (NAFLD), where bromelain enhanced lipid clearance and reduced hepatic inflammation through modulation of metabolic regulators, including PPARα, CPT1α, ACOX1, PGC-1α, LXRα, and ABCA1[37]. Overall, the therapeutic effects observed in our experimental model can be attributed to bromelain's robust suppression of inflammatory and oxidative pathways, notably involving NF-κB, MAPK, MLCK, TNF-α signaling, and modulation of cytokine-mediated inflammation, thereby confirming its potential role in managing ischemia-reperfusion injury.

López-Pedrouso et al. highlighted the potent antioxidant activity of bromelain-derived hydrolysates obtained from porcine liver, noting significant reductions in lipid peroxidation markers, including DPPH, ABTS, FRAP, and ORAC[39]. Their peptidomic analysis identified specific bioactive peptides such as GLNQALVDLHALGSAR and ALFQDVQKPSQDEWGK, strongly correlated with antioxidant activity, suggesting a peptide-mediated antioxidative mechanism consistent with our findings of reduced TOS, OSI, and improved tissue integrity. Similarly, bromelain demonstrated robust protective effects in aluminum-induced testicular injury, evidenced by reductions in oxidative stress markers (TBARS, H_2O_2) and enhancements in the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferase (GST)[36]. These results parallel our findings of improved oxidative balance and tissue preservation in ischemia-reperfusion (IR) injury. Further supporting bromelain's antioxidative potential, Gwozdzinski et al. demonstrated its beneficial effects in human endothelial cells from umbilical and varicose veins[24]. Their study showed bromelain's capability to significantly reduce reactive oxygen species (ROS), superoxide anion radicals, and modulate nitric oxide (NO) generation, aligning with our observations of diminished oxidative stress and improved vascular integrity during IR injury. Bakare and Owoyele expanded the therapeutic profile of bromelain by highlighting its antinociceptive and neuroprotective effects in a neuropathic pain model induced by sciatic nerve ligation[35]. They observed significant reductions in pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), oxidative stress markers (MDA, NO), and enhanced antioxidant enzyme activities—findings that strongly correlate with our results regarding bromelain's protective effects in skeletal muscle IR injury. In a study conducted by Pekas et al., bromelain combined with anthocyanins notably improved endothelial function, muscle oxygenation, and total antioxidant capacity by reducing oxidative stress and inflammation[25]. These mechanisms offer additional insights supporting our findings, which similarly demonstrated enhanced vascular function and reduced tissue damage in our experimental IR model. Paksoy et al. evaluated bromelain's systemic effects in experimental periodontitis, observing reductions in pro-inflammatory cytokines (TNF-α, IL-6) and bone resorption biomarkers (RANKL, M-CSF, MMP-8), while antioxidant enzymes (GPx, SOD) and anti-inflammatory mediators such as osteoprotegerin (OPG) were enhanced[30]. Similarly, El-Demerdash et al. demonstrated the nephroprotective potential of bromelain in aluminum chloride-induced oxidative kidney injury, identifying significant reductions in lipid peroxidation (TBARS, H_2O_2) and increased antioxidant enzyme activities (SOD, CAT, GPx, GR, GST)[22]. Bromelain treatment also normalized glutathione (GSH) levels and improved renal function biomarkers (urea, creatinine), lipid profiles, and renal histopathology, partly through its metal-chelating and antioxidant properties.

In the context of our findings, the proposed signaling involvement (NF-κB, MAPK, MLCK, Nrf2, and Akt/FOXO) represents plausible mechanistic interpretations consistent with previous reports, rather than pathways directly quantified in the current experiment[18,21,29,31,32]. To our knowledge, this is the first experimental study to demonstrate bromelain's combined antioxidant and anti-inflammatory protective effects in a rat model of lower-limb ischemia-reperfusion injury, providing a novel mechanistic link between systemic oxidative balance and local tissue protection. Although the antioxidant and anti-inflammatory effects of bromelain have been described in other organ systems, the present study is the first to demonstrate these dual protective effects in a rat model of lower-limb ischemia-reperfusion injury. These findings, while confirmatory in nature, provide novel experimental evidence supporting bromelain's translational potential in peripheral tissue reperfusion injury. The protective mechanisms observed in our study align closely with previous research, suggesting multiple molecular pathways through which bromelain exerts its beneficial effects. El-Demerdash et al. reported that bromelain significantly ameliorated oxidative stress markers (TBARS, H_2O_2), normalized antioxidant enzyme activities—including SOD, CAT, GPx, GR, and GST—and downregulated inflammatory mediators (TNF-α, IL-1β, MMP9) in aluminum-induced hepatic injury[21]. Moreover, bromelain administration was shown to upregulate Nrf2 expression, enhancing antioxidant responses and promoting tissue regeneration[21]. This mechanism of Nrf2 pathway activation could similarly underlie bromelain's antioxidant and anti-inflammatory effects in our ischemia-reperfusion model, given the parallel improvements observed in oxidative stress and inflammatory markers. Additionally, Juhasz et al. provided further insights by demonstrating bromelain's cardioprotective effects against myocardial ischemia-reperfusion injury in rats[29]. In their study, bromelain improved myocardial function, reduced infarct size, and attenuated apoptosis through activation of the Akt/FOXO signaling pathway, specifically via enhanced phosphorylation of Akt and FOXO3A[29]. This pathway activation aligns closely with our observations regarding bromelain's capacity to reduce oxidative stress, inflammation, and apoptosis in skeletal muscle ischemia-reperfusion injury. Thus, the mechanisms highlighted in these studies—including activation of the Nrf2 antioxidant response pathway, modulation of Akt/FOXO signaling, reduction of oxidative mediators, and suppression of inflammatory cytokines—provide strong support for our findings and suggest plausible molecular pathways underlying bromelain's beneficial effects in ischemia-reperfusion injury.

The present study has several limitations that should be considered when interpreting the findings. Firstly, this research was conducted using an animal model, specifically a rat lower limb ischemia-reperfusion model, which may not entirely replicate the complex pathophysiological processes observed in clinical human conditions. Thus, direct translation of these findings to clinical practice requires caution. Secondly, the sample size was relatively limited (six animals per group), which may influence the generalizability and robustness of the observed results. Further studies using larger sample sizes are necessary to validate our conclusions. Additionally, our investigation focused predominantly on short-term effects of bromelain administration on oxidative stress, inflammation, and histopathological changes. Longer-term outcomes and the durability of bromelain's protective effects were not assessed, highlighting the need for future studies to evaluate chronic effects and possible long-term benefits or adverse reactions. Furthermore, this study did not explore dose-response relationships or the efficacy of different routes and timings of bromelain administration, aspects crucial for optimizing therapeutic application. Finally, although multiple key pathways such as NF-κB, MAPK, MLCK, Nrf2, and Akt/FOXO were suggested as mechanisms underlying bromelain's protective effects, detailed molecular analyses (protein expression levels, phosphorylation status, gene expression profiles) were not conducted. Therefore, future research incorporating comprehensive molecular and signaling pathway assessments is recommended to clarify bromelain's precise mechanisms of action in ischemia-reperfusion injury.

The study included a limited sample size ($n=6$ per group), consistent with previous validated ischemia–reperfusion models, which may restrict the statistical power. The experiment was designed to evaluate acute-phase ischemia-reperfusion injury; therefore, long-term outcomes such as tissue regeneration or functional recovery were not assessed. Future studies with larger cohorts and extended observation periods are planned to confirm and expand these findings.Although inflammation represents a central pathophysiological component of ischemia-reperfusion injury, the present study did not include direct measurement of inflammatory cytokines (IL-1β, IL-6, TNF-α) or other immunological mediators. Future studies in our laboratory are planned to incorporate ELISA-based cytokine profiling and molecular assays to further delineate the anti-inflammatory mechanisms of bromelain in ischemia-reperfusion injury. Although the present study demonstrates clear biochemical and histopathological protective effects of bromelain, its translational applicability is limited by the use of a single experimental dose and the absence of pharmacokinetic or cytokine-level validation. Future studies will aim to explore dose-dependent responses, alternative administration routes, and molecular assays to enhance the clinical relevance and translational value of these findings. Moreover, although the discussion refers to several potential molecular mechanisms (NF-κB, MAPK, MLCK, Nrf2, Akt/FOXO), these were not experimentally validated in the present study through protein or gene expression analyses such as Western blotting, ELISA, or RT-PCR. These pathways were mentioned solely to contextualize our biochemical and histopathological findings within the framework of previously established bromelain mechanisms. Future investigations in our laboratory are planned to include molecular-level verification of these pathways to substantiate the mechanistic basis of bromelain's protective effects. This study primarily focused on histological and biochemical outcomes to establish the baseline protective efficacy of bromelain in ischemia-reperfusion injury. Functional and molecular endpoints, including cytokine profiling and tissue enzyme activity assays, were beyond the present scope but are planned for future investigations to complement and extend these findings.

Although the present study demonstrates histological and biochemical protection, functional assessments such as limb perfusion, contractile strength, and locomotor activity were not performed. Future experiments are planned to include these parameters—using laser-Doppler imaging, muscle-force testing, and behavioral gait analysis—to better correlate biochemical and histopathological improvements with functional recovery, thereby enhancing the translational relevance of the findings.

Although TAS, TOS, and OSI provide a validated overview of systemic oxidative status, they are nonspecific indicators that do not directly reflect particular oxidative or antioxidant pathways. Future studies are planned to include more specific biochemical markers such as MDA, GSH, SOD, and CAT to further delineate the redox-modulating effects of bromelain.

The present study did not include molecular analyses such as Western blot, qPCR, or immunohistochemistry, which would allow direct assessment of signaling pathways related to oxidative stress and inflammation. While TAS, TOS, and OSI provide validated systemic indices of oxidative balance, they do not specifically identify molecular mediators. Future research will incorporate protein and gene-expression analyses (e.g., NF-κB, Nrf2, MAPK, and cytokine panels) to confirm the biochemical and mechanistic pathways underlying bromelain's protective effects.

Conclusion

In conclusion, our study demonstrated that bromelain effectively mitigates skeletal muscle ischemia-reperfusion (IR) injury by significantly reducing oxidative stress markers (TOS, OSI), enhancing antioxidant defenses (TAS), and attenuating inflammation. Histopathologically, bromelain treatment improved muscle architecture, evidenced by reduced muscle fiber atrophy, degeneration, leukocyte infiltration, and structural disruptions. These protective effects are likely mediated through multiple mechanisms, including the inhibition of critical inflammatory pathways (NF-κB, MAPK, MLCK), suppression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), modulation of nitric oxide production, and activation of key antioxidant signaling pathways (Nrf2, Akt/FOXO). Given these multifaceted beneficial outcomes, bromelain emerges as a promising therapeutic agent for mitigating oxidative and inflammatory damage associated with ischemia-reperfusion injury, warranting further clinical investigation to confirm its potential application in clinical settings.

Data availability

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

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Author contributions

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

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

Ethical approval for the study was obtained from the Animal Research Committee of Gazi University (Ankara, Turkey; Approval number: G.Ü.ET-25.025, date: 03.03.2025). The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1986). The study was conducted and reported in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments, https://arriveguidelines.org) guidelines.

Additional information

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