Effect of rivaroxaban on DNA damage in an ischemia-reperfusion model: Evaluation of 8-OHdG levels

CEREN SAYARER[1], ABIDIN CENK ERDAL[1], TUĞRA GENÇPINAR[1], ÖZLEM GÜRSOY DORUK[2] and TUNCAY KÜME[2]

[1]Department of Cardiovascular Surgery, Dokuz Eylül University, Izmir 35340, Turkey; [2]Department of Medical Biochemistry, Dokuz Eylül University, Izmir 35340, Turkey

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Abstract. Ischemia-reperfusion (I/R) injury is a notable cause of tissue damage, particularly in patients with peripheral artery disease. Rivaroxaban is a novel oral anticoagulant that can reduce cardiovascular events. However, its potential antioxidant properties remain poorly understood. Therefore, the present study aimed to investigate the effect of rivaroxaban on oxidative DNA injury in a rat model of I/R injury by measuring 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels, a key biomarker of oxidative DNA injury. A total of 21 female Wistar albino rats were randomly divided into three groups, namely the sham, control (I/R) and rivaroxaban treatment (3 mg/kg/day) groups. Following treatment for 10 days, hind limb ischemia was induced in rats for 1 h, followed by reperfusion for 2 h. Subsequently, blood and skeletal muscle samples were collected and analyzed for oxidative stress markers, including 8-OHdG, glutathione (GSH), oxidized GSH and malondialdehyde (MDA), using ELISA and high-performance liquid chromatography. The results demonstrated that compared with those in the sham group, rats in the control group exhibited significantly elevated 8-OHdG, GSSG and MDA levels, coupled with decreased GSH levels. By contrast, treatment with rivaroxaban notably reversed the elevated 8-OHdG and MDA levels whilst restoring GSH levels compared with those in the control group, indicating an improved oxidative status. Overall, these findings suggested that in addition to its established anticoagulant properties, rivaroxaban can also protect against I/R-induced oxidative DNA injury.

Correspondence to: Dr Ceren Sayarer, Department of Cardiovascular Surgery, Dokuz Eylül University, 1606 Mithatpaşa Cad, Inciraltı, Balçova, Izmir 35340, Turkey
E-mail: ceren.sayarer@deu.edu.tr

Abbreviations: I/R, ischemia-reperfusion; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; GSH, glutathione; GSSG, oxidized GSH

Key words: rivaroxaban, oxidative stress, 8-OHdG, ischemia-reperfusion, DNA damage, rat model, antioxidant, GSH, MDA

Introduction

Peripheral artery disease (PAD) is becoming a global health concern, affecting >200 million individuals worldwide (1) Despite the implementation of antiplatelet therapy, patients with PAD remain at high risk of atherosclerotic complications (2). In particular, acute thromboembolic occlusions commonly occur in this population. Current treatment strategies primarily focus on restoring perfusion in the ischemic limb and mitigating ischemic injury (3). Thromboembolism is considered to be the primary etiology of acute limb ischemia, where several clinical factors, including abdominal aortic surgery, trauma, revascularization surgery and orthopedic procedures, can be involved in ischemia-reperfusion (I/R) injury. I/R injury is a well-documented complication in vascular surgery and contributes markedly to postoperative morbidity and mortality (3).

In clinical practice, during revascularization, tissues perfused by compromised arterial systems typically experience varying durations of ischemia. The resulting burst of oxidative and inflammatory mediators can induce both local and systemic organ injury (4-6). Experimental evidence has previously demonstrated that I/R can activate oxidative and inflammatory molecular pathways, eventually leading to the excessive generation of reactive oxygen species and cellular damage, partly through dysregulation of the nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated antioxidant response (7). Furthermore, I/R can disrupt redox homeostasis, promote lipid peroxidation and deplete endogenous antioxidants [such as glutathione (GSH)], as evidenced in preclinical studies (3,8,9). As the predominant tissue in the limb, the skeletal muscle exhibits marked susceptibility to ischemic stress, underscoring the critical need for prophylactic strategies to limit I/R-induced injury in clinical settings such as acute limb ischemia, lower extremity revascularization procedures and major vascular surgery (3).

Among the molecular hallmarks of I/R injury, oxidative DNA damage is of particular relevance. The product of DNA oxidation, 8-hydroxy-2'-deoxyguanosine (8-OHdG), is a well-established biomarker of oxidative DNA modifications

that has been extensively studied in diseases associated with elevated oxidative stress, including cardiovascular disorders and malignancies (10). Elevated 8-OHdG levels indicate oxidative genomic instability, contributing to mutagenesis and the progression of chronic diseases, including cancer, atherosclerotic cardiovascular disease and neurodegenerative disorders (10). The key pathophysiological role of oxidative DNA damage in I/R injury highlights the necessity of therapeutic interventions targeting oxidative stress pathways.

Rivaroxaban is a direct oral anticoagulant that selectively inhibits factor Xa and exhibits pleiotropic properties beyond its antithrombotic action. Emerging evidence has suggested that rivaroxaban can exert anti-inflammatory and antioxidative effects by modulating protease-activated receptor signaling (11). In addition to its established role in thromboprophylaxis, rivaroxaban could reduce ischemic complications in patients with PAD, thereby conferring protective effects against I/R-induced tissue injury (4). Notably, rivaroxaban has been investigated for its safety and efficacy in oncologic populations, a cohort frequently predisposed to hypercoagulability and oxidative stress-related complications (12). Its ability to mitigate oxidative stress and DNA damage could therefore have broader clinical implications, particularly in conditions characterized by intertwined thrombo-inflammation mechanisms.

Therefore, the present study aimed to investigate the effects of rivaroxaban on skeletal muscle I/R injury, with emphasis on oxidative DNA damage. To comprehensively assess oxidative stress and antioxidant defense mechanisms, the levels of malondialdehyde (MDA), a marker of lipid peroxidation, in addition to reduced GSH and oxidized GSH (GSSG) as key indicators of the endogenous antioxidant defense system, were assessed (13,14). Additionally, 8-OHdG levels were quantified in both tissue and serum samples to determine the extent of oxidative DNA damage and to reveal the potential role of rivaroxaban in I/R-induced oxidative stress (10).

Materials and methods

Blinding and experimental design. The present study was a single-blind experimental study. Although the investigator involved in treatment administration was aware of the group allocations, all biochemical analyses, including ELISA and high-performance liquid chromatography (HPLC), were performed by an independent researcher blinded to the experimental groups. All samples were anonymized and coded prior to analysis to ensure objective data evaluation.

Animal ethics and study approval. The experimental protocol was approved by the Local Ethics Committee for Animal Experiments of Dokuz Eylül University (approval no. 12/2021; Izmir, Turkey). All animal procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (15). The total duration of the experiment was 17 days, including a 7-day acclimatization period, 10 days of drug administration and a 3-h I/R protocol.

Experimental groups. A total of 21 female Wistar albino rats aged 10-12 weeks (weight, 200-250 g) were obtained from the Dokuz Eylül University Multidisciplinary Animal Experimentation Laboratory. Only female rats were used because the institutional breeding colony available at the time of the experiment predominantly contained female animals of required age and weight range. The required sample size was calculated using the G*Power software (version 3.1.9.7; http://www.gpower.hhu.de) for one-way ANOVA across three independent groups. An effect size (f) of 0.45 was assumed based on a previously published experimental study evaluating 8-OHdG as the primary oxidative stress outcome, with a significance level (α) of 0.05 and a power (1-β) of 0.80 (10). The calculated total sample size was 21 rats (n=7 per group). This rat model was selected based on its availability, high reliability and reproducibility in I/R studies (3,16,17). Rats were maintained under controlled environmental conditions, with a 12-h light/dark cycle, a temperature of 22±2˚C and a relative humidity of 50±10% with *ad libitum* access to standard pellet feed. All animals were housed in standard rat cages, where all experiments were conducted under the same laboratory conditions. Humane endpoints were predefined according to ARRIVE guidelines and animals were to be euthanized if any of the following occurred: >20% weight loss, severe hypoactivity or inability to ambulate, respiratory distress, hypothermia (<30˚C), self-mutilation or uncontrollable bleeding. No animals reached these humane endpoints. A total of 21 animals entered the study, where all completed the planned procedures and all were euthanized at the scheduled experimental endpoint under deep anesthesia. No unplanned deaths occurred and no animal died due to procedural complications.

The primary outcome measured was the quantification of 8-OHdG levels in serum and skeletal muscle tissues as a biomarker of oxidative DNA damage following I/R and its modulation by rivaroxaban (Xarelto®; Bayer AG) treatment. The rats were randomly allocated into the following three groups (n=7 each): i) Group 1 (sham group), where no I/R procedure was applied, but rats received anesthesia, limb shaving and antiseptic skin preparation to ensure comparable procedural stress; ii) group 2 (control group), where rats were subjected to I/R injury without treatment; and iii) group 3 (rivaroxaban treatment group), where rats received 3 mg/kg/day rivaroxaban by oral gavage for 10 days prior to I/R induction.

The rivaroxaban dosing regimen was selected based on previous studies employing similar animal models (17,18). Rivaroxaban was diluted in distilled water to a final concentration of 1 mg/ml and administered daily at a volume of 1 ml per 250 g body weight to ensure accurate and consistent dosing across animals. Following 10 days of treatment, rats were administered ketamine (50 mg/kg; Ketalar®; Pfizer, Inc.) and xylazine (10 mg/kg Xylazine®; Bayer AG) by intraperitoneal injection to achieve anesthesia and analgesia. The left hind limb was then shaved, disinfected and a tourniquet was applied at the level of the hip joint using a standardized elastic tension. Ischemia was induced by tightening the elastic band with three turns and secured with consistent tension, where adequate occlusion was verified by the absence of Doppler flow signals in the posterior tibial artery using a portable Doppler ultrasound device (Getinge AB). This form of ischemia was maintained for 1 h. The tourniquet method was selected based on a previous experimental study indicating that simple femoral artery ligation is insufficient to induce lower extremity ischemia due to robust collateral circulation in the rat hind limb (16). After 1 h of ischemia, the tourniquet

was removed, the animals were allowed to recover from anesthesia and a reperfusion period of 2 h was allowed. At the end of the reperfusion period, whilst under deep anesthesia (ketamine 50 mg/kg and xylazine 10 mg/kg), ~3-4 ml blood was collected by intracardiac puncture. Euthanasia was subsequently completed by exsanguination. Death was confirmed by cessation of cardiac and respiratory activity. Skeletal tissue samples were obtained from the medial head of the left gastrocnemius muscle at the central portion, washed in 100 ml 0.9% NaCl and placed on ice for transport to the biochemistry laboratory. The tissue samples were stored in Eppendorf tubes at -80˚C for subsequent analysis. Blood samples were centrifuged at 450 x g for 10 min at 4˚C to separate serum, which was then stored in Eppendorf tubes at -80˚C for preservation.

Tissue homogenization and HPLC procedure. Skeletal muscle tissue samples were stored in Eppendorf tubes at -80˚C until homogenization. Prior to homogenization, samples were thawed on ice, weighed and homogenized in chilled PBS at a tissue-to-buffer ratio of 1:10 (w/v; 100 mg tissue in 1 ml PBS) to ensure consistent homogenate concentrations. Subsequently, metal beads were added and the tissues were homogenized using the TissueLyser II QIAGEN® device (Qiagen, Inc.). The homogenates were then centrifuged at 10,000 x g for 15 min at 4˚C. Following centrifugation, the supernatant was transferred into new Eppendorf tubes, whilst the remaining pellet and beads were discarded. On the same day, a portion of the supernatant was used to determine protein content with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The results for MDA, GSH/GSSG and 8-OhdG assays were normalized to total tissue protein levels. Tissue and serum levels of MDA and GSH/GSSG were quantified using an HPLC system (Shimadzu Prominence HPLC system; Shimadzu Corporation) with commercial assay kits (ImmuChrom GmbH). This method was selected for its high analytical precision and reproducibility in biochemical marker quantification (13). Chromatographic separation was carried out using reversed-phase columns maintained at 30˚C, namely Bischoff Prontosil Eurobond (5 μm; 125x4 mm; BİSCHOFF Chromatography) for MDA analysis and MZ Inertsil ODS (5 μm; 125x4 mm; MZ-Analysentechnik GmbH) for GSH/GSSG analysis. The mobile phase was delivered at a flow rate of 0.8 ml/min. Samples were injected at a volume of 20 μl, and the total run time for each analysis was 4 min. Fluorescence detection was performed with an excitation wavelength of 515 nm and an emission wavelength of 553 nm for MDA and 385/515 nm for GSH/GSSG. All HPLC analyses were performed by an external service (Delta Analiz Laboratuvar Hizmetleri).

ELISA. Quantification of 8-OHdG levels in tissue and serum samples was performed using the BT Lab Rat 8-Hydroxy-deoxyguanosine ELISA® kit (cat. no. E0031Ra; BT LAB Bioassay Technology Laboratory; Shanghai Korain Biotech Co., Ltd.) in strict accordance with the manufacturer's protocol based on the sandwich ELISA principle. Colorimetric detection was carried out at 450 nm using a microplate reader. The amount of 8-OHdG in the samples was determined by comparing the measured absorbance values to a standard curve generated from known concentrations of 8-OHdG, where the concentration of 8-OHdG is expressed in ng/ml. The measurement range provided by the kit was 0.05-20 ng/ml, with a lower detection limit of 0.027 ng/ml.

Statistical analysis. All statistical analyses were performed using IBM SPSS version 29 (IBM Corp.). The data are expressed as the mean ± standard deviation. The normality of distribution was assessed using the Shapiro-Wilk test and serum GSH levels did not initially show normal distribution ($P<0.05$). Therefore, $\log_{10}$ transformation was applied prior to analysis. Log_{10} transformation was chosen for serum GSH to reduce positive skewness, stabilize variance and allow the use of parametric models, which are more powerful in small experimental samples when their assumptions are satisfied. These assumptions include approximate normality of the data distribution and homogeneity of variances. All other variables, including tissue parameters, satisfied normality assumptions. Following transformation, all variables met the criteria for normal distribution (Shapiro-Wilk $P>0.05$), allowing for parametric analysis using one-way ANOVA. Homogeneity of variances was assessed using Levene's homogeneity test. For variables with homogeneous variances, one-way ANOVA followed by Bonferroni's post-hoc test was performed. For variables with heterogeneous variances, Welch's ANOVA was used together with Tamhane's T2 post-hoc test, which is recommended for unequal variances and small sample sizes. $P<0.05$ was considered to indicate a statistically significant difference. To assess the robustness of the findings, non-parametric analyses (Kruskal-Wallis followed by Dunn's post-hoc test) were also performed for the non-normally distributed serum biomarker (GSH), where this yielded a significance pattern consistent with the parametric analyses.

Results

Model of oxidative stress. In the present study, GSH levels were significantly reduced in blood samples in the control (I/R) group compared with those in the sham group ($P=0.046$). By contrast, GSSG, MDA and 8-OHdG levels were significantly higher in the control (I/R) group compared with those in the sham group ($P<0.001$, $P=0.006$ and $P<0.001$, respectively; Fig. 1). These findings suggest an increase in oxidative markers (GSSG, MDA and 8-OHdG) and depletion of antioxidant markers (GSH) following I/R injury. Consistently, in skeletal muscle tissues, GSH levels were found to be significantly reduced in the control group compared with those in the sham group ($P=0.009$), whereas GSSG, MDA and 8-OHdG levels were significantly elevated ($P=0.003$, $P<0.001$ and $P<0.001$, respectively; Fig. 2). These results indicated that I/R-related oxidative stress was successfully induced in the current experimental model.

Effect of rivaroxaban on circulating oxidative biomarkers. Pairwise comparisons revealed that rivaroxaban treatment significantly increased blood GSH levels compared with those in the untreated control group ($P<0.001$). By contrast, circulating GSSG, MDA and 8-OHdG levels were significantly lower in the rivaroxaban group compared with those in the control group ($P<0.001$, $P=0.010$ and $P<0.001$, respectively). These findings indicated that rivaroxaban could exhibit antioxidant

Table I. Serum levels of oxidative stress markers.

Group	GSH, μM/l ($\log_{10}$ transformed)	GSSG, μM/l	MDA, μM/l	8-OHdG, ng/ml
Control	0.34±0.11	27.3±3.5	0.55±0.08	3.00±0.10
Sham	0.79±0.22	17.5±1.5	0.27±0.14	2.36±0.77
Rivaroxaban	0.66±0.15	17.5±1.5	0.28±0.09	2.55±0.15

8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; GSH, glutathione; GSSG, oxidized glutathione.

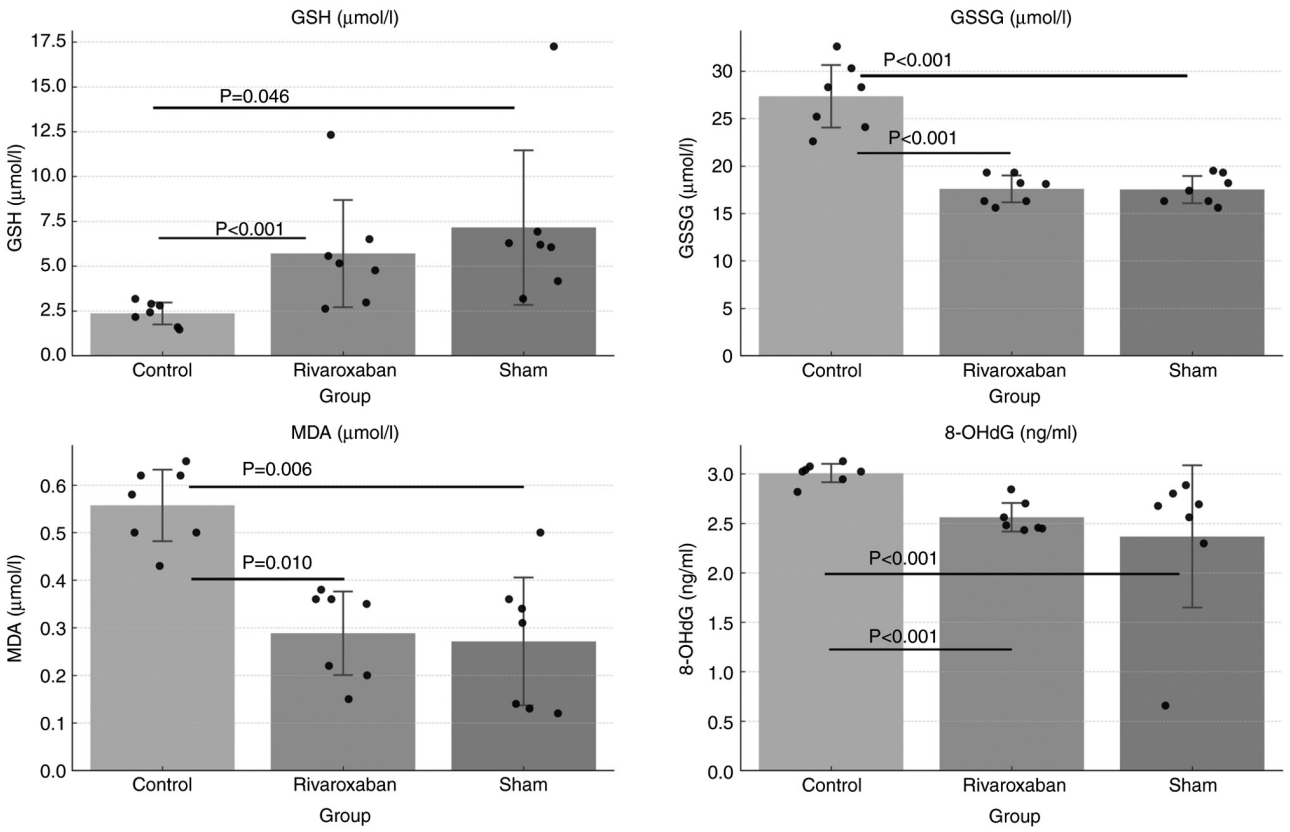

Figure 1. Serum levels of GSH, GSSG, MDA and 8-OHdG across all groups. Rivaroxaban significantly reduced oxidative stress markers compared with those in the ischemia/reperfusion control group. Data are presented as mean ± standard deviation, with individual values shown as dots. Exact P-values are indicated in the figure. P<0.05 was considered to indicate a statistically significant difference. GSH, GSSG and MDA levels were measured using high-performance liquid chromatography, while 8-OHdG levels were measured using ELISA. 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; GSH, glutathione; GSSG, oxidized glutathione.

and potential cytoprotective effects against I/R-induced oxidative stress, highlighting its ability to mitigate oxidative stress (Fig. 1; Table I).

Skeletal muscle oxidative stress markers. In skeletal muscle tissues, GSH levels were significantly higher in the rivaroxaban group compared with those in the control group (P=0.028), whereas GSSG (P=0.003), MDA (P<0.001) and 8-OHdG (P<0.001) levels were significantly lower (Fig. 2; Table II), thus verifying that rivaroxaban not only attenuated oxidative damage but it was also involved in maintaining redox homeostasis at the tissue level. The observed reduction in oxidative products and restoration of antioxidant defenses in both serum and tissue samples supported that rivaroxaban could exert a protective effect against I/R-induced oxidative stress.

Discussion

PAD remains a major cause of morbidity and mortality worldwide, where the optimal medical therapeutic strategy following revascularization continues to be a subject of debate. I/R injury is a critical concern in PAD management, since it exacerbates tissue injury and contributes to both local and systemic complications (1,19). The present study aimed to investigate the potential effects of rivaroxaban, a direct factor Xa inhibitor, on skeletal muscles subjected to I/R injury. The results demonstrated that rivaroxaban could significantly attenuate oxidative stress and DNA damage in both serum and skeletal muscles, thereby supporting its potential therapeutic role in mitigating I/R-related complications.

Table II. Skeletal muscle tissue levels of oxidative stress markers.

Group	GSH, nM/100 mg protein	GSSG, nM/100 mg protein	MDA, nM/100 mg protein	8-OHdG, pg/mg protein
Control	370.2±79.0	1,823.0±518.0	0.79±0.11	325±35
Sham	967.5±338.0	694.0±67.0	0.37±0.11	194±39
Rivaroxaban	539.4±117.0	695.0±98.0	0.39±0.10	198±29

8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; GSH, glutathione; GSSG, oxidized glutathione.

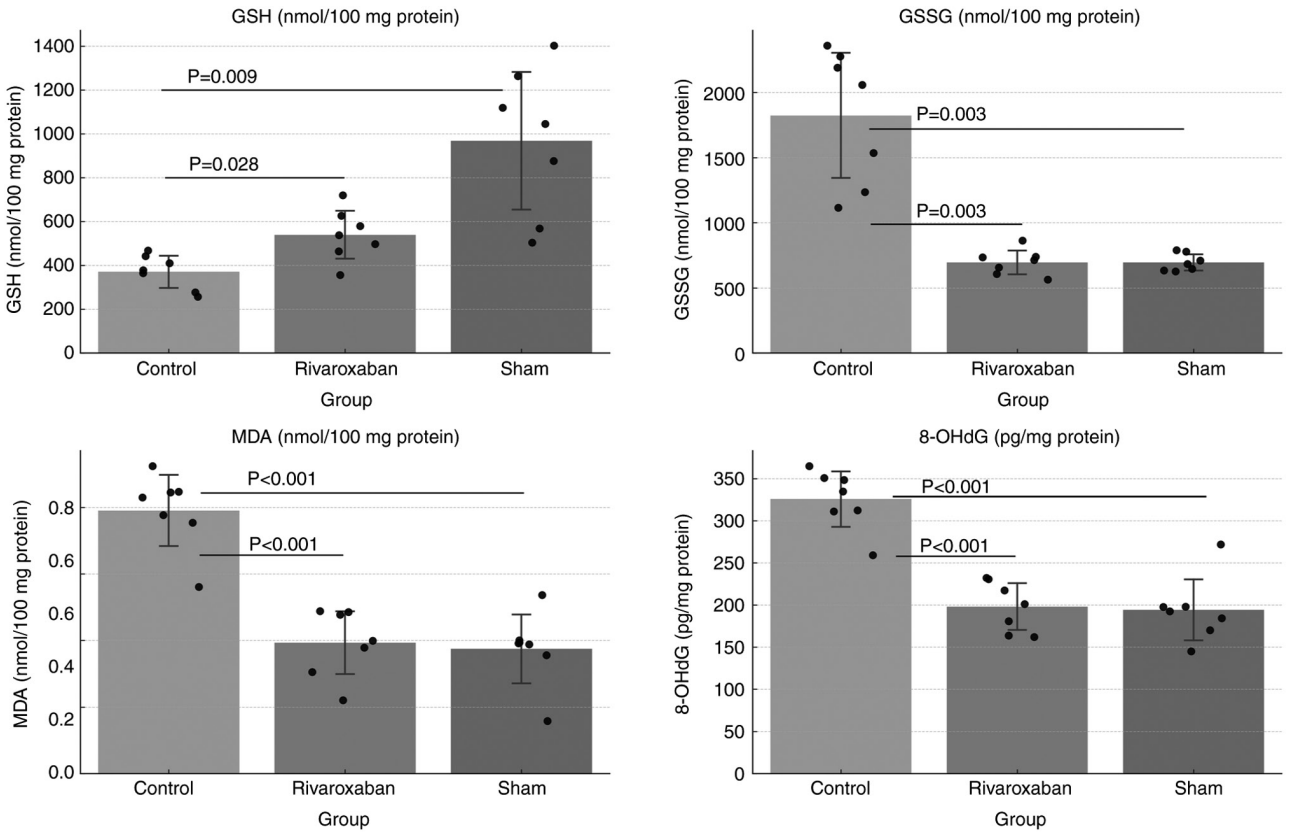

Figure 2. Skeletal muscle levels of GSH, GSSG, MDA and 8-OHdG in the three study groups. Rivaroxaban-treated rats showed significantly lower oxidative stress and DNA damage markers compared with those in the ischemia/reperfusion control group. Data are presented as mean ± SD with individual values shown as dots. Exact P-values are indicated in the figure. P<0.05 was considered to indicate a statistically significant difference. GSH, GSSG and MDA levels were measured using high-performance liquid chromatography, while 8-OHdG levels were measured using ELISA. 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; GSH, glutathione; GSSG, oxidized glutathione.

To the best of our knowledge, only a few experimental studies have evaluated 8-OHdG as a primary biomarker of oxidative DNA damage in lower extremity I/R models. In the present study, the results showed that even a brief period of I/R could induce a significant increase in 8-OHdG levels, reinforcing its value as a sensitive and quantifiable indicator of oxidative DNA injury. Previous experimental studies have demonstrated that I/R injury is closely associated with oxidative stress-mediated inflammation, apoptosis and functional tissue impairment using alternative oxidative stress markers and experimental endpoints (9,20). Furthermore, the significant reduction in 8-OHdG levels observed in the rivaroxaban-treated group in the present study suggested that rivaroxaban could mitigate DNA oxidative damage, potentially contributing to the preservation of cellular integrity under oxidative stress conditions.

The antioxidant properties of rivaroxaban observed in the present study were further supported by the decrease in MDA and GSSG levels, accompanied by the preservation of GSH concentrations. These markers reflect lipid peroxidation and redox balance, respectively, and are commonly used to quantify oxidative stress in experimental models (7,8). The consistent improvements detected in both serum and skeletal muscle tissues indicated that rivaroxaban could exert systemic protective effects against I/R-induced oxidative stress.

Although the present study did not directly investigate molecular signaling mechanisms, prior evidence indicated that rivaroxaban can modulate key oxidative stress-related

pathways. Specifically, a previous study demonstrated that inhibition of the protease-activated receptor-2 and NF-κB pathways was associated with attenuated inflammation and oxidative stress in experimental cellular and *ex vivo* tissue models (21). Additionally, oxidative stress responses are regulated by the Nrf2 pathway, which regulates the expression of antioxidant enzymes and may be relevant in the context of reduced oxidative stress following factor Xa inhibition (22). However, these pleiotropic effects warrant further investigation in mechanistic studies.

While rivaroxaban was chosen based on its documented non-anticoagulant properties, other oral anticoagulants, including apixaban and dabigatran, have also been studied for their potential antioxidant or endothelial-protective effects. These effects were demonstrated in various *in vitro* endothelial cell models exposed to inflammatory or oxidative stimuli, highlighting the shared endothelial-protective and antioxidant actions of these agents (23). However, comparative evidence remains limited, where rivaroxaban continues to be the most extensively investigated agent in both clinical and experimental models of PAD, likely due to its earlier approval, extensive evaluation in large-scale trials and its dual capacity to inhibit both factor Xa and thrombin generation, mechanisms that are particularly relevant to thrombosis and vascular inflammation in PAD (4,5,11,17,21,24).

Results of the present study are in line with clinical evidence from various trials, such as VOYAGER-PAD and COMPASS, which demonstrated that low-dose rivaroxaban reduced limb-related events (such as acute limb ischemia, major amputation and repeat revascularization) in patients with PAD undergoing revascularization (5,25). However, it should be noted that in the present study an acute I/R injury model was employed in healthy rats, which differs markedly from the chronic and comorbid conditions observed in human PAD. Consequently, the translational relevance of these findings should be interpreted with caution. Future studies involving chronic ischemia models or animals with comorbid models could provide additional insights.

The present study there were several limitations. Experiments were conducted in a healthy rat model using an acute I/R protocol, which may not fully reproduce the complex pathophysiology of chronic PAD in human. The I/R period was relatively short, where long-term outcomes, such as tissue regeneration or functional recovery, were not evaluated. In addition, the analysis was limited to oxidative stress markers, whereby histological or molecular analyses were not performed to verify tissue-level damage or repair. Future studies in chronic I/R models and clinical settings are warranted to validate the translational relevance of these findings. Furthermore, only female rats were used, which may introduce sex-related biological bias and limit the generalizability of the findings to male subjects.

Current guidelines for PAD management remain inconclusive regarding the optimal medical therapy following revascularization. The 2016 American College of Cardiology and American Heart Association guidelines recommend monotherapy with either aspirin or clopidogrel, whereas the 2021 Turkish National Guidelines for Peripheral Artery and Venous Disease suggest that low-dose rivaroxaban combined with aspirin could provide additional benefit in high-risk patients (6,26) Despite these recommendations, the use of dual antiplatelet therapy or anticoagulant-antiplatelet combinations remains common in clinical practice.

Overall, the findings of the present study contributed to the growing body of preclinical evidence supporting the potential role of rivaroxaban in the management of PAD, particularly under experimental conditions of acute I/R injury, which simulate a high ischemic risk state. The observed reduction in oxidative stress and DNA damage suggests that rivaroxaban may confer additional protective effects beyond its anticoagulant action, potentially improving long-term outcomes following revascularization.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CS conceived and designed the study, conducted the animal experiments and drafted the manuscript. TG assisted with the animal experiments and sample collection. ÖGD and TK performed the biochemical analyses. ACE contributed to statistical analysis and interpretation of data. CS and ACE confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the Local Ethics Committee for Animal Experiments of the Dokuz Eylül University (approval no. 12/2021; Izmir, Turkey) and were performed in accordance with the National Institutes of Health Guide for the care and use of laboratory animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work, AI tools were used to improve the readability and language of the manuscript. Specifically, ChatGPT (OpenAI-GPT 5.1) was used for text refinement. Subsequently, the authors revised and edited the content refined by the AI tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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