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ORIGINAL ARTICLE

# The effect of tourniquet reperfusion interval on malondialdehyde (MDA) level and skeletal muscle damage in the treatment of long bone fractures

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## ABSTRACT

**Aim** Tourniquets are commonly used during surgery to control bleeding, however, their application can lead to complications, including ischemic reperfusion injury. Post-tourniquet syndromes such as pain, swelling, and muscle weakness may persist for up to six weeks. The aim of this study was to investigate the reperfusion interval duration to prevent muscle tissue damage.

**Methods** This experimental study involved 48 male white rats (*Rattus norvegicus*) with induced tibial fracture. The rats were divided into two groups, A and B, with each group receiving four different treatments, resulting in a total of eight groups. The control groups (A1 and B1) had the tourniquet applied for 3 hours, while other groups had it used for 2 hours, followed by 5, 10, and 15-minute reperfusion intervals. Group A was sacrificed one hour post-deflation, and Group B was sacrificed on day 14. Malondialdehyde (MDA) level and muscle histology were analysed using one-way ANOVA.

**Results** In group A, significant difference was found between the 10 and 15-minute reperfusion intervals. In group B, the 10-minute reperfusion interval showed the most favourable outcome, with a 42.63% reduction in injury for group A and a 32.27% reduction for group B. Significant differences in MDA level were found between the control and reperfusion groups in both groups A and B.

**Conclusion** The 10-minute reperfusion interval effectively reduces ischemic reperfusion injury in muscle tissue, as indicated by lower MDA level and less muscle damage. This interval optimally restores aerobic metabolism and prevents excessive reactive oxygen species (ROS) production.

**Keywords:** malondialdehyde, reperfusion injury, tourniquet

## INTRODUCTION

Harvey Cushing introduced the pneumatic tourniquet in 1904, revolutionizing orthopaedic surgery by providing a method to maintain a clean surgical field and enhance precision during procedures on the extremities (1). While tourniquet use has clear benefits in facilitating surgical access and minimizing blood loss, it is not without complications. Among the most concerning is ischemic reperfusion injury, which occurs when blood flow is restored to previously ischemic tissues, triggering inflammation, oxidative stress, and tissue damage (2,3). Clinically, ischemic reperfusion injury is a significant concern, occurring in approximately 1 in 6,000 cases of upper extremity surgery and 1 in 3,700 lower extremity surgeries (4). This underscores the need to optimize tourniquet protocols to minimize patient morbidity.

The pathophysiology of ischemic reperfusion injury involves a cascade of metabolic disruptions, oxidative stress, and cellular damage. Muscle tissue is particularly vulnerable due to its high oxygen demand, with irreversible injury beginning after just 3 hours of ischemia, compared to nerves (8 hours) and bone (4 days) (5,6). During ischemia, adenosine triphosphate (ATP) depletion and lactate accumulation initiate cellular damage and activate inflammatory mediators (7,8). Upon reperfusion, the sudden influx of oxygen triggers reactive oxygen species (ROS) formation, which exacerbates tissue injury through lipid peroxidation (9). Malondialdehyde (MDA), a secondary product of lipid peroxidation, is commonly used as a biomarker to assess oxidative stress and tissue damage (10,11).

Ischemic reperfusion injury is not limited to muscle tissue. Endothelial cells and microvasculature are highly susceptible, leading to increased vascular permeability, enema, and leukocyte adhesion, which further exacerbate tissue injury and delay recovery (12,13). Prolonged ischemia has been shown to cause

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significant microvascular damage, observable upon reperfusion (14,15). These findings highlight the importance of not only controlling the duration of ischemia but also managing the reperfusion interval, which may critically influence the extent of tissue damage.

Although much of the clinical focus has historically been on ischemia duration, recent studies suggest that the timing of reperfusion itself can significantly affect muscle recovery (16). Short reperfusion intervals, such as 10 minutes, have been reported to enhance metabolic recovery, reduce ROS formation, and maintain ATP levels, thereby mitigating ischemic reperfusion injury (17,18). Despite these promising findings, the optimal reperfusion interval for reducing oxidative damage in skeletal muscle remains unclear, and evidence directly comparing different intervals is limited.

The aim of this study was to investigate the effects of different reperfusion intervals on skeletal muscle MDA levels and histopathological changes following tourniquet-induced ischemia in a rat model assessing 5-, 10-, and 15-minute reperfusion intervals to determine how variations in reperfusion timing influence oxidative stress and muscle tissue recovery, providing novel insights that may guide safer tourniquet application in clinical practice.

## MATERIALS AND METHODS

### Materials and study design

This research was conducted between January and March 2023 in several locations. We raise, treat, and sacrifice the experimental animals at the Parasitology Laboratory, Faculty of Medicine, Universitas Brawijaya, Indonesia. The MDA level measuring was performed at the Physiology Laboratory, Faculty of Medicine, Universitas Brawijaya. The Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Brawijaya, was used for the staining process and organ histology assessment.

An experimental study on male white rats (*Rattus norvegicus*) was used to see the effect of reperfusion interval on the use of tourniquets on the systemic effect of reperfusion ischemic injury in skeletal muscle in the treatment of long bone fractures by analysing MDA biomarkers and histopathological observations of skeletal muscle.

The sample size was calculated using the Federer formula (19) to estimate the required number of subjects based on power analysis. The formula used was as follows:

(n - 1) (t - 1)≥15

where: n = required sample size per group, t = number of treatment groups

$$(n-1)\,(8-1)15$$

$$(n-1)\,(7)15$$

$$7n-715$$

$$7n22$$

$$n \geq \frac{22}{7}$$

$$n3.14$$

Eight groups in this study included:

- Group P1A-O1A: No reperfusion interval, the rats were sacrificed 1 hour after tourniquet deflation
- Group P2A-O2A: A 5-minute reperfusion interval, the rats were sacrificed 1 hour after tourniquet deflation.
- Group P3A-O3A: A 10-minute reperfusion interval, the rats were sacrificed 1 hour after tourniquet deflation.
- Group P4A-O4A: A 15-minute reperfusion interval, the rats were sacrificed 1 hour after tourniquet deflation.
- Group P1B-O1B: No reperfusion interval, the rats were sacrificed 14 days after the treatment.
- Group P2B-O2B: A 5-minute reperfusion interval, the rats were sacrificed 14 days after the treatment.
- Group P3B-O3B: A 10-minute reperfusion interval, the rats were sacrificed 14 days after the treatment.
- Group P4B-O4B: A 15-minute reperfusion interval, the rats were sacrificed 14 days after the treatment.

To avoid a lack of sample size, each group consists of 6 rats, therefore 48 rats were included in the study.

The inclusion criteria were as follows: male *Wistar* strain white rats (*Rattus norvegicus*), aged 3 to 4 months, with a body weight between 180 and 200 grams, and in good health, as indicated by active movement and the absence of any extremity deformities. The exclusion criteria included rats that were ill or showed signs of compartment syndrome.

All protocols were approved by the Health Research Ethics Commission of the Faculty of Medicine, Universitas Brawijaya Malang, with ethical approval number 70B/EC/KEPK-PPDS/02/2023.

### Methods

**Acclimatization**. Rats were acclimatized for seven days in laboratory conditions, housed in standard cages (30x20 cm). The animals were given access to food and distilled water ad libitum, with the bedding changed daily to maintain cleanliness.

**Fracture**. The experimental animals fasted for 3 hours before termination. Anaesthesia was first administered through ketamine hydrochloride (Ketalar) at a dosage of 40 mg/kg, followed by the prophylactic antibiotic cefazolin at 5 mg/kg intramuscularly. The operating field was prepared by shaving the area, followed by cleaning with Savlon, 70% alcohol, and povidone-iodine, and then covered with a sterile sheet. Once anaesthesia was achieved, indicated by the rat's eyes beginning to close and its movements slowing, the surgery proceeded. The operator, wearing sterile gloves and a gown, ensured aseptic conditions throughout the procedure. A closed fracture of the middle segment of the tibia was treated. Following the fracture, a mini circular cast was applied to immobilize the injured segment.

**Tourniquet.** To induce ischemia, an orthodontic rubber band (4.5 oz) was tied around the groin of each rat's leg. The tourniquet was applied uniformly to all rats, ensuring consistent ischemia. Rats in the control group were subjected to a 3-hour ischemic period without reperfusion, while rats in the experimental group received different reperfusion intervals of 5, 10, or 15 minutes after 2 hours of ischemia. A 1-hour reapplication followed the initial reperfusion period to simulate the clinical use of a tourniquet during surgeries with varied reperfusion intervals.

**Fixation and post-surgical care.** Plaster of Paris was applied to the tibia (lower leg) using the long leg cast method (20), extending from one-third of the middle of the thigh to the an-

kle, ensuring that the leg was in a straight position with full extension. Following the procedure, the experimental animals were placed into their respective cages and provided daily food according to their usual feeding habits. If any signs of lethargy, difficulty eating, or shivering were observed, the rats were administered paracetamol at a dosage of 100 mg/kg as an anti-pain medication.

**Specimen collection.** In group A, rats were sacrificed 1 hour after tourniquet deflation, in group B rats were sacrificed 14 days post-treatment using the cervical dislocation technique. Following this, skeletal muscle samples were taken distal to the tourniquet location for tissue MDA examination and anatomical pathology histology preparations. Once the organs for the study were removed, the rats were confirmed dead. The carcasses were then placed in a basin, and subsequently buried in the ground at a minimum depth of 50 cm, with an area of 0.25 $m^2$ per burial site. To prevent the carcasses from being unearthed by other animals, such as cats, each hole was used to bury no more than ten rats. The hole was then covered with soil and compacted to ensure the odour of the carcasses did not escape. The specimen replication obtained from these procedures was then examined for measurements at the Physiology Laboratory, Faculty of Medicine, Universitas Brawijaya.

**Measurements of malondialdehyde (MDA) level.** Skeletal muscle samples were ground with the assistance of liquid nitrogen and weighed to 50-100 mg. The samples were then placed in a Petri dish, and one cc of phosphate buffer was added. Following this, one cc of 100% TCA (Trichloroacetic Acid), one cc of 1 N HCl (Normal Hydrochloric Acid), and one cc of 1% Na-thiobarbiturate were added sequentially. The mixture was heated in a water bath at 100 °C for 25 minutes. After heating, the solution was centrifuged at 2000-3000 rpm for 15 minutes. The supernatant was then collected and diluted with water to a final volume of 3 cc. The sample was then analysed using spectrophotometry at a wavelength of 532 nm (Shimadzu Corporation, Kyoto, Japan).

**Histological examination.** After taking the rat's skeletal muscle tissue, the tissue was taken to the Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Brawijaya, to prepare for staining and histological examination. The tissue samples were soaked in a medium containing 10% formaldehyde. Dehydration was then performed using a series of alcohol solutions: 70% alcohol for 1 hour, followed by 80% alcohol for 1 hour, 90% alcohol for 1 hour, 95% alcohol for 1 hour, 99% alcohol for 1 hour, and 100% alcohol for 1 hour. The clearing process was conducted by placing the dehydrated material in xylol solution for two 30-minute intervals. Following this, the embedding process was carried out. The block was then placed on the rotary microtome, and thin longitudinal cuts of 3-5 µm were made. The cut sections were transferred to a water bath to allow proper expansion, after which they were placed onto a labelled glass slide. Staining was performed with Hematoxylin & Eosin (HE), and the slides were covered with a cover glass. Histological observations of the skeletal muscles were carried out by quantitatively counting the fields of view in 10 small sections using an Olympus BX-51 dot Slide microscope, equipped with an Olympus XC10 camera (Olympus Corporation, Tokyo, Japan), at 400x magnification. The results were then analysed for further evaluation.

## Statistical analysis

The normality of the data was assessed using the Kolmogorov-Smirnov test. If the data were normally distributed, a parametric test performed; otherwise, non-parametric tests were used. Homogeneity of variances was assessed using Levene's test to ensure equal variances between the groups. For comparing between-group differences, a one-way analysis of variance (ANOVA) was conducted. This test assessed whether there were significant differences in the measured outcomes. If ANOVA indicated significant differences, Tukey's HSD test was used for pairwise comparisons between the groups, allowing for identification of which groups significantly differed from each other. A paired t-test was used for within-group comparisons at the two time points (1 hour and 14 days) to assess changes over time within each group.

## RESULTS

The results of the normality test using the Kolmogorov-Smirnov test showed significance values of p=0.280 and p=0.251 for the percentage of injury, and p=0.291 and p=0.287 for MDA. Since the p-value was greater than 0.05, we accepted Ho, indicating that the data followed a normal distribution. Before testing using ANOVA, the data obtained for each treatment were analysed for homogeneity of variance using the homogeneity of variance test (Levene test) to determine whether the data used had the same variance (Table 1).

**Table 1. Homogeneity of variance test using Levene for the percentage of injury (PI) and malondialdehyde (MDA) levels across different groups**

| Parameter | Levene statistic | Significance (p-value) |
|---|---|---|
| PI | 0.395 | 0.758 |
| PI-14 | 0.196 | 0.754 |
| MDA | 0.187 | 0.058 |
| MDA-14 | 2.819 | 0.065 |

The test results showed the value of the Levene test for the Percentage of Injury, with p=0.758 and p=0.754, while MDA had p=0.058 and p=0.065. Because the p-value was greater than 0.05, Ho was accepted, and it could be concluded that the data used had a homogeneous variance. Thus, ANOVA could be used to test. A one-way ANOVA test was conducted to determine whether there was a significant difference between the treatments (Table 2). Based on the ANOVA analysis, the p-values for the percentage of injuries, the 14-day period, and MDA were all p=0.000.

**Table 2. One-way ANOVA test between percentage of injury (PI) and malondialdehyde (MDA) levels**

| Parameter | p | Description |
|---|---|---|
| PI | 0.000 | Significance |
| PI-14 | 0.000 | Significance |
| MDA | 0.000 | Significance |
| MDA-14 | 0.000 | Significance |

The results of comparing each group after the One-way ANOVA test were further analysed using the Tukey test. The results of the Tukey test that had different group mean values were shown if they had a $p<0.05$. Tukey's test results on percentage of injury

**Table 3. Paired t-test percentage of injury (PI) and malondialdehyde (MDA) levels between the first day and the 14th day**

| Parameter | Mean | p | Description |
|---|---|---|---|
| PI | 58.90 | 0.000 | Significance |
| PI-14 | 47.87 | | |
| MDA | 261.70 | 0.000 | Significance |
| MDA-14 | 361.67 | | |

(PI) showed that the control group differed significantly from the 10-minute and 15-minute groups. A comparison between the 5-minute reperfusion interval groups showed a significant difference between the 10-minute and 15-minute groups. Meanwhile, the 10-minute reperfusion interval group showed no difference from the 15-minute reperfusion interval group.

Table 3 presents the results of testing the percentage of injuries on the first day showed p=0.000 for 14 (Table 3). Thus, it could be concluded that there was a significant difference between the percentage of injuries on the first and 14th day, where the percentage of injuries on the first day was higher than on day 14. The results of testing the levels of MDA on the first and 14th days showed p=0.000 which was <0.05. Thus, it could be concluded that there was a significant difference between the MDA on the first day and the MDA on day 14, where the MDA level on day 14 was higher than on the first day (Figures 1, 2).

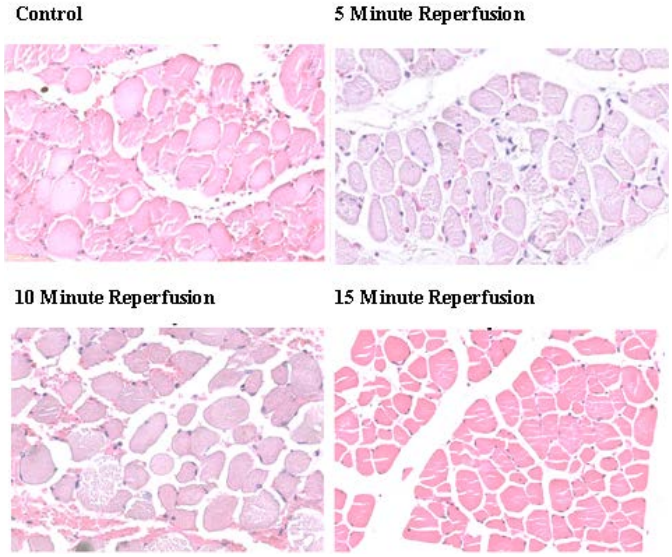


**Figure 1. Histology of percentage injury in Control, 5, 10, and 15-minute reperfusion groups** (Faculty of Medicine, Universitas Brawijaya, 2023)

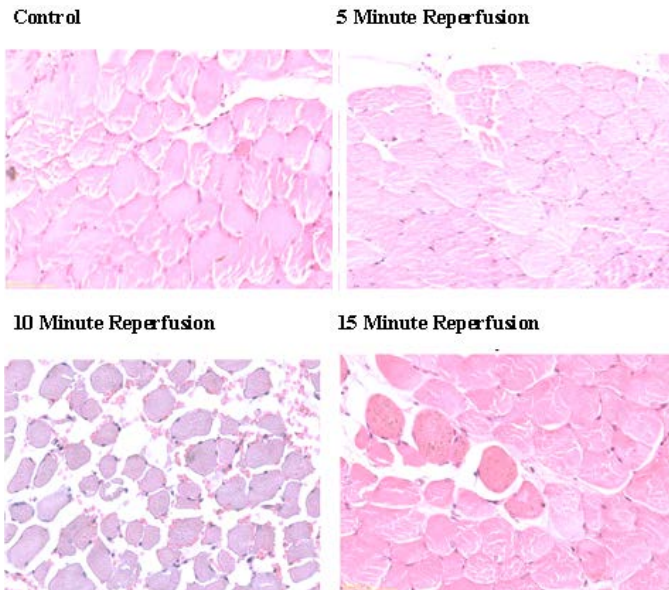


**Figure 2. Histology of percentage injury day-14 in Control, 5, 10 and 15- minute reperfusion groups** (Faculty of Medicine, Universitas Brawijaya, 2023)

## DISCUSSION

Tourniquets to assist haemostasis have been commonly used clinically in trauma cases (1). Initially used in wartime to stop bleeding from war wounds, tourniquets are now standard in orthopaedics to reduce bleeding and maintain a clear operating field during surgeries for fractures (21). However, the return of blood circulation to ischemic tissue after tourniquet deflation can lead to ischemic reperfusion injury (5). Previous research using Wistar rats in a 3-hour leg ischemia model demonstrated an increase in serum levels of urea, creatinine, SGOT, SGPT, LDH, TNF-α, and IL-6 in the group given reperfusion for 1, 2, and 3 hours (22).

In our study, the measurement of MDA levels in Group A, in which rats were sacrificed 1 hour after tourniquet deflation, revealed a significant difference between the control group and those receiving reperfusion intervals of 5 minutes, 10 minutes, and 15 minutes. Interestingly, while there were no significant differences between the 5- and 15-minute reperfusion groups, the 10-minute reperfusion interval showed significantly lower MDA level when compared to both the 5-minute and 15-minute reperfusion groups. This finding is consistent with previous studies, which have shown that shorter reperfusion intervals may not provide sufficient recovery, leading to higher oxidative stress levels and muscle damage (18).

Our results align with those of a study investigating circulatory discontinuation and its impact on oxidation during healing in rats. Femoral artery clamping for 4.5 hours after tibial fracture significantly increased MDA levels on days 3, 7, and 14 (23). This is in line with our findings, where a longer reperfusion interval (10 minutes) improved recovery compared to shorter intervals, further reducing oxidative stress as measured by MDA level. Our study also supports the idea that a 10-minute reperfusion interval facilitates better metabolic recovery compared to 5-minute reperfusion intervals, which have been shown to result in extensive metabolic breakdown and poor recovery (13).

Furthermore, several studies suggest that reperfusion intervals help reduce muscle damage when used after prolonged ischemia (more than 2 hours) (17). Other research showed that the presence of a reperfusion interval in the experimental group of rats, for 20 minutes after experiencing ischemia for 1.5 hours, showed better blood flow in skeletal muscle than the group without a reperfusion interval (24). It was shown that a group of experimental animals that had received reperfusion intervals showed a decrease in the degree of cell damage and a decrease in serum creatinine phosphokinase levels (16). A study suggested that to reduce the complications of using a tourniquet, you can give a perfusion time interval with tourniquet deflation for a specific duration, with a reperfusion interval of 10 minutes or 30 minutes after using a tourniquet for 2 hours (13).

Ischemic conditions, caused by tourniquet use, disrupt the bloodstream, leading to tissue damage and organ dysfunction (25). The tissue undergoes hypoxia, resulting in decreased ATP

levels and activation of anaerobic glycolysis, which accumulates lactate and other toxic byproducts (6). These changes activate phospholipase A2, which converts phospholipids into arachidonic acid, leading to the production of inflammatory mediators such as leukotrienes and prostaglandins (26). These metabolites increase leukocyte adhesion to the vascular endothelium and enhance permeability of post-capillary venules (27). Additionally, ischemia induces the conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO), which, along with hypoxanthine accumulation, produces oxidants (28). The reduction in ATP also impairs calcium pumps, raising intracellular calcium levels and causing mitochondrial damage (6). During the reperfusion phase, the return of blood flow provides an adequate blood supply.

Oxygen in ischemic tissue through blood vessels restores tissue metabolism to aerobic, but at the same time, triggers the formation of ROS. The formation of excess ROS due to the interaction of XO and hypoxanthine causes oxidative stress and lipid peroxidation, activation of transcription factors such as NF-B, and increased proinflammatory mediators. Lipid peroxidation is produced by the product malondialdehyde (MDA) (3). In addition, antibody complexes also appear in the reperfusion phase, which will damage cell membranes (3). Complement activation that occurs also causes degranulation of mast cells and the release of histamine and other chemical mediators that can lead to multiple organ failure, resulting in postoperative mortality and morbidity (8).

Oxidative stress due to increased ROS in the reperfusion phase is triggered by the return of oxygen supply (reoxygenation), which can cause damage to cellular macromolecules such as nucleic acids, proteins, and lipids, especially in endothelial cells (7). Under physiological conditions (low lipid peroxidase level), cells can maintain balance through the antioxidant defence system (29). On the other hand, in conditions of high lipid peroxidase level, the oxidative damage that occurs exceeds cell repair ability, so cells will begin to undergo apoptosis or necrosis (30). Hydrogen peroxide forms in tissues exposed to ischemic reperfusion injury and is a relatively stable oxidant. At concentrations of 10-100 M, $H_2O_2$ can cause endothelial dysfunction, increased expression of adhesive molecules against leukocytes, and increased inflammatory mediators. One of the parameters that can be used to assess oxidative stress conditions is measuring MDA levels (31).

This study demonstrated that ischemic-reperfusion injury causes damage to muscle organs, primarily due to the return of an abundant oxygen supply during reperfusion, which triggers a surge in ROS production and leads to oxidative stress. This condition is capable of damaging muscle cell structure, as previously described (32). Statistical tests showed higher MDA levels in group B (the group that was sacrificed on day 14); in that group, the MDA levels in the control group were much higher than the treatment group; this indicates that the reperfusion interval can reduce oxidative stress conditions. The injury process is still ongoing until day 14; it can be correlated with the post-tourniquet syndrome process in patients who have undergone surgery using tourniquets, which can occur up to the sixth week postoperatively. Histopathological analysis of muscle organs in group B showed a significantly lower PI value than in group A, indicating the reversibility of the injury and the process of repair and regeneration of skeletal muscle cells (4).

The 10-minute reperfusion interval in this study was proven to prevent excessive ROS formation, thereby reducing muscle tissue damage in the distal part of the tourniquet. However, further research is necessary to investigate oxygen levels in the distal tortuous during both ischemia and reperfusion in real-time. Additionally, studies are needed on the oxygen levels required by distal tourniquet tissue to maintain vital functions, as well as on the use of antioxidants as initial therapy to prevent the effects of ROS. Furthermore, additional research is needed to investigate the effects of longer reperfusion intervals and study durations on the persistence of oxidative stress following tourniquet use.

This study demonstrates that administering reperfusion intervals during tourniquet use significantly reduces MDA levels in skeletal muscle on both the 1st and 14th days post-injury. The application of reperfusion intervals also alleviates ischemic reperfusion injury, as evidenced by the reduction in skeletal muscle damage (percentage injury) at both time points. These findings suggest that incorporating a 10-minute reperfusion interval following tourniquet application can help mitigate muscle damage and improve tissue recovery during and after orthopaedic surgeries. In clinical practice, these results could guide surgeons in adjusting reperfusion strategies during surgeries to minimize ischemic reperfusion injury and enhance postoperative recovery, particularly in patients undergoing procedures involving tourniquets.

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## TRANSPARENCY DECLARATION

Conflicts of interest: None to declare.

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