

# Experimental study comparing the effects of cold and warm applications on oxidative stress markers in a rat extremity ischemia-reperfusion injury model

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## ABSTRACT

**BACKGROUND:** After successful restoration of limb perfusion following limb ischemia, a series of secondary damage mechanisms, collectively known as reperfusion injury, is triggered. This study investigated the comparative effects of warming, a technique commonly used in clinical settings after extremity revascularization, and cooling, in a rat model of ischemia-reperfusion injury.

**METHODS:** Sprague Dawley rats aged 12 to 14 weeks and weighing between 250-350 g were used. The study included four experimental groups: Sham, Control, Cold Application, and Warm Application. Ischemia was induced by occluding the femoral artery with an atraumatic vascular clamp for three hours. Cold and warm treatments were applied for two hours by immersing the hind limbs in water at specific temperatures. In the cold application group, water temperature was maintained at  $12\pm 2^\circ\text{C}$  using ice cubes to lower the temperature as needed. In the warm application group, the water temperature was kept at  $38\pm 2^\circ\text{C}$  by adding hot water when necessary. Twenty-four hours after the injury model was established, the animals underwent a second surgical procedure to obtain tissue samples for analyses of malondialdehyde (MDA), myeloperoxidase (MPO), poly(ADP-ribose) polymerase (PARP), catalase, and superoxide dismutase (SOD).

**RESULTS:** Compared to the control group, the warm application group showed no statistically significant differences in levels of MDA ( $p=0.910$ ), MPO ( $p=0.527$ ), PARP ( $p=0.192$ ), catalase ( $p=0.999$ ), or SOD ( $p=0.987$ ). In contrast, the cold application group exhibited a significant reduction in MDA, MPO, PARP, catalase, and SOD levels ( $p<0.001$  for all).

**CONCLUSION:** To minimize reperfusion injury following limb ischemia, cold application may provide greater benefits than warming the extremity. Further studies are necessary to explore the clinical relevance and applications of this finding.

**Keywords:** Amputation; cold; cooling; crush; hypothermia; microsurgery; revascularization; warm.

## INTRODUCTION

Injuries that cause peripheral ischemia, such as traumatic crush injuries, major vascular trauma, and amputations, damage living tissues through two distinct mechanisms.<sup>[1,2]</sup> The primary injury disrupts distal circulation, leading to tissue loss

in areas that cannot sustain their metabolic activities due to inadequate blood supply—a phenomenon known as ischemic injury. When ischemia is subsequently resolved and extremity perfusion is restored, a cascade of secondary damage mechanisms, collectively referred to as reperfusion injury, is triggered. During this phase, some tissues that initially survived

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the ischemic period may still be lost due to the harmful effects of reperfusion.<sup>[3-6]</sup>

The ischemic injury phase is characterized by biochemical processes including adenosine triphosphate (ATP) depletion, increased anaerobic metabolism, lactate accumulation, and acidosis. On the other hand, the reperfusion injury phase involves mitochondrial dysfunction, intracellular calcium accumulation, normalization of pH, elevated production of reactive oxygen species, and activation of regulated cell death pathways.<sup>[7]</sup>

Treatment principles for injuries causing extremity ischemia should be guided by the underlying damage mechanisms. The primary goals include restoring extremity perfusion as quickly as possible, maintaining homeostasis, and minimizing the loss of viable tissue by mitigating reperfusion injury to the greatest extent possible.<sup>[8]</sup>

Research on the impact of end-organ temperature following ischemia on reperfusion injury has primarily focused on the use of hypothermia in traumatic brain injury and its effects on various biochemical pathways.<sup>[9-11]</sup> This approach has been translated into clinical studies for the management of traumatic brain injury. However, the influence of end-organ temperature on reperfusion injury in extremity trauma involving ischemia-reperfusion remains poorly understood. It is still unclear whether the benefits of warming the extremity, aimed at preventing vasoconstriction, outweigh those of cooling the end-organ to reduce metabolic activity.<sup>[12,13]</sup> To address this gap, the present study investigated the comparative effects of warming (a practice commonly used in clinical settings following extremity revascularization, unlike in brain injuries) versus cooling, using a rat model of ischemia-reperfusion injury. The goal was to determine which approach more effectively minimizes end-organ damage.

## MATERIALS AND METHODS

This study was conducted as experimental research using an animal model. Ethical approval was obtained from the Ege University Local Ethics Committee for Animal Experiments under protocol number 2017-017. All experiments complied with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act of 1986 and its associated guidelines, as well as the EU Directive 2010/63/EU on the protection of animals. The study utilized 12- to 14-week-old Sprague Dawley rats, weighing between 250 and 350 grams, regardless of sex. The animals were supplied by the Ege University Laboratory Animal Application and Research Center, where all experimental procedures were performed.

Animals were housed under standardized environmental conditions, including a 12-hour light/dark cycle, a controlled temperature range of 20-24°C, and relative humidity between

50% and 60%. They were kept in cages of two to three animals per cage and provided with ad libitum access to food and water. A total of 28 animals were included in the study, evenly distributed into four experimental groups, with seven animals in each group. Surgical procedures were performed under intraperitoneal anesthesia using a combination of ketamine and xylazine. Ketamine hydrochloride (50 mg/kg; Ketalar®, Parke-Davis, Istanbul, Türkiye) and xylazine hydrochloride (10 mg/kg; Rompun®, Bayer, Istanbul, Türkiye) were administered intraperitoneally. If the anesthetic effect diminished before the required five-hour duration in the study groups, additional doses of anesthesia were administered to maintain the desired level of anesthesia.

The study included four experimental groups. The first group served as the sham group, in which animals underwent surgical incision and femoral artery dissection following anesthesia but did not undergo vascular clamping. A five-hour anesthesia period was completed without inducing ischemia. The second group served as the control group, in which ischemia was induced and maintained for three hours without any additional intervention. The third group was designated as the cold application group (first treatment group). In this group, the affected hind limb was exposed to cold for two hours following a three-hour ischemia period. The fourth group was the warming group (second treatment group), in which the affected hind limb was warmed following the ischemia period.

The ischemia model was applied to the right hind limb of each animal. Following appropriate preparation steps, including shaving, antiseptic application, and fixation, a 2 cm oblique incision was made along the groin to expose the femoral neurovascular bundle. The femoral artery was dissected and separated from the surrounding neurovascular structures. Ischemia was induced by occluding the femoral artery using an atraumatic vascular clamp (Vascu-Stat® Plus Approximator-Mini), effectively halting distal circulation. Simultaneously, a tourniquet was applied at the proximal thigh using an Esmarch bandage to prevent venous return and block arterial supply from collateral circulation. The duration of ischemia was standardized at three hours. After this period, both the vascular clamp and tourniquet were removed to restore extremity reperfusion. The incision site, including the skin and underlying tissues, was closed using absorbable sutures.

Cold and warm applications were administered by immersing the animals' hind limbs in water tanks maintained at specific temperatures. These interventions were carried out following incision closure, after the hind limbs had been subjected to three hours of ischemia. Both cold and warm treatments were applied for a duration of two hours. Water temperature was continuously monitored using a digital thermometer (TFA Digital Laboratory Thermometer 30.1034) to ensure it remained within the predetermined range. In the cold application group, the water temperature was main-



**Figure 1.** Experimental setup showing cooling and warming procedures applied to the hind limb.

tained at  $12 \pm 2^\circ\text{C}$ , with ice cubes added as needed to sustain the desired level. In the warm application group, the water temperature was kept at  $38 \pm 2^\circ\text{C}$  by adding hot water when necessary. In both setups, only the designated hind limb was exposed to the temperature intervention, while the rest of the animal's body remained at room temperature ( $25 \pm 2^\circ\text{C}$ ). To prevent direct contact between the limb and the water, the treated hind limb was enclosed in a latex glove during the intervention (Fig. 1). Postoperative analgesia was provided by adding paracetamol (500 mg/500 mL; Tamol®, Sandoz, Türkiye) to the animals' drinking water.

Following the ischemia-reperfusion model, the animals were reoperated 24 hours later to obtain tissue samples. For the 24-hour analyses, a similar surgical setup was performed, and a 1.5 cm incision was made on the posterior cruris to excise the gastrocnemius muscle. The collected muscle samples were stored at  $-80^\circ\text{C}$  until the day of analysis.

Tissues designated for biochemical analysis were homogenized in a phosphate buffer (1:10 w/v) without allowing thawing. Malondialdehyde (MDA) levels, a marker of lipid peroxidation, were determined using the Thiobarbituric Acid Reactive Substances (TBARS) method. Superoxide dismutase (SOD) levels, a key antioxidant, were measured using a colorimetric method, while catalase levels, which are protective against oxidative stress and reactive oxygen species (ROS), were assessed spectrophotometrically. Myeloperoxidase (MPO) levels were measured using the Rat Myeloperoxidase Enzyme-Linked Immunosorbent Assay (ELISA) Kit (MyBio-source, catalog no: MBS046496), and poly ADP ribose polymerase (PARP) levels, associated with DNA damage, were quantified using the Rat PARP ELISA Kit (SunRed, catalog no: 201-II-0224).

## Statistical Analysis

Statistical analysis was performed using SPSS version 25.0 (IBM Corporation, Armonk, NY, USA). For comparisons of quantitative variables, one-way analysis of variance (ANOVA) was used for parametric data, followed by the Games-Howell test for post hoc analysis. For non-parametric data, the Kruskal-Wallis test was applied, with Dunn's test used for post hoc comparisons. A p-value of  $<0.05$  was considered statistically significant.

## RESULTS

In the comparative analysis of tissue MDA levels at 24 hours, a significant increase was observed in both the injury group and the warm application group compared to the sham group ( $p < 0.001$  for both). In contrast, MDA levels in the cold application group were effectively suppressed and comparable to those of the sham group ( $p = 0.401$ ). When comparing the treatment groups to the control group, MDA levels were significantly lower in the cold application group ( $p < 0.001$ ), whereas no significant difference was found in the warm application group ( $p = 0.910$ ) (Table 1).

In the comparative analysis of MPO levels at 24 hours, effective suppression was observed in the cold application group, with levels comparable to the sham group ( $p = 0.327$ ). In contrast, MPO levels were significantly elevated in both the control and warm application groups ( $p < 0.001$  for both). When the treatment groups were compared to the control group, MPO levels were significantly lower in the cold application group ( $p < 0.001$ ), while no significant difference was observed in the warm application group ( $p = 0.527$ ) (Table 1).

PARP levels showed a significant increase in the control group and in both treatment groups compared to the sham group ( $p < 0.001$  for all). When comparing the treatment groups to the control group, PARP levels were significantly lower in the cold application group ( $p < 0.001$ ), whereas no significant difference was observed in the warm application group ( $p = 0.192$ ) (Table 1).

In the 24-hour analyses, catalase levels were significantly elevated in the control group, as well as in the cold and warm application groups, compared to the sham group ( $p < 0.001$ ,  $p = 0.041$ , and  $p < 0.001$ , respectively). When the treatment groups were compared to the control group, catalase levels were significantly lower in the cold application group ( $p < 0.001$ ), whereas no significant difference was observed in the warm application group ( $p = 0.999$ ) (Table 1).

In the evaluation of SOD enzyme activity levels, a significant increase was observed in the control and treatment groups compared to the sham group ( $p < 0.001$  for all). When comparing the treatment groups to the control group, SOD activity levels were significantly reduced in the cold application group ( $p < 0.001$ ), whereas no significant difference was found in the warm application group ( $p = 0.987$ ) (Table 1).

**Table 1.** Levels of malondialdehyde (MDA), myeloperoxidase (MPO), poly(ADP-ribose) polymerase (PARP), catalase, and superoxide dismutase (SOD) across groups

| Group            | MDA<br>(nmol/mg protein) | MPO<br>(U/mg protein) | PARP<br>(ng/mg protein) | Catalase<br>(U/mg protein) | SOD<br>(U/mg protein) |
|------------------|--------------------------|-----------------------|-------------------------|----------------------------|-----------------------|
|                  | Median (Range)           | Median (Range)        | Mean (±SD)              | Mean (±SD)                 | Mean (±SD)            |
| Sham             | 0.11 (0.07-0.23)         | 4.50 (3.37-5.21)      | 0.70 (±0.14)            | 2.36 (±0.36)               | 1.18 (±0.31)          |
| Control          | 1.02 (0.31-1.81)         | 20.26 (19.96-23.45)   | 20.88 (±1.83)           | 13.19 (±1.61)              | 13.10 (±1.36)         |
| Cold             | 0.16 (0.13-0.46)         | 6.45 (5.15-7.01)      | 3.21 (±0.40)            | 3.14 (±0.46)               | 2.49 (±0.29)          |
| Warm             | 0.83 (0.46-1.49)         | 22.66 (22.13-25.88)   | 18.59 (±1.55)           | 13.30 (±1.23)              | 13.56 (±1.43)         |
| p value          | p<0.0011                 | <0.0011               | <0.0012                 | <0.0012                    | <0.0012               |
| Sham vs. Control | <0.001                   | <0.001                | <0.001                  | <0.001                     | <0.001                |
| Sham vs. Cold    | 0.401                    | 0.327                 | <0.001                  | 0.041                      | <0.001                |
| Sham vs. Warm    | <0.001                   | <0.001                | <0.001                  | <0.001                     | <0.001                |
| Control vs. Cold | 0.001                    | 0.001                 | <0.001                  | <0.001                     | <0.001                |
| Control vs. Warm | 0.913                    | 0.527                 | 0.192                   | 0.999                      | 0.987                 |
| Cold vs. Warm    | 0.001                    | <0.001                | <0.001                  | <0.001                     | <0.001                |

MDA: Malondialdehyde; MPO: Myeloperoxidase; PARP: Poly(ADP-ribose) polymerase; SOD: Superoxide dismutase. <sup>1</sup>Kruskal-Wallis test (Monte Carlo); Post Hoc Test : Dunn's test., <sup>2</sup>One-way analysis of variance (ANOVA) (Robust statistic: Brown-Forsythe); Post Hoc test: Games-Howell. Med: Median; Min: Minimum; Max: Maximum; SD: Standard deviation.

## DISCUSSION

The most notable finding of our study is that secondary cell death following reperfusion after ischemia is more effectively suppressed by cold application than by warm application. This result raises questions about the routine use of warming practices in clinical settings, particularly following minor replantation procedures. Given that different types and severities of injury may lead to varied clinical outcomes, it is essential to carefully weigh the potential benefits and drawbacks of warming in procedures involving reperfusion.

The protective effect of cold application against reperfusion injury is not a novel finding; previous studies have demonstrated its ability to mitigate oxidative stress-induced damage in reperfused organs across various injury models, including extremity ischemia.<sup>[14-17]</sup> However, our study highlights a critical contradiction: the widely practiced use of warming, particularly in extremity crush injuries and replantation, appears to conflict with this established evidence. In such cases, arterial thrombosis remains the most common cause of failure, and the vasorelaxant effects of warming are often employed to reduce this risk. However, warming also influences numerous biological processes beyond vasorelaxation, many of which remain poorly understood. Although the findings of our study are not sufficient to draw definitive clinical conclusions, they provide valuable preliminary data on the potential drawbacks of warming, particularly the missed opportunity to reduce reperfusion injury through cooling interventions. These findings underscore the need for further research to better understand the broader implications of warming in

these contexts.

Although the findings of our study are discussed using general terms such as "warming" and "cooling," it is important to recognize that different outcomes may arise depending on the intensity and duration of these interventions. The warming and cooling temperatures used in our study were selected based on the average body temperature of rats (37.5°C); however, this variable should be taken into account when interpreting the results. Currently, there are no established thresholds defining what constitutes "cooling" or "warming," nor is there a consensus on the optimal duration of these applications across different experimental models. A similar ambiguity exists in clinical practice, where the application of warming is often empirical rather than systematic and may vary depending on individual patient factors.<sup>[12,13]</sup> Varying the duration and temperature parameters could serve as an important focus for future research in both experimental and clinical settings. If more standardized, reproducible, and measurable data can be generated on this topic, it may pave the way for establishing a more optimal and evidence-based approach to temperature management in clinical practice.

In our experimental setup, the combined use of a tourniquet and arterial clamping to induce ischemic injury is a technique previously described in the literature.<sup>[18]</sup> The vascular clamp was used to reduce arterial inflow in a standardized manner, while the tourniquet was applied to obstruct venous return and block collateral circulation. Tourniquet application alone was not preferred for inducing ischemia, as it would not allow for adequate standardization. Despite its limitations, we



believe this method is appropriate for the design of a preliminary study aimed at addressing our research question.

The findings of our study demonstrate that cold application provides greater protection against oxidative stress compared to warming, as evidenced by the evaluation of oxidative stress markers in the reperfused extremity. However, it is also noteworthy that warming does not appear to exacerbate oxidative stress, as the measured markers were comparable to those of the control group. This suggests that while warming the reperfused extremity may not mitigate reperfusion injury, it may be considered safe in terms of not worsening it. These results raise the question of whether alternative warming protocols could offer potential benefits. Recent data from other injury models suggest that gradual warming and transient heat stress may play a role in reducing reperfusion injury.<sup>[19,20]</sup> Considering all these factors, the possibility of developing new warming modalities following extremity reperfusion warrants further investigation in future studies.

## CONCLUSION

To minimize reperfusion injury following limb-related ischemic events, cold application may offer greater benefits compared to warming the extremity. Further studies are needed to explore the clinical relevance and applications of this finding.

**Ethics Committee Approval:** This study was approved by the Ege University Scientific Research and Projects Committee (Date: 29.03.2017, Decision No: 2017-TIP-028/102).

**Peer-review:** Externally peer-reviewed.

**Authorship Contributions:** Concept: K.E., L.K.; Design: K.E., E.Y.S., L.K.; Supervision: E.S.Y., L.K.; Resource: K.E., L.K.; Materials: A.V., E.E., Data collection and/or processing: A.V., E.E., K.E.; Analysis and/or interpretation: A.V., E.E., K.E.; Literature review: A.V., E.E., K.E.; Writing: A.V., K.E.; Critical review: E.E., L.K., E.S.Y.

**Conflict of Interest:** None declared.

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## DENEYSEL ÇALIŞMA - ÖZ

**Sıçan ekstremite iskemi-reperfüzyon yaralanması modelinde soğuk ve sıcak uygulamanın oksidatif stres belirteçleri üzerindeki etkilerini karşılaştırılması: Deneysel çalışma**

**AMAÇ:** İskemi sonrası uzuv perfüzyonunun başarılı bir şekilde restore edilmesinin ardından, reperfüzyon hasarı olarak adlandırılan bir dizi ikincil hasar mekanizması devreye girer. Bu çalışmada, ekstremite revaskülarizasyonundan sonra klinik uygulamada kullanılan bir yaklaşım olan ısıtma ile ve iskemi-reperfüzyon yaralanması sıçan modelinde uygulanan soğutmanın karşılaştırmalı etkileri araştırılmıştır.

**GEREÇ VE YÖNTEM:** Çalışmada, 250-350 gr ağırlığında, 12 ila 14 haftalık Sprague Dawley sıçanları kullanıldı. Çalışmaya dört deney grubu (Sham, Kontrol, Soğuk Uygulama, Sıcak Uygulama) dahil edilmiştir. İskemi, atravmatik bir vasküler klempt kullanılarak femoral arterin 3 saat boyunca oblitere edilmesiyle indüklenmiştir. Soğuk ve sıcak uygulamalar, hayvanların arka uzuvlarının belirlenmiş sıcaklıklarda suya 2 saat süreyle daldırılmasıyla uygulanmıştır. Yaralanma modelinin oluşturulmasını takiben, hayvanlar malondialdehit (MDA), miyeloperoksidaz (MPO), poli-ADP-riboz-polimeraz (PARP), katalaz ve süperoksit dismutaz (SOD) seviyelerinin analiz edileceği kas örneklerinin alınması için 24. saatte sakrifiye edilmiştir.

**BULGULAR:** Tedavi grupları kontrol gruplarıyla karşılaştırıldığında, sıcak uygulama grubunda MDA seviyeleri ( $p=0.910$ ), MPO seviyeleri ( $p=0.527$ ), PARP seviyeleri ( $p=0.192$ ), katalaz seviyeleri ( $p=0.999$ ) ve SOD seviyeleri ( $p=0.987$ ) açısından anlamlı bir fark gözlenmedi. Buna karşılık, soğuk uygulama grubu MDA, MPO, PARP, katalaz ve SOD seviyelerinde anlamlı bir baskılanma gösterdi (hepsi için  $p<0.001$ ).

**SONUÇ:** Uzuvların iskemik yaralanmalardan sonra reperfüzyon hasarını en aza indirmek için, soğuk uygulama ekstremiteye uygulanan ısıtmaya kıyasla daha fazla fayda sağlayabilir. Bu çıkarımın klinik önemini ve uygulamalarını araştırmak için daha fazla çalışmaya ihtiyaç vardır.

**Anahtar sözcükler:** Ampütasyon; ezilme; hipotermi; mikrocerrahi; revaskülarizasyon; sıcak; soğuk; soğutma.

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