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Original Article / Özgün Makale

How important is the damage to the liver after lower limb ischemia-reperfusion? An experimental study in a rat model

Alt ekstremite iskemi reperfüzyonu sonrası karaciğer hasarı ne kadar önemlidir? Sıçan modelinde deneysel bir çalışma

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ABSTRACT

Background: The aim of this study was to compare the effect of lower extremity ischemia reperfusion on the liver and the effect of ischemia-reperfusion on the liver itself in a rat model.

Methods: Thirty Sprague-Dawley male rats were randomly divided into three groups including 10 in each group: sham (Group 1), lower limb ischemia-reperfusion (Group 2), and liver ischemia-reperfusion (Group 3). In Group 2, one hour of left lower limb ischemia was performed. In Group 3, one hour of ischemia in the liver was performed, followed by 24 hours of reperfusion. After reperfusion, the liver tissues were removed, and the groups were evaluated biochemically and histologically.

Results: The liver malondialdehyde levels were significantly higher in Groups 2 and 3 than in the sham group ($p<0.001$). In Group 2, the malondialdehyde levels were significantly higher than in Group 3 ($p=0.019$). The glutathione levels in the liver were significantly lower in Groups 2 and 3 than in the sham group ($p<0.001$). However, the glutathione levels were significantly higher in Group 2 than in Group 3 ($p=0.005$). In the histological evaluation, although the liver damage score was higher in Group 3 than in Group 2 ($p=0.015$), there was no significant difference between the two groups in TUNEL(+) cell number ($p>0.05$).

Conclusion: Reperfusion injury in the liver after lower limb ischemia-reperfusion is as important as ischemia-reperfusion injury which is specifically induced in the liver. This should be taken into account, particularly in reperfusion surgeries following vascular trauma or in cases of leg tourniquets to stop bleeding after lower limb vascular trauma.

Keywords: Ischemia, reperfusion, limb, liver.

ÖZ

Amaç: Bu çalışmada bir sıçan modelinde alt ekstremite iskemi reperfüzyonunun karaciğer üzerindeki etkisi ile karaciğerin spesifik iskemi reperfüzyonunun karaciğer üzerindeki etkisi karşılaştırıldı.

Çalışma planı: Otuz adet Sprague-Dawley tipi erkek sıçan 10'arlı olarak rastgele üç gruba ayrıldı: kontrol grubu (Grup 1), alt ekstremite iskemi reperfüzyon grubu (Grup 2) ve karaciğer iskemi reperfüzyon grubu (Grup 3). Grup 2'de sol alt ekstremiteye bir saat süreyle iskemi uygulandı. Grup 3'te karaciğere bir saat süreyle iskemi ve ardından 24 saat süreyle reperfüzyon uygulandı. Reperfüzyon sonrası karaciğer dokuları çıkarıldı ve gruplar biyokimyasal ve histolojik olarak değerlendirildi.

Bulgular: Karaciğer malondialdehit düzeyleri Grup 2 ve Grup 3'te kontrol grubuna kıyasla anlamlı olarak yüksek idi ($p<0.001$). Grup 2'de malondialdehit düzeyleri Grup 3'e kıyasla anlamlı olarak yüksek idi ($p=0.019$). Karaciğer glutatyon düzeyleri Grup 2 ve Grup 3'te kontrol grubuna kıyasla anlamlı olarak düşük idi ($p<0.001$). Ancak, Grup 2'de glutatyon düzeyleri Grup 3'e kıyasla anlamlı olarak yüksek idi ($p=0.005$). Histolojik değerlendirmede karaciğer hasar skoru Grup 3'te Grup 2'ye kıyasla daha yüksek olmakla birlikte ($p=0.015$), TUNEL(+) hücre sayısı açısından iki grup arasında anlamlı bir fark yoktu ($p>0.05$).

Sonuç: Alt ekstremite iskemi reperfüzyonu sonrası karaciğerde reperfüzyon hasarı, spesifik olarak karaciğerde meydana getirilen iskemi reperfüzyon hasarı kadar önemlidir. Özellikle vasküler travma sonrası yapılan reperfüzyon ameliyatlarında veya alt ekstremite vasküler travma sonrası kanamayı durdurmak için ayak turnikelerinde bu durum göz önünde bulundurulmalıdır.

Anahtar sözcükler: İskemi, reperfüzyon, ekstremite, karaciğer.

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The number of patients exposed to trauma constitute a significant portion of emergency department visits. The severity and region of the trauma may affect vital functions, particularly for those patients with vascular injuries who are exposed to lower limb ischemia due to tourniquet applications before surgery or arterial clamping during surgical intervention. Ischemia-reperfusion (IR) injury, skeletal muscle injury, and associated secondary end-organ injury may occur following the revascularization of an extremity after prolonged tourniquet-induced ischemia.[1] The main mechanism which initiates IR injury is the activation of the inflammatory response. This can lead to a complex cytokine cascade or storm that serves to perpetuate inflammatory reactions in remote organs, which can clinically manifest as multiple organ dysfunction (i.e. acute liver injury and acute lung injury).[1-3]

Reperfusion injury in remote organs induced by lower limb IR has been experimentally demonstrated in several studies.[1-4] However, in most of the studies, reperfusion injury is examined in a specific organ in which IR is applied.[5-7] To illustrate, reperfusion injury in liver tissue can be examined both after lower limb IR and specifically after IR injury in the liver.[1,3,7] However, there is no study investigating whether the liver tissue develops more damage with the lower limb IR or the specific IR of the liver.

It is critical to show that lower limb IR can yield as much liver damage as liver IR. Establishing that not only the relevant limbs, but also other distal organs are affected at the same rate can help in developing new treatment strategies, particularly in emergency services after tourniquet applications to stop bleeding or vascular injuries after reperfusion surgery. Therefore, in the present study, we aimed to compare the effect of lower limb IR on the liver and to examine specific IR of the liver in a rat model.

MATERIALS AND METHODS

Thirty randomly selected male Sprague-Dawley rats weighing 400 and 520 g were used in our study between March 2018 and May 2018. All animals were individually housed in plastic cages and kept on a 12-hour light/dark cycle with unlimited access to food (standard rat food) and fresh water. The study protocol was approved by the Dokuz Eylul University, Faculty of Medicine, Animal Experiments Local Ethics Committee.

Intravenous access (IV) catheters were preoperatively placed in the rats' tail veins. Intramuscular ketamine at a dose of 50 mg/kg (Ketalar®, Pfizer, Inc., Istanbul, Turkey) and intramuscular xylazine (Rompun®, Bayer Healthcare AG, Leverkusen, Germany) at a dose of 5 mg/kg were administered as the anesthesia protocol. To ensure standardization due to subjects undergoing vascular clamping, all groups received IV heparin at a dose of 100 IU/kg. Intramuscular xylazine was administered at a dose of 2.5 mg/kg as an analgesic to all postoperative rats. For prophylaxis, the rats were given antibiotherapy with cefazolin at a dose of 50 mg/kg. To provide better exposure during surgery, the subjects' surgical areas were shaved and disinfected with povidone-iodine. The rats were randomly divided into three groups. After the anesthesia protocol as described above, a midline laparotomy was performed in the first group (Group 1: sham, n=10), and the abdomen was closed without any other procedure to achieve standardization. After 24 hours, the rats were sacrificed with 150 mg/kg of pentothal, and the liver tissues were removed. After the anesthesia protocol, the left femoral regions of the subjects in the second group (Group 2: lower limb IR, n=10) were tourniqueted after heparinization, as described by Gokalp et al.,[2] and allowed one hour of ischemic time. The cessation of the femoral artery flow was confirmed with sonic hand Doppler. To achieve standardization with the other groups, the laparotomy procedure of the abdomen was applied to this group during the lower limb ischemia. However, there was no further intervention in the abdomen, and the subjects were closed after reperfusion of the limbs. To minimize the loss of heat and fluid from the peritoneal cavity, the abdominal incision was temporarily covered with warm, wet gauze during left lower limb ischemia. After the clamp in the femoral artery was removed and the surgical site was closed, the rats in this group were given the same medications for prophylaxis and analgesia as described above. The rats received anesthesia after 24 hours and were sacrificed with 150 mg/kg of pentothal, and the liver tissues were harvested in the same fashion. The subjects in the third group (Group 3: Liver IR, n=10) were carefully dissected after laparotomy to expose the hepatic artery, portal vein, and bile duct, as described in the studies of Taha et al.[7] After standard heparinization, the hepatic artery was obliterated with a vascular clamp for one hour. At the end of the hour, the clamp was removed, and the abdomen was closed. In this group, to minimize the heat and fluid loss, the abdomen was covered with warm, wet gauze during the ischemia. The postoperative antibiotherapy and analgesia protocol was administered in the same fashion as in the other groups. After 24 hours, 150 mg/kg of pentothal was administrated, decapitation

was applied for scarification, and the liver tissues were harvested.

Tissue preparation

At the end of the study, all rats were sacrificed, and their liver tissues were removed. The tissue samples were weighed after cleaning with a physiological saline solution and homogenized with phosphate buffer saline (pH 7.4) using an ultrasonic homogenizer (Bandelin Sonopuls, Germany). The homogenates were stored at -80ºC for the measurement of glutathione peroxidase (GPx) activity and malondialdehyde (MDA) and glutathione (GSH) levels.

Biochemical analysis

The MDA levels were measured by the spectrophotometric (T80, PG instruments, UK) method using the Bioxytech MDA-586 kit (Oxis International, USA). The kit method is based on the reaction of MDA with a chromogenic reagent at 45ºC. The MDA levels were determined from the standard curve by measuring the absorbance at 586 nm. The results were expressed in μM.

The GSH levels were determined using the Bioxytech GSH-420 (Oxis International, USA) kit. The kit method is based on the formation of a chromophoric thione. The oxidized GSH is converted into a reduced form by adding the reducing agent to the supernatant mixed with the buffer. After adding the chromogen, the pH is increased to form a chromophoric thione. The GSH levels were determined by measuring the absorbance at 420 nm. The results were expressed in μM/mg protein.

The GPx activity was determined using the Bioxytech GPx-340 kit (Oxis International, Inc., Portland, OR, USA). The GPx catalyzes the GSH oxidation with tert-butyl hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, oxidized glutathione (GSSG) is converted to a reduced form GSH, while NADPH is oxidized to NADP+. The GPx activities were determined by measuring the decrease in absorbance at 340 nm using a spectrophotometer. The results were expressed in mU/mL.

Histological analysis

The liver tissues were fixed in 10% buffered formalin solution and embedded in paraffin. Serial sections of 5-μm thickness were taken from the paraffin blocks with a microtome (Thermo Finesse M+). The sections were stained with hematoxylin-eosin (H-E) and Masson's trichrome stains for examination under a light microscope. Liver damage was evaluated by vascular congestion, mononuclear cell infiltration, pyknotic nucleus, hemorrhage, and sinusoidal dilatation. The samples were analyzed semi-quantitatively and graded as follows: no damage (0, -), slight damage (1, +), moderate damage (2, ++), and severe damage (3, +++). [8]

To demonstrate apoptotic cells in the liver tissue, the paraffin sections were stained using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method. A TUNEL assay was performed with the In Situ Cell Death Detection Kit (Roche, Germany) according to the manufacturer's instructions. The TUNEL-positive cells were analyzed by an image analysis system (CellSens Entry 1.7, Olympus) consisting of a microscope (Olympus CX-41) and a video camera (Olympus DP25). In each section, apoptotic cells were counted in 10 different areas, and the percentage was determined.[9]

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 22.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were shown in mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) followed by a post-hoc least significant difference test was carried out to analyze significant differences between the groups. A p value of <0.05 was considered statistically significant.

RESULTS

The mean MDA levels of all groups are shown in Figure 1. The MDA values of the hepatic and limb IR groups significantly increased compared to those of the sham group (p<0.001). The MDA levels of the limb IR group significantly increased compared to those of the hepatic IR group (p=0.019).

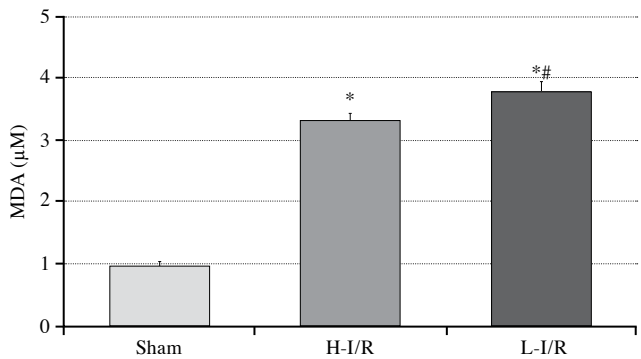

Figure 1. Malondialdehyde levels in rat livers.

MDA: Malondialdehyde; Data are given in mean ± SEM; H-I/R: Hepatic I/R group; L-I/R: Limb I/R group; * p<0.001 compared to sham group; # P<0.05 compared to hepatic IR group.

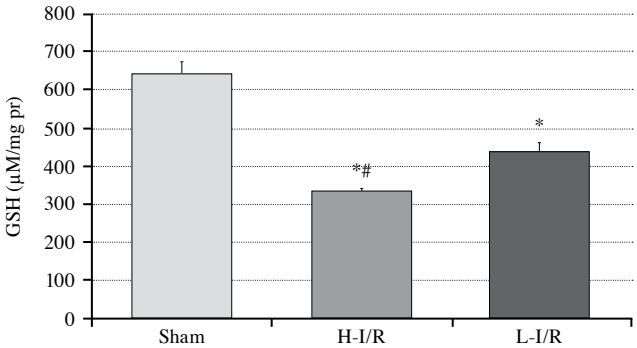

Figure 2. Glutathione levels in rat livers.

GSH: Glutathione; Data are given in mean ± SEM; H-I/R: Hepatic IR group; L-IR: Limb IR group; * P<0.001 compared to sham group. # P<0.05 compared to limb IR group.

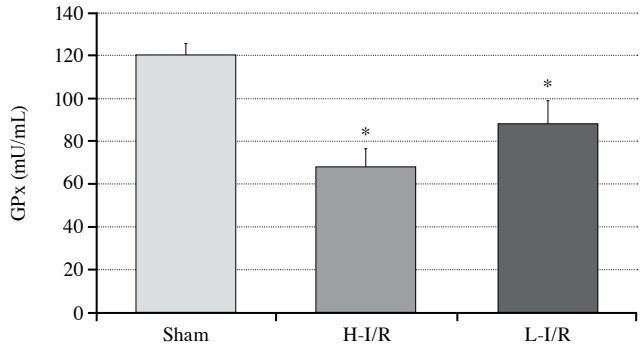

Figure 3. Glutathione peroxidase activities in rat livers.

GPx: Glutathione peroxidase; Data are given in mean ± SEM; Hepatic IR group; L-IR: Limb IR group; * P<0.05 compared to sham group.

The mean GSH levels of all groups are shown in Figure 2. The GSH values of the hepatic and limb IR groups were significantly lower than those of the sham group (p<0.001). The GSH values in the hepatic IR group were significantly lower than those in the limb IR group (p=0.005).

The mean GPx activities of all groups are shown in Figure 3. The GPx levels of the hepatic and limb IR groups significantly decreased compared to those of the sham group (p<0.001 and p=0.017, respectively). There was no significant difference between the hepatic and limb IR groups (p>0.05).

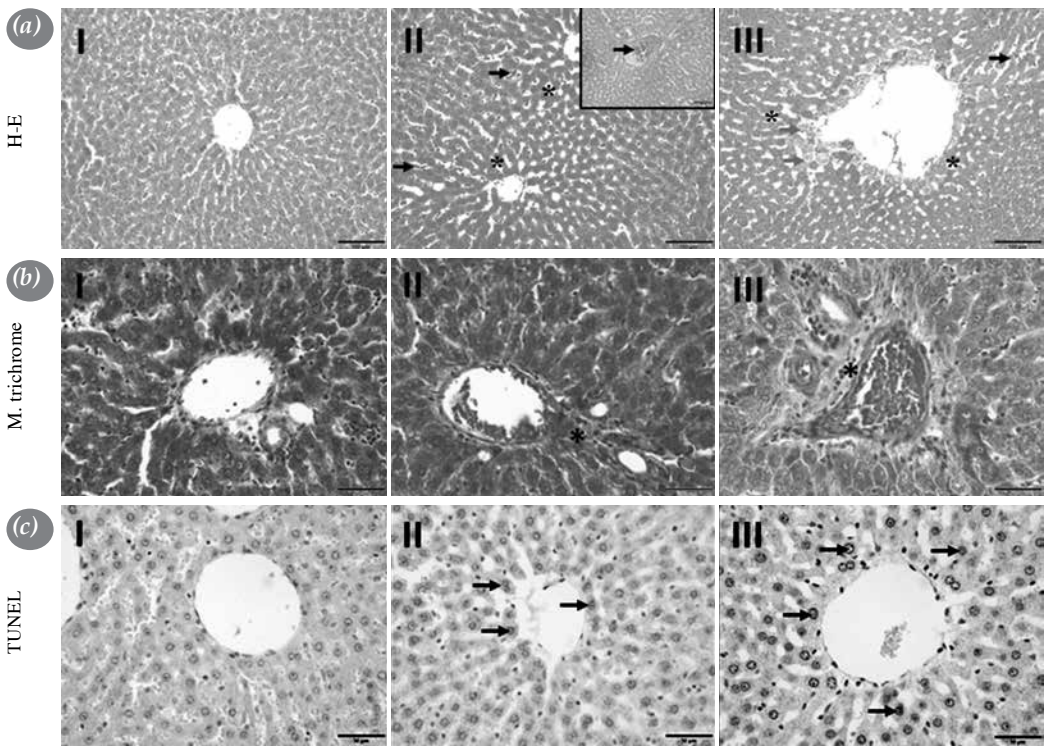

Figure 4. Light microscopic images of rat liver sections.

I; Sham; II; Limb IR; III; Hepatic IR group; **Upper (a)** Hematoxylin-Eosin (H-E) stained sections. The morphology of liver in the sham group was normal. In IR groups, sinusoidal dilatation (stars), sinusoidal and central vein (inset) congestion (black arrows) and necrosis (blue arrows) can be seen. **Middle (b)** Masson's trichrome stained sections. Collagen deposits (stars). **Lower (c)** TUNEL staining. Representative photomicrographs of TUNEL-positive cells (arrows).

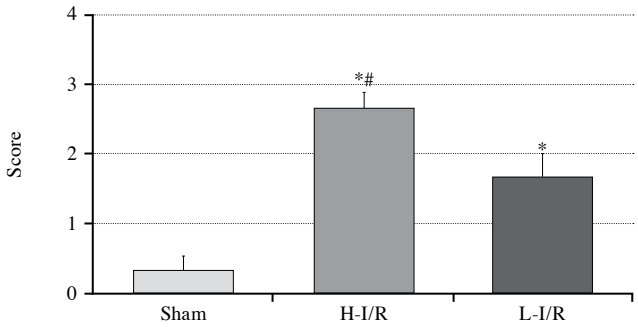

Figure 5. Liver damage score.

Data are given in mean ± SEM; H-IR: Hepatic I/R group; L-IR: Limb IR group; * P<0.05 compared to sham group. # P<0.05 compared to limb IR group.

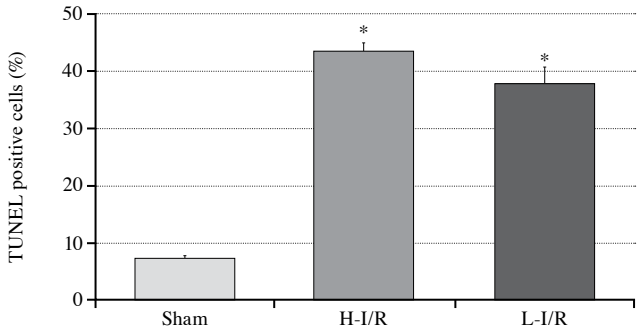

Figure 6. The percentage of TUNEL-positive cells of rat livers.

Data are given in mean ± SEM. H-IR: Hepatic IR group; L-IR: Limb IR group; * P<0.001 compared to sham group.

Histological analysis

The liver images stained with H-E are presented in Figure 4a. While histological appearance was normal in the control group (Figure 4aI), the liver histology was found to be impaired in the IR groups. Dilatation, congestion, and hemorrhagic areas were observed more frequently in the hepatic IR group (Figure 4-aIII). In the sections stained with the Masson's trichrome, there was no interstitial fibrosis in the sham group (Figure 4-bI), whereas there were increased collagen deposits around the central vein and portal areas in the IR groups (Figure 4-bII, III). The liver damage score was significantly higher in the hepatic and limb IR groups than in the sham group (Figure 5, $p<0.001$ and $p=0.002$, respectively). In the hepatic IR group, the damage score was significantly higher than in the limb IR groups ($p=0.015$).

The TUNEL-stained liver images are presented in Figure 4c. In the IR groups, the number of TUNEL-positive cells was significantly higher than in the sham group (Figure 4cII, III). The TUNEL-positive cell percentage was significantly higher in the hepatic and limb IR groups than in the sham group ($p<0.001$, Figure 6). There was no significant difference between the hepatic and limb IR groups ($p>0.05$).

DISCUSSION

In this experimental study, we compared the effect of lower limb IR on the liver and specific IR of the liver in rats and found that there was reperfusion injury in the liver tissue after IR of the lower limb as much as the specific IR formed in the liver.

One of the standard hemostatic procedures applied to patients who are exposed to vascular injuries is the application of a tourniquet to the injury area or a more proximal area in emergency departments.[10-12] Although there are debates on its effectiveness, this practice is still actively used in both military and civilian medicine.[10-12] The most common complications of this application are muscle cell necrosis, myoglobinuria, renal failure, and even death due to limb ischemia, particularly in prolonged tourniquet applications.[13,14] Definitely, this phenomenon, which may also affect the distant organs, is another problem that can arise from reperfusion injury, which may be a result of prolonged tourniquet applications. The underlying mechanism of IR injury is a very complex situation involving many different cell types and various factors. The severity of reperfusion injury varies according to the affected organ and the duration of the ischemia. Indeed, reperfusion of acutely ischemic tissues induces the release of very powerful oxygen free radicals and cytokines, which stimulate a natural immune response with subsequent leukocyte recruitment, endothelial dysfunction, and tissue damage.[1,15] Although reperfusion injury in the lower limb after IR is an expected condition, the more complicated and remarkable finding is reperfusion injury in other distant organs after the lower limb IR. Therefore, in this study, we attempted to understand which is remarkable: is IR damage in the liver more severe when the liver is a remote organ after lower limb IR or when the liver is specifically damaged with IR? First, we observed that there was a reperfusion injury in the liver compared to the sham group in both models (lower limb IR and specific liver IR). Specifically, post-IR injury in the liver has already been demonstrated in many studies.[16-18] This damage is mainly due to the rapid release of reactive oxygen species (ROS) and the overproduction of many inflammatory cytokines.[18] Increased ROS, activates Kupffer cells, resulting in more ROS and cytokine production. Meanwhile,

nitric oxide levels are reduced, and an imbalance arises between endothelin-1 and nitric oxide synthase, affecting the production of nitric oxide, which leads to vasoconstriction of the sinusoids.[18,19] Vasoconstriction of the sinusoids causes compression of platelets and neutrophils. In a different pathway, hepatocellular necrosis and apoptosis increase hepatocyte injury.[18,20] The controversial issue herein is the liver damage that occurs after lower limb IR. In one of the few studies on this topic, Mansour et al.[3] reported that reperfusion injury developed in the lungs after upper limb IR, although there was no change in the liver or kidney.[3] However, other studies have shown reperfusion injury in the liver after lower limb IR.[2,4,21-24] While trying to understand the cause of this damage, many mechanisms have been mentioned, particularly, severe inflammatory response. It has been shown in some studies that leukocyte recruitment is responsible for liver damage after lower limb IR.[21,23] The microvascular location of such recruitment may be different between organs. Inflammation within the skeletal muscle and mesentery results in leukocyte-endothelial cell interactions within the postcapillary veins. Leukocyte accumulation within the liver occurs not only in the collecting venules (post-sinusoidal venules), but also in the hepatic sinusoids in inflammation and infection models.[21] However, the basic mechanism often suggested is similar to the mechanism of reperfusion injury, which occurs specifically after IR in the liver. In other words, the cause of reperfusion injury is the ROS that emerge after IR and the lipid peroxidation products triggered by these radicals. As a result, all these mechanisms are part of a severe inflammatory process, which is also responsible for organ damage.

As seen, whether after lower limb ischemia or after specific liver ischemia, similar mechanisms induce reperfusion damage in the liver. In our study, we examined which of these two techniques resulted in more liver damage. As a result of our study, the blood MDA levels were found to be statistically higher in the lower limb IR group, compared to the liver IR group. In addition, the GSH levels were higher in the lower limb IR group than in the liver IR group.

In other words, after lower limb IR, the liver had higher biochemical reperfusion injury markers, but a higher antioxidant activity was also observed in this group. In the histopathological evaluation, the liver damage scores were higher in the liver IR group compared to the lower limb IR group, although no statistically significant difference was found between the two groups in terms of the TUNEL(+) cell count. Thus, similar reperfusion injury occurred in the liver with both methods.

The first limitation of this study is its small sample size. The second one is that the methodology was solely constructed on histological materials. In addition, we are unable to naturally demonstrate the clinical results of the study, as the study was experimentally designed.

In conclusion, in the lower limb tourniquet or reperfusion surgery frequently performed on vascular trauma patients admitted to the emergency departments, reperfusion injury of the lower limb is usually the main focus; however, reperfusion injury of the distant organs is often neglected. Our study shows that lower limb IR affects the liver as much as reperfusion injury in the related limb. Distal organ damage should never be ignored. However, there is a need for a prospective, controlled clinical studies in which biochemical inflammatory processes are studied.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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