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In which period of skeletal muscle ischemia-reperfusion injury is local hypothermia more effective?

İskelet kası iskemi-reperfüzyon hasarının hangi evresinde lokal soğuk uygulaması daha etkilidir?

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Amaç: Sıçan modelinde lokal hipotermi uygulamasının iskemi-reperfüzyon hasarının farklı evrelerindeki etkileri incelendi.

Çalışma planı: Grasilis kası iskemi-reperfüzyon modeli için, her birinde altı sıçan olan dört grup oluşturuldu. Femoral arterin altı saat süreyle klemplenmesiyle iskemik hasar oluşturulan kaslara sadece iskemik dönemde (İH), sadece dört saatlik reperfüzyon döneminde (RH) veya iskemi ve reperfüzyon dönemlerinde (İRH) 10 °C'de lokal hipotermi uygulandı. Kontrol grubuna hipotermi uygulanmadı. Yirmi dört saatlik perfüzyon dönemi sonunda yaşamları sonlandırılan deneklerin grasilis kasları çıkarıldı ve örneklerde kas ödemi (yaş/kuru ağırlık oranı), polimorfonükleer lökosit (PMNL) sayımı ve nekroz yüzdesi incelendi.

Sonuçlar: Kontrol grubuyla karşılaştırıldığında (193.7 ±38.9), PMNL sayıları İH grubunda (111.5±36.2; p<0.004), RH grubunda (82±18.6; p<0.002) ve İRH grubunda (54.5±21.8; p<0.002) anlamlı derecede düşük bulundu. Hipotermi grupları arasında PMNL açısından anlamlı tek fark İRH ve İH grupları arasında idi (p<0.004). Doku nekroz oranları, kontrol grubuyla (%22.5±5.2) karşılaştırıldığında, hipotermi gruplarının hepsinde anlamlı derecede düşük bulundu (İH: %11.7±5.2, p<0.004; RH: %10.8±3.8, p<0.004; İRH: %6.7±2.6, p<0.002). Kas ödemi, kontrol grubuyla (2.89±0.46) karşılaştırıldığında, hipotermi gruplarında anlamlı derecede düşük düzeylerdeydi (İH: 2.01±0.26, p<0.001; RH: 1.98±0.34, p<0.001; İRH: 1.97±043, p<0.001). Nekroz oranları ve kas ödemi açısından hipotermi grupları arasında anlamlı fark yoktu.

Çıkarımlar: Bulgularımız, iskemi ve reperfüzyon süreçlerinde uygulanan lokal hipoterminin iskemi-reperfüzyon hasarını azaltmada belirgin yararı olduğunu göstermiştir.

Anahtar sözcükler: Hipotermi oluşturma; iskemi; kas, iskelet/kan desteği; sıçan; reperfüzyon hasarı/önleme ve kontrol.

Objectives: We investigated the potential beneficial effects of local hypothermia applied during different periods of ischemia-reperfusion injury in a rat model.

Methods: An isolated gracilis muscle model of ischemia-reperfusion injury was used consisting of four groups, each with six rats. Ischemic injury was induced by clamping the femoral artery for six hours. Local hypothermia at 10 °C was applied during only ischemia (IH), during reperfusion (RH) for four hours, and during both ischemia and reperfusion (IRH). The control group remained untreated. After 24 hours of perfusion, the rats were sacrificed and the gracilis muscles were removed to determine muscle edema (wet-to-dry weight ratio), polymorphonuclear leukocytes (PMNL), and the percentage of necrosis.

Results: Compared to the control group (193.7 ±38.9), the PMNL count was significantly lower in the IH, RH, and IRH groups (111.5±36.2, p<0.004; 82±18.6, p<0.002; 54.5±21.8, p<0.002, respectively). The only significant difference in the PMNL count was between the IH and IRH groups (p<0.004). In all the hypothermia groups, the percentage of necrosis was found significantly less than that of the control group (22.5±5.2% *vs* 11.7±5.2% in IH, p<0.004; 10.8±3.8% in RH, p<0.004; 6.7±2.6% in IRH, p<0.002).Similarly, muscle edema was significantly decreased in the study groups (control, 2.89±0.46 *vs* 2.01±0.26 in IH, p<0.001; 1.98±0.34 in RH, p<0.001; 1.97±043 in IRH, p<0.001). There were no significant differences between the three hypothermia groups with respect to the percentage of necrosis and muscle edema.

Conclusion: Our results show that local hypothermia applied during ischemic and reperfusion periods is significantly effective in reducing ischemia-reperfusion injury.

Key words: Hypothermia, induced; ischemia; muscle, skeletal/blood supply; rats; reperfusion injury/prevention & control.

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Even though there are numerous studies conducted against the mortality and major morbidity of the acute-ischemia of the lower or upper extremity developed from vascular trauma or arterial obstruction, these issues are still facts of the life. When revascularization is realized after high levels of ischemia is detected, high levels of mortality and amputation, %48 and % 52 respectively, is expected.[1] It is a known fact that, the post reperfusion edema, which occurs as a result of late-revascularization, may cause a compartment syndrome, a reperfusion trauma and as a result a lost extremity. Pathophysiology of the vascular ischemia-reperfusion trauma is not yet identified in detail and still disputable. Even knowing the problem, there still are difficulties in clinical applicability of the researches that are conducted for the prevention of reperfusion traumas.

The effects of local hypothermia application in order to protect ischemia extremity, Euro-Collins solution, Wisconsin University solution, independent radical scavengers and various medical agents are identified in the literature.[2-6] This methods can be divided into two main groups as simple-protection with or without the application of hypothermia and continuous perfusion. Application of hypothermia and simple-protection in ischemic period is practical and has achieved success in numerous clinical cases. [7] However, evaluation of the vitality of muscles is yet difficult. Allen [8] has proved on an experimentally modeled ischemic extremity that hypothermia is reducing the mortality and extremity lose rates. Acute extremity ischemia can be tolerated safely with short-term freeze or optimal temperature reductions. Duncan [9] has approved this result with the comparison of hypothermic protection and normothermic protection of muscle edema in crush injury of extremity model.

Hamel and Moe [10] observed less loose of extremity and edema in extremities that they have applied hypothermia. They were comparing 18 degree local hypothermia and normothermic tourniquet ischemia in ischemia model that had created by 5 hours of tourniquet applied to the backside extremities of a dog. Brunelli [11], meanwhile, reported successful replantation of wholly functional extremities after 24 hours of hypothermic ischemia. In a recent research, Akahene et al. [12] have investigated the efficiency of simple hypothermic protection of maximum 6 hours. The research has also been covering the determination of the gene expression level of ischemic muscles. The research showed that, in clinical extremity resuscitation, hypothermic environment is efficient till six hours in the protection of extremity that suffers from vascular injury. Numerous studies in the literature evaluated the effect of hypothermia before the reperfusion. Most of the researchers reported local inflammatory response frequency and tissue edema reduction in hypothermia application during the ischemia.[13-21] Application of hypothermia during the reperfusion phase in ischemia-reperfusion injuries is still disputable. There is a common belief that application of hypothermia during the reperfusion in injured extremities could be resulted in a vasoconstriction. On the other hand, most of the experiments on animals showed positive effects of hypothermia on cerebrovascular obstruction or cardiac bypass models during the reperfusion.[20-26] It is stated that light hypothermia between 28-35 degrees during the early reperfusion, is resulted in cellular neuroprotective effects.[22-28] In today, unification of the damage, major vascular injury and extremity contusion, is considerably common in extremity traumas. Vascular injuries, accompanied with broken bones can cause late vascular rehabilitation and, compartment syndrome as a result. In this study, we aimed to compare the potential benefits of local hypothermia in different process of ischemia and early reperfusion. Its main target is to light the way for clinical studies dedicated to prevent fatal results of reperfusion injuries.

Appliance and method

Subject choice and surgical approach

We have used 24 Wistar rats which are weighted between 300 and 370 grams. Intravascular ketamine (50 mg/ml[Ketalar]) 50 mg/kg and 4 mg/kg xylazyne and anesthesia were applied to the animals. Anesthesia continued by an additional half dose in every 90 minutes.

Clamping the femoral-arter

The femoral arter was carefully dissected on the distaline of branches of epigastric arter under the inguinal ligament. Inguinal ligament were hooked with caudal hook. Femoral arter were clamped with microclemp under the inguinal ligament. (Roboz,Washington DC). It is observed that superficial and deep femoral veins were fully blocked during the clamping process.

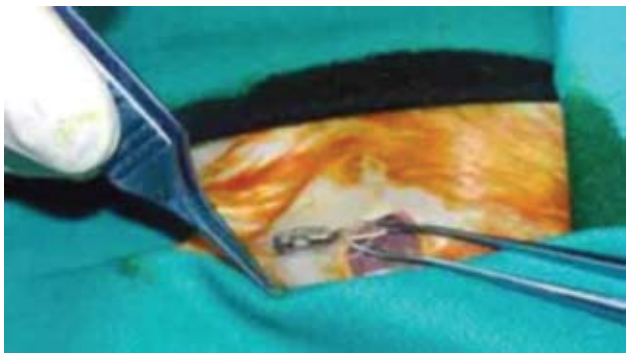

Figure 1. Clamping of femoral arter under the inguinal ligament (Roboz,Washington DC)

Hypothermia procedure

The cooling system that had been introduced by Mowlavi et al.[29] to the literature were applied. Shortly; the gracillis muscle is coiled with a glass tube which is 6 mm in diameter. In this way, an hypothermia of 10° C was provided. The cold touch was provided by the continuous irrigation of the water (10° C cold) in the glass tube, pumped with a thermoplastic pomp. Room temperature was fixed in 25° C and the body temperature of rats was monitored by rectal probe. The temperature of Gracillis muscle was checked with a pinpointed thermometer. (picture 2a-b)

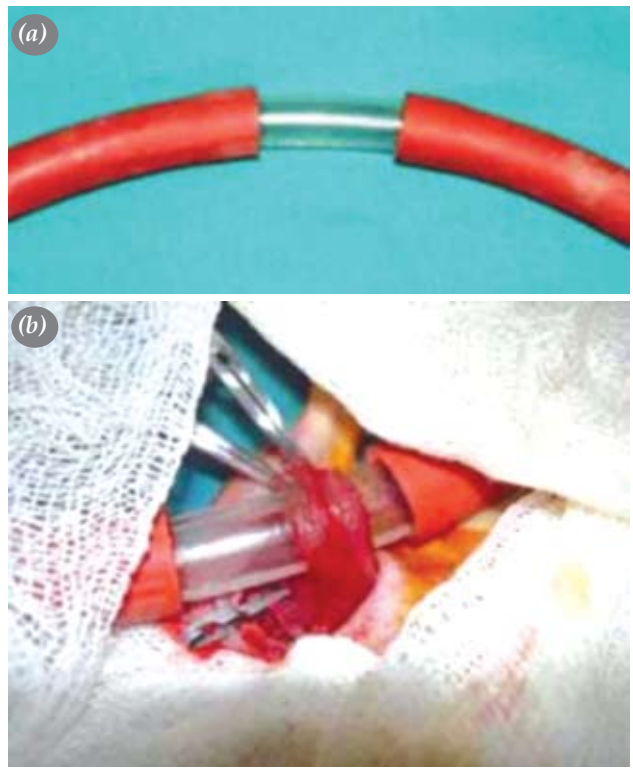

Figure 2. (a) Cooling Process; Cooling via continuously washing with 10° C cold water. Pumping it with thermoplastic pomp thorough the glass tube. **(b)** The position of glass tube surrounded with Gracillis muscle.

Experimental model

The subjects were divided into four experiment groups according to hypothermia applied during the ischemia and reperfusion periods. (Table 1).

Anticipating that the ischemic muscles will be cooled down to the room temperature, the room temperature were fixed at 25°C and the general body temperature of rats (37°C) were observed in order to prevent any possible systemic hypothermia. Micro vascular clamps were opened and following to 6 hours of ischemia, adequate blood flow in both surface and deep femoral arters was inspected in enlarged scales. Gracillis muscle phelebs were sutured with 0-5 Ethibond after a reperfusion period of 4 hours. In this process, paid attention not to cause more damage to the muscle. Incisions occluded with 4-0 Prolene. For the rest of the perfusion period, animals were let to awake. In order to evaluate muscle inflammation/necrosis, muscle phelebs were inspected under microscope after 24 hours of perfusion followed by a four hours of reperfusion.

Interstitial tissue edema, inflammation type and intensity, localization of inflammation, PMNL type and quantity were evaluated. After the completion of ischemia and reperfusion applications, all muscles were fixed anatomically and perfuse in body temperature (37°C). After 24 hours of perfusion,

Table 1. Detailed distribution of control and experiment groups according to hypothermia application periods.

	Control group (°C) (n=6)	Ischemia-Hypothermia group (°C) (n=6)	Reperfusion-Hypothermia group (°C) (n=6)	Ischemia-Reperfusion-Hypothermia Group (°C) (n=6)
6 hours Ischemia	25	10	25	10
4 hours reperfusion	37	37	10	10
24 hours perfusion	37	37	37	37

the experiment was ended and, anesthesia with pentobarbital (42mg/intraperitoneal) was applied to the subjects and euthanasia applied with 1-ml intracardiac lidocaine injection. Tissues sampled from bilateral Gracilis muscles collected for the evaluation. In each group, non-ischemic contra lateral muscles token as an internal reference for microscopic investigation.

Histopathologic inspection

Tissues were detected in 10% formaldehyde solution, installed in paraffin, 6 cross sections were token and painted with hematoxylin-eosin. These cross sections were inspected blindly by two pathologists under the light-microscope. The pathologic findings inspected, evaluated within the perspective of muscular necrosis and neutrophil infiltration. Necrosis of the muscular tissue were evaluated within the perspective of; the hyalinosis, striation loss, granular appearance on sarcolemma and inflammatory cells infiltrated with the fragmentation of muscle fibers. The degree of histological muscle necrosis evaluated with regard to percent level indicating the histological damage level of cross sections. The infiltrated neutrophil quantity calculated as the total number of neutrophils in 3 areas in x400 size.

Muscle edema

At the end of the reperfusion period, gracilis muscles were divided into two equal pieces for muscle edema and histopathological inspection. For muscle weight measurement, the wet weight of half of a muscle were measured (Shimatzu® Magnet Balance), later, the muscle was re-measured after 36 hours of dehydration in drying oven at 28C (dry weight). The proportion of wet weight to dry weight calculated for measurement of muscle edema.

Statistical analysis

Findings analyzed with SPSS program and average values indicated as “average standard drift”. For comparing differences between groups, Kruskal-Wallis Variance Analysis, Ki-kare Test and Mann-Whitney U test were used.

Results

PMNL infiltration

Comparing with the normothermic control group(193.7 ±38.9), the MNL infiltration in study group (IH group111.5±36.2, p <0.004, RH group 82±18.6, p< 0.002, IRH group 54.5±21.8, p<0.002) was observed as meaningfully low. The comparison between study groups indicated that, PMNL infiltration in IRH group is meaningfully low regarding to IH group (Comparison between IRH and IH p<0.004). Another noteworthy result is that in terms of PMNL infiltration, there is no important difference between the application of hypothermia in ischemic period and the application of hypothermia in pre-reperfusion period. (p=0.093). Figure 3 indicates the PMNL infiltration distribution of the groups.

It is pathologically indicated that the PMNL infiltration localization is limited in the perifacial area in local hypothermia applied groups while in control groups PMNL infiltration reached as far as the endomysium.

Tissue necrosis

Comparing with the nomothermic groups, the tissue necrosis proportions in all study groups were regarded meaningfully low. (IH group 11.7±5.2, p <0.004, RH group10.8±3.8, p< 0.004, IRH group 6.7±2.6, p<0.002). However, no differences observed between study groups in terms of necrosis proportions. (p=0.093 IH group and IRH group, p=0.937 RH group and IH group, p=0.093 RH group and IRH group).

Muscle edema

Comparing with normothermic control groups, an important decrease in the muscle edema is observed. (IH group 1.98±0.34, p <0.001, RH group 1.99±0.34, p< 0.001, IRH group 1.97±043, p<0.001). However, no differences between study groups in terms of wet and dry weight proportions.(p=0.192 IH group and IRH group, p=0.337 RH group and IH group , p=0.223 RH group and IRH group).

Discussion

In this study, the effect of local hypothermia applied in ischemia and reperfusion periods have been inspected by observing basic inflammatory reactions with pathological methods in an model simulating the ischemia in an extremity with damaged major arterials. The first result obtained from this study is that the local cooling application is remarkably effective in preventing ischemia reperfusion traumas. Another important result of the study is; taking basic parameters like muscle edema and necrosis into consideration, it is observed that cooling application in different phases has similar positive effects. Similarly, regarding the PMNL infiltration in control group, a remarkable dec-

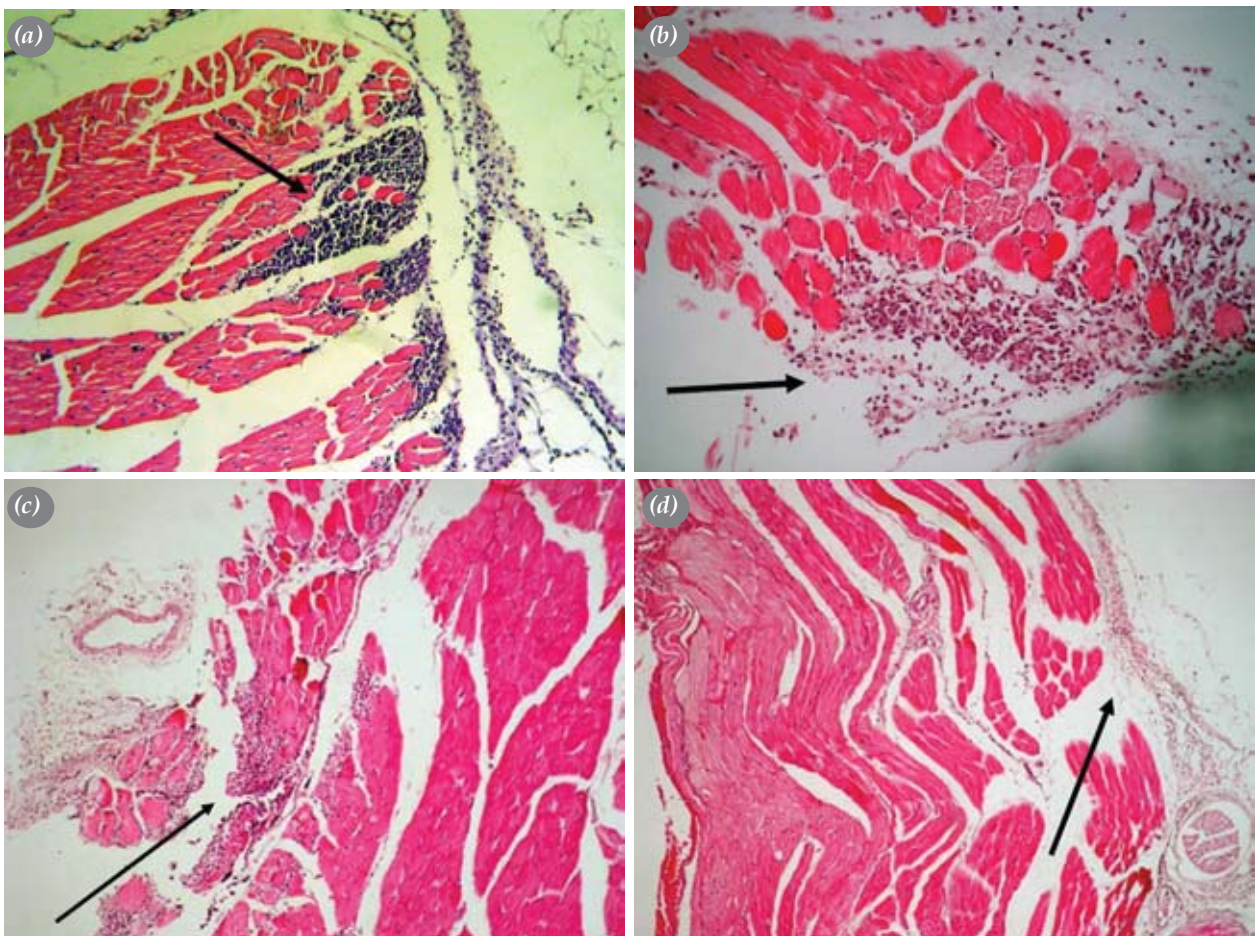

Figure 3.(a) The massive infiltration of PMNL in control group towards perimysium and endomysium. **(b)** Moderately infiltration of PMNL in IH group into the perimysium and endomysium. **(c)** Slight PMNL infiltration in RH group. Limited in Perifacial area of muscle. , **(d)** PMNL infiltration. In IRH group, the infiltration is slight and limited in perifaical area.

rease in PMNL infiltration in all study groups has observed. Comparing study groups between themselves, similar PMNL infiltration between group RH and two other groups was observed while a major difference between the group IH and IRH (in favor of the group IRH) was observed.

Reperfusion damage was known as "Declamping Shock" in the 60s. Reperfusion damage was clinically determined in the post-replacement aortic valve myocardial tissue inspected by Cerra et al. in a necropsy study at 1975.[30] Today, whether the cornerstones of the reperfusion damage have been identified by various clinical and experimental studies, there still is a need for studies for preventing the damage.

Reperfusion damage is a complex inflammatory reaction. If restoration of circulation is realized after the cell damage phase, it will cause leukocyte endothelial reactions and immune complex activations. Moreover, reperfusion damage especially release free oxygen radicals which cause lipid peroxydation in endothell cells. In next phase, these free radicals trigger vasoconstriction and aggregation by affecting leukocytes and trombocytes. As a result, inflammation increases the cell damage and may cause loss of extremity after the compartement syndrome. [31]

In order to protect the devascularised tissue and to reduce the ischemic damage, Hypothermia, hyperbaric oxygen, free radical cleansers, Euro-Collin solution, Wisconsin University Solution, perfolorocarbons (perflorochemicals) are used [3-6]. Hypothermia application helps the protection of muscular viability by reducing the leukocyte infiltration (accordingly reducing the appearance of free oxygen radicals) towards the tissues during and after the ischemia.[29, 32,33] Despite the fact that the hypothermia is the mostly studied method in

the field, its application timing is still disputable.[11,29] The effects of hypothermia during the ischemia is known, however, its effects in the post-ischemia period has not discussed much.

Wright et al. worked on local effects of hypothermia on post-ischemic reperfusion of dog gracilis models in vivo conditions.[34-35] They had tried to show that the post ischemic hypothermia increase the interstitial muscle PH and comparing with perfuse muscles in body temperature, it can reduce the post-reperfusion edema without reducing the vascular permeability of albumin. In the same study, it is determined that the oxygen consumption, muscle destruction and infarct area are decreased. Moreover, in spite of the common opinion, they have tried to reveal the protective effect of vasoconstriction derived from hypothermia. The weakness of this study was that it cannot conclude clear results on positive effects of local hypothermic reperfusions during ischemia and reperfusion periods.

In a recent study, Movlavi et al.[22] inspected the benefits of isolated local hypothermia against muscle viability, muscle edema, neutrophil integration and infiltration and neutrophil oxidation in ischemia and reperfusion damages on rat skeleton-muscle phleb. As the result of their study, they have reported that local hypothermia applied in early reperfusion averts CD 11b expression and suppresses neutrophil oxidative potential and neutrophil infiltration and so protect the skeletal muscle from damage.

In our study, we have preferred six hours of ischemic process because ischemia-related structural changes happens in the first four hours period and irreversible muscle necrosis as a part of which histological changes like disappearance of glycogen granules, irregularity in z band, mitochondrial distention and disorganization of cristas, distention of sarcoplasmic reticulum and destruction of sarcolemma membrane happen in 6-8 hours period.[36-38] Sapega et al. suggested that local temperature should be 10 Co during the ischemia in order to protect the muscle tissue through hypothermia. At this temperature, local tissue PH was at the most stable state and ATP and phosphocreatine level were measured at the highest level.[32] If the local temperature were further reduced down to -5 Co, a paradoxical answer would had been occur: an increased lactate level and decreased ATP and phosphocreatine level. [22,32,39] For all these reasons, we applied 10 Co hypothermia in our study. According to study of Busto et al. [40] , local hypothermia should be provided in the first 3 hours period of reperfusion. They have indicated that 2 hours of hypothermia has positive effects while more than three hours of hypothermia has no beneficial effects on reperfusion damages.

In our study, we have observed that, in terms of reducing the PMNL infiltration, application of hypothermia during both ischemia and resuscitation periods are more effective than its application only in the ischemia period. In this perspective, no major differences between IRH group and RH group seems to be a noteworthy result. As seen from this aspect, application of hypothermia in the early reperfusion period, even if no hypothermia is applied during the ischemia period, would become a partial make up for a lost chance.

In this study, existence of muscle edema and evaluation of its intensity was important because clinical reflections of interstitial tissue edema are muscle necrosis and compartment syndrome. PMNL infiltration in inflamed muscle tissue causes interstitial tissue edema with various mechanisms. Hypoxic tissue excretes chemical mediators for lipoxygenase and cyclooxygenase tracts and leukocytes. Arachidonic acid derives, especially leukotrienes, increase the adhesion of leukocytes (which are characteristics of reperfusion damage) to vascular endothelium and permeability of post capillary venules.[32,41,42] Mowlavi et al.[22] and Cornejo et al. [43] reported that the protective effect of hypothermia during the early reperfusion occurs as a repression on selective neutrophil suppression related to the edema formation. However, in our study, we have observed that local hypothermia has positive effects at same level on muscle edema and necrosis of all three study group. According to this result, we have proved that the application of local hypothermia in early reperfusion period does not lead an additional vasoconstriction and so increase the intensity of edema and necrosis, but in contrast, it has a protective effect.

In clinical practices, surgeons encounter with extremities subjected to ischemia but not with well-protected tissues. In some cases the loss of extremity is inevitable. Destructive results of a late revascularization after a major arterial injury on an extremity are all well-known and for this reason practical and simple protection measurements are important in order to protect the ischemic tissue. Although there are numerous methods identified, hypothermia application is the most practical and most confidential known method.

The results of our study reveal that hypothermia applied in the early reperfusion period and local hypothermia applied in both ischemia and early reperfusion periods equally protect the extremity from reperfusion injuries. In the light of these findings, development of practical methods that will adept basic hypothermia techniques to clinical applications is a necessity.

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