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The Effect of "Carvacrol" on Ischemia-Reperfusion Injury in the Skeletal Muscles of Rats

Ratlarda "Karvakrol"ün İskelet Kasında İskemi-Reperfüzyon Hasarı Üzerine Etkisi

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ABSTRACT

Objective: Ischemia is characterized by an inadequate supply of nutrients and oxygen due to reduced blood circulation to specific tissues or organs. Reperfusion injury refers to the detrimental effects of abrupt exposure of the ischemic tissue to elevated oxygen levels and subsequent generation of reactive oxygen derivatives inside the tissue. In surgical procedures characterized by frequent occurrences of ischemia-reperfusion (I/R), such as transplant and cardiovascular surgery, it is crucial to prioritize the preservation of tissue integrity and mitigation of associated damage. In this study, we formulated a hypothesis suggesting that carvacrol (Car), an aromatic hydrocarbon present in some plant species, might decrease I/R injury by implication of its antioxidant, anti-apoptotic, and anti-inflammatory functions.

Methods: We planned our study with 18 rats, randomly divided into three groups: control group, I/R group, and I/R + Car group. In the control group, laparotomy was performed only, and blood and lower limb muscle tissue samples were taken. In the I/R group, after laparotomy, lower extremity ischemia is achieved for 60 min, and reperfusion is achieved for another 60 min. In I/R + Car, rats were administered 100 mg/kg Car via intraperitoneal injection 30 min after ischemia. Then, 60 min of ischemia and 60 min of reperfusion were achieved. Lower limb muscle samples were examined both histologically and biochemically. We evaluated the levels of malondialdehyde (MDA), glutathione-S transferase (GST), and catalase (CAT). In addition, serum ischemia-modified albumin (IMA) levels were measured.

Results: Our study showed that the findings of I/R injury decreased prominently in the I/R + Car group's samples. GST, MDA, CAT, and IMA levels were significantly higher in the I/R group than in the control

ÖZ

Amaç: İskemi, herhangi bir doku veya organa giden kan akımının azalması veya kesilmesi sonucunda ortaya çıkan besin ve oksijen yetersizliği durumudur. Reperfüzyon hasarı ise iskemiye uğramış bir yapının ani olarak yüksek oksijene maruz kalması ve ortaya çıkan reaktif oksijen türevlerinin dokuda oluşturduğu hasara verilen isimdir. Özellikle transplant cerrahisi, kalp ve damar cerrahisi gibi iskemi-reperfüzyon (İ/R) olaylarının sık görüldüğü cerrahilerde, dokuyu İ/R'den mümkün mertebe korumak, oluşan hasarı en aza indirmek gibi konular önem kazanmaktadır. Çalışmamızda bazı bitkilerde bulunan bir aromatik hidrokarbon olan karvakrol (Car) molekülünün, antioksidan, anti-apoptotik ve anti-enflamatuvar etkileri sayesinde İ/R hasarını önleyebileceği hipotezini kurguladık.

Yöntemler: 200-250 gram ağırlıktaki 18 adet Wistar albino rat rastgele olarak 3 eşit gruba ayrılıp, bu gruplardan birincisi kontrol, ikincisi İ/R, üçüncüsü İ/R + Car grubu olarak belirlendi. Kontrol grubunda sadece laparotomi yapıldı, kan ve alt ekstremite doku örnekleri alındı. İ/R grubunda laparotomi sonrası 60 dakika süreyle alt ekstremite iskemisi yaratıldı ve sonraki 60 dakika boyunca reperfüzyon sağlandı. İ/R + Car grubunda ise 100 mg/kg Car, iskemi yaratılmasından 30 dakika önce intraperitoneal olarak uygulandı. Akabinde 60 dakika iskemi ve 60 dakika reperfüzyon oluşturuldu. Ratların alt ekstremite kas dokusu örneklerinde malondialdehid (MDA), glutatyon S-tranferaz (GST) ve katalaz (CAT) düzeyleri ölçülüp, histopatolojik inceleme yapıldı. Kan örneklerinde ise iskemi modifiye albümin (İMA) düzeyleri ölçüldü.

Bulgular: İ/R + Car grubunun örneklerinde, İ/R hasarını gösteren bulguların istatistiksel olarak anlamlı şekilde azaldığı gösterilmiştir. GST, MDA, CAT ve İMA düzeylerinin; İ/R grubunda, kontrol ve İ/R +

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ABSTRACT

and I/R + Car groups. (p=0.024, p=0.010, p=0.030 and p<0.0001; respectively). Histopathological examination revealed no degeneration in the control group. Conversely, contraction, hypertrophy, nucleus degeneration, necrotic fibers, and hyalinization were observed in the I/R group. In the I/R + Car group, inflamed areas were less frequent than in the I/R group, and vascular dilatation of myofibre was noted.

Conclusion: This study demonstrates that the Car molecule protects against I/R injury. Therefore, we contend that more experimental cohort investigations are needed to examine the impact of the Car molecule on preventing I/R injury.

Keywords: Lower extremity, ischemia-reperfusion, carvacrol, malondialdehyde, catalase

ÖZ

Car gruplarına göre anlamlı düzeyde yüksek olduğu görüldü (sırasıyla; p=0,024, p=0,010, p=0,030 ve p<0,0001). Histopatolojik incelemede, kontrol grubunda yer alan kas liflerinde herhangi bir dejenerasyona rastlanmazken, İ/R grubunda miyofibrillerde kontraksiyon, bazı liflerde hipertrofi, nükleuslarda dejenerasyon, nekrotik miyofibriller, hyalinizasyon gibi çizgili kas hasarına dair bulgular izlenmiştir. İ/R + Car grubunda ise bu hasarlı alanların İ/R grubuna göre kısmen daha az olduğu ve miyofibrillerin içerisinde yer alan vasküler yapılarda dilatasyon olduğu gözlenmiştir.

Sonuç: Yaptığımız çalışmada Car molekülünün İ/R hasarını önlemeye yardımcı bir madde olduğu deneysel ve istatistiksel olarak ortaya konulmuştur. Karvakrole dair çeşitli organ ve dokular üzerinde yapılan bazı diğer çalışmalar incelendiğinde benzer sonuçlar alındığını görmekteyiz. Bu nedenle daha geniş deney gruplarında ve daha ileri düzey çalışmalar ile karvakrol molekülünün İ/R hasarını önlemeye yönelik etkisinin araştırılması gerektiğini düşünmekteyiz.

Anahtar Sözcükler: Alt ekstremite, iskemi-reperfüzyon, karvakrol, malondialdehid, katalaz

INTRODUCTION

Ischemia is the insufficiency of oxygen and nutrients in tissues caused by the cessation of blood flow. This condition may develop acutely because of several factors, including vasospasm, embolism, trauma, or chronic conditions such as atherosclerosis, tumor compression, and intimal hyperplasia. Ischemia leads to reduced oxidative metabolism processes in the affected cells. At the same time, alterations in mitochondria and cell membranes result in reversible or permanent cell damage. Furthermore, this condition evokes apoptosis in adjacent cells, leading to significant destruction at both organ and tissue levels (1).

Following the removal of ischemia, the reperfusion phase begins. Reperfusion is the whole of the cellular responses to the sudden increase in oxygen and nutrients that occurs when tissue is restricted from oxygen and nutrients and reaches blood support again.

Developing ischemia-reperfusion (I/R) injury to the skeletal muscle is a possible medical condition. Inadequate or no blood supply for muscle tissue causes ischemia. When blood supply is restored, reperfusion may unexpectedly increase the ratio of mortality and morbidity due to extended inflammation and necrotic and apoptotic events caused by reactive oxygen species (ROS) (2).

The primary target of ROS is polyunsaturated fatty acids in the cell membrane. The modifications that occur when ROS specifically targets these fatty acids are essential mechanisms responsible for cellular damage. Lipid peroxide radicals are produced by the interaction between fatty acids and ROS. Metals present in surroundings can catalyze processes involving lipid peroxides, forming breakdown components such as propanal, hexanal,4-hydroxynonenal, and malondialdehyde (MDA) (3).

Another parameter we measured in our manuscript, ischemia-modified albumin (IMA), which can be defined as a molecule related to injury, is increased in plasma after ischemia due to changes in the N-terminal structure. It is assumed that the fundamental reason for this change is increased hydroxyl in reperfusion (4).

glutathione-S transferase (GST) is an enzyme that removes some toxic compounds that cells encounter or make during metabolism by glucuronidation. This mechanism is especially crucial in tissues with high metabolic activity, such as the liver and kidney. Mitochondrial levels play a vital role in cells affected by I/R damage and can save the cell from death (5).

Catalase (CAT) facilitates the transformation of hydrogen peroxide (H_2O_2) molecules, which are present in peroxisomes in various tissues, particularly in the liver, and released due to the action of enzymes like xanthine oxidase and urate oxidase in these organelles, into water (H_2O) and oxygen (O_2) molecules. If tissue levels of CAT are insufficient after I/R injury, protection against the detrimental impacts of H_2O_2 will be difficult cellular damage.

Carvacrol (Car) is a monoterpenoid phenol compound present in different amounts in plants like red bergamot (monarda didyma), black cumin (nigella sativa), and corn lavender (lavandula multifida), particularly in thyme species (origanum spp.). It is also found in other aromatic compounds related to the terpene and phenol groups. It is primarily present in the oil of oregano (O. vulgare), thyme (T. vulgris), pepperwort (lepidium flavum), wild bergamot (citrus aurantium var. bergamia loisel), and other plants (6). The literature emphasizes its anti-oxidan (7), anti-inflamatory (8), anti-bacteriel (9), and pro-apoptotic (10) in a few cancer species and anti-apoptotic (11) in cells affected by environmental conditions.

In our study, we aimed to determine the effects of Car on I/R injury by measuring specific cell damage markers mentioned in rats.

MATERIALS AND METHODS

The experimental methods were appropriately implemented with the approval granted by our institution, the Gazi University Local Animal Care and Use Committee (approval number: E.184884, date: 27.12.2017). Car was obtained from Sigma-Aldrich in a sterile glass container at a concentration of >98% (product number: W224502-100G-K).

Study Design and Experimental Protocol

A total of 18 male Wistar albino rats, weighing 200-250 gr, were fed and kept at a temperature of 20-21 °C, considered a light cycle. Their food and water supplies were not interrupted until two hours before the experiment began. The rats were randomized to three groups (n=6); the Control group (C), the I/R group, and the I/R + Car group.

Before the surgical procedure, the rats were subjected to an intraperitoneal injection of 50 mg/kg ketamine and 10 mg/kg xylazine while in a supine position and exposed to a warm lamp. A dosage of 100 mg/kg of Car was administered to the I/R + Car group via intraperitoneal injection 30 minutes before laparotomy. Rats with aseptically prepared skin underwent midline laparotomy. In the I/R and I/R + Car groups, the aorta was carefully explored at the infrarenal level after the intestines were removed with wet gauze. A non-traumatic microvascular clamp was placed in the infrarenal abdominal aorta (IAA). The microvascular clamp in IAA was removed after 60 min, and reperfusion was achieved for 60 min. The absence of a pulse in the distal aorta throughout the clamping procedure confirmed aortic ischemia. In contrast, the recurrence of a pulse in the distal aorta after removing the clamp confirmed aortic reperfusion. In the control group, laparotomy was the sole procedure, and I/R was not performed. Saline was applied to the peritoneal cavity at the appropriate temperature to reduce heat and fluid loss from the abdomen during periods of I/R, and the abdominal incision was temporarily covered with wet gauze after the clamp was placed and removed from the IAA. In the final stage of the study, adequate anesthesia and analgesia were provided, tissue samples were collected, and the rats were euthanized via intracardiac blood collection.

Histopathological and Biochemical Analyses

Muscle tissue samples from the lower extremities were preserved in a 10% formaldehyde solution for histological analysis, and the presence of muscle atrophy-hypertrophy, degeneration, congestion, leukocyte cell infiltration, muscle nuclei-oval-pyknotic nucleus, fragmentation, hyalinization, and apoptosis were examined under a light microscope.

Lower extremity muscle tissue samples were taken for biochemical examination and stored at 80 °C, and the MDA, GST, and CAT levels were evaluated in the tissues using ELISA.

The blood samples were stored at +4 °C and then centrifuged. IMA levels were then determined by spectrophotometry.

Statistical Analysis

The data were analyzed using variance analysis in the Statistical Package for the Social Sciences (Chicago, IL, USA) 22.0 program for Windows statistical software. The Kruskal-Wallis test was used to evaluate biochemical and histological markers. The value of $p<0.05$ was considered statistically significant.

RESULTS

In GST, there was a significant difference among groups. (p=0.024). GST enzyme activity was remarkably higher in the I/R group than in the control and I/R + Car groups (p=0.009, p=0.046, respectively) (Table 1).

Similarly, we also found a notable discrepancy in CAT (p=0.030). CAT levels were significantly lower in the control and I/R + Car groups than in the I/R group (p=0.016, p=0.028, respectively) (Table 1).

Another important difference was in MDA (p=0.010). Similarly, MDA levels were significantly increased in the I/R group compared with the control and I/R + Car groups, as shown in Table 1 (p=0.003, p=0.033, respectively).

A significant difference was observed when the groups were compared on the basis of IMA levels (p<0.0001). In the I/R group, IMA levels were significantly higher than those in the control and I/R + Car groups, significantly (p<0.0001, p=0.001, respectively) (Table 1).

In Figures 1-6, normal lower extremity muscle tissue is shown. Histopathological examination revealed no degeneration in the control group. On the other hand, contraction, hypertrophy, nucleus degeneration, necrotic fibers, and hyalinization were prominently observed in the I/R group, as shown in Figure 7-12. In the I/R + Car group, these inflamed areas were subtle compared with the I/R group, and vascular dilatation of myofibre was also noted (Figure 13-18).

DISCUSSION

In the present study, we hypothesized that the parameters of cell damage, GST, MDA, IMA, and CAT, would decrease in the I/R + CAR group. Our results are in accordance with our hypothesis.

In the literature, IMA has proven to be used in assessing the efficacy of specific substances applied in clinical settings to prevent I/R injury (12). Regarding other parameters we measured, GST and MDA also had results similar to those of previous research (13,14).

Research on various age groups has indicated that they may have increased vulnerability to damage caused by I/R due to lower levels of CAT activity and quantity at an early age (15). It has also been reported that CAT replacement using biotechnology products, which increases intracellular CAT activity, can reduce cellular damage (16).

Based on the current understanding, there is a limitation of research examining the impact of Car on I/R injury in the skeletal muscles of

Table 1. Muscle tissue anti-oxidant enzyme activities and oxidant (MDA) levels [mean ± S.E.]

	Control group, (n=6)	I/R group, (n=6)	I/R + Car group, (n=6)	p**
GST (mIU/mg-protein)	0.14±0.02*	0.22±0.02	0.16±0.02*	0.024
CAT (IU/mg-protein)	520.33±53.23*	705.00±58.90	538.33±26.22*	0.030
MDA (nmol/mg-protein)	0.99±0.18*	1.75±0.14	1.23±0.14*	0.010
IMA (ΔA)	0.12±0.01*	0.18±0.01	0.14±0.01*	<0.0001

*P<0.05: Compared to group I/R, **Significance level with Kruskal-Wallis test p<0.05. MDA: Malondialdehyde, GST: Glutathione-S transferase, CAT: Catalase, IMA: Ischemia-modified albumin, S.E.: Standard error, I/R: Ischemia-reperfusion, Car: Carvacrol.

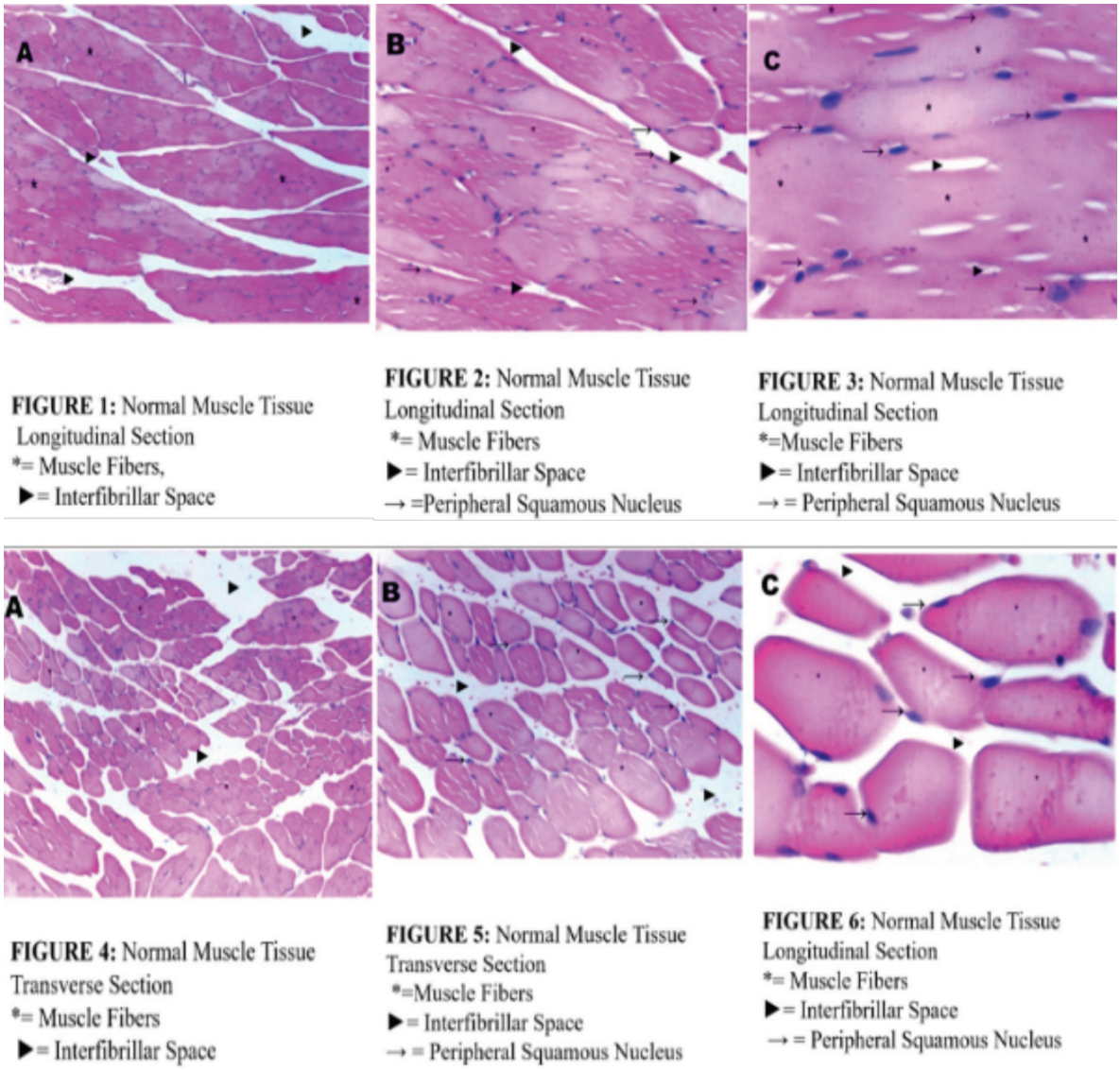

Figure 1-6. Lower extremity muscle tissue.

the lower extremities. However, additional research on Car's effects on other organs and tissues has revealed that outcomes compatible with our research have been achieved (1,17-19).

Metabolic and structural changes are observed in cells during the ischemia phase. When blood flow to tissue terminates, cellular oxidative phosphorylation declines, reducing the production of high-energy phosphates like adenosine 5'-triphosphate (ATP) and phosphocreatine. The release of energy reserves in cells inhibits the cell membrane's Na^+/K^+- ATPase pump. Subsequently, the concentrations of Na^+ and Ca^{+2} within the cells increased. Elevating the concentration of Ca^{+2} ions within the cell is detrimental to cell viability. At the same time, it triggers the activation of many enzymes, including phospholipase, protease, and endonuclease, which begin the cascade of events leading to apoptosis. During this period, the ion concentration of cells changes, relating to increased production of proinflammatory cytokines. At the same time, there is a decrease in the secretion of antioxidant enzymes. The immune system's antioxidant processes generally eliminate ROS from the surroundings, maintaining a balance inside the organism. However, because of earlier phases, during reperfusion, ROS are disseminated throughout the body via systemic circulation (20,21).

Despite the accumulation of data, the precise process of I/R injury still needs to be better understood. As mentioned before, I/R injury may be observed in numerous clinical situations. It is important to note that although these situations might have similar pathogenesis, essential differences exist in their treatment strategies regarding the primary condition. In reperfusion, it is vital to have a hedge to protect cells against I/R injury due to leukocyte migration and its inflammatory process. In some clinical circumstances, such as transplant surgery, immunosuppressive treatments are commonly used to suppress immune responses. However, immunosuppressive

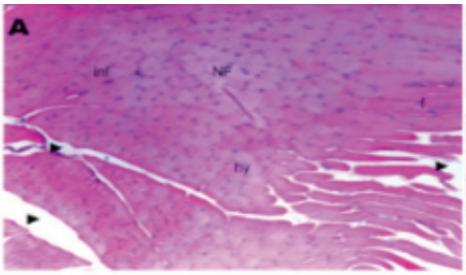

FIGURE 7: I/R Group Muscle Tissue
Longitudinal Section
►= Interfibrillar Space
NF= Necrotic Fibrils
hy= Hyalinization
f= Fragmantetion
inf= Infiltration

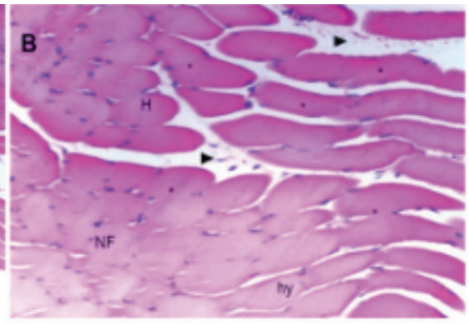

FIGURE 8: I/R Group Muscle Tissue
Longitudinal Section
►=Interfibrillar Space
NF=Necrotic Fibrils
hy= Hyalinization
H= Hypertrophy

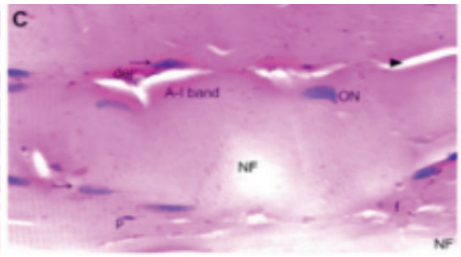

FIGURE 9: I/R Group Muscle Tissue
Longitudinal Section
►= Interfibrillar Space
NF= Necrotic Fibrils
f= Fragmentation
p= Picnotic Nucleus
ON= Oval Nucleus
dej= Degeneration
→ = Peripheral Squamous Nucleus
A-I Band= Sarcomer Bands

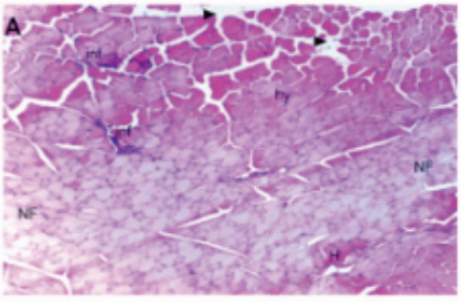

FIGURE 10: I/R Group Muscle Tissue
Transverse Section
►= Interfibrillar Space
NF= Necrotic Fibrils
H= Hypertrophy
dej= Degeneration
hy=Hyalinization
inf=Infiltration

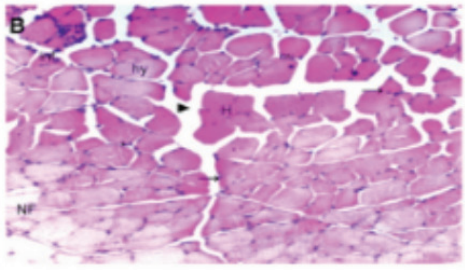

FIGURE 11: I/R Group Muscle Tissue
Transverse Section
*=Muscle Fibers
►= Interfibrillar Space
NF= Necrotic Fibrils
inf= Infiltration
ON= Oval Nucleus
H= Hypertrophic
hy=Hyalinization
→ =Peripheral Squamous Nucleus

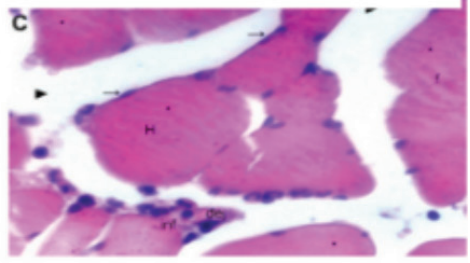

FIGURE 12: I/R Group Muscle Tissue
Transverse Section
*=Muscle fibers
►=Interfibrillar Space
f= Fragmentation
inf=Infiltration
ON= Oval Nucleus
dej= Degeneration
H= Hypertrophy
→ =Peripheral Squamous Nucleus

Figure 7-12. Lower extremity I/R group muscle tissue.
I/R: Ischemia-reperfusion.

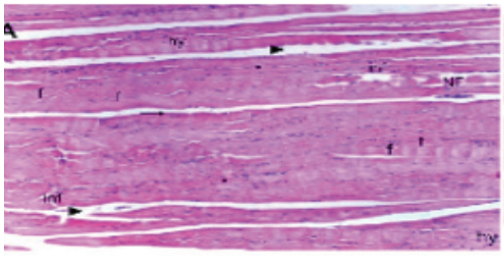

FIGURE 13: I/R+Car Group Muscle Tissue
Longitudinal Section
*=Muscle Fibers
► Interfibrillar Space
f= Fragmentation
inf Infiltration
NF=Necrotic Fibrils
hy Hyalinization
→ =Peripheral Squamous Nucleus

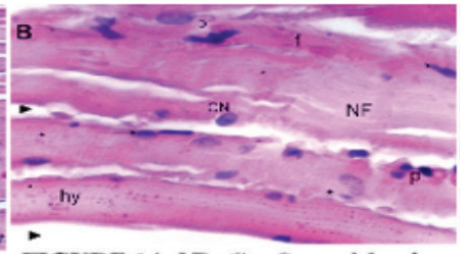

FIGURE 14: I/R−Car Group Muscle
Longitudinal Section
*=Muscle Fibers
►= Interfibrillar Space
f= Fragmentation
NF= Necrotic Fibrils
hy Hyalinization
ON=Oval Nucleus
p Picnotic Nucleus
H=Hypertrophy
→ = Peripheral Squamous Nucleus

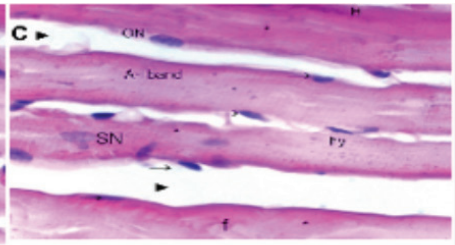

FIGURE 15: I/R+Car Group Muscle
Longitudinal Section
*=Muscle Fibers
► Interfibrillar Space
f= Fragmentation
hy= Hyalinization
ON= Oval Nucleus
H= Hypertrophy
→ A-I Band Sarcomer Bands.
SN(CN)= Central Nuclues

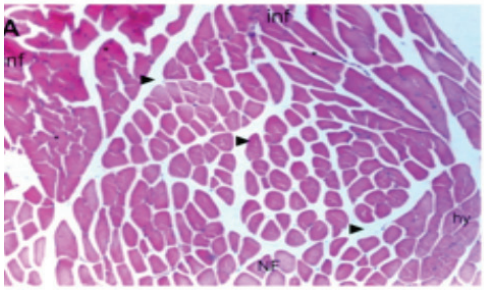

FIGURE 16: I/R+Car Group Muscle
Transverse Section
*= Muscle fibers
►= Interfibrillar Space
inf= Infiltration
NF= Necrotic Fibrils
hy= Hyalinization

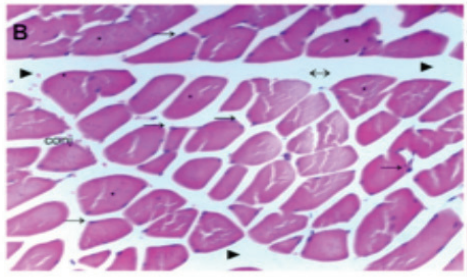

FIGURE 17: I/R+Car Group Muscle
Transverse Section
*=Muscle Fibers
►=Interfibrillar Space
H= Hypertrophy
→ = Peripheral Squamous Nucleus
conj=Conjestion
↔ =Vasodilatation

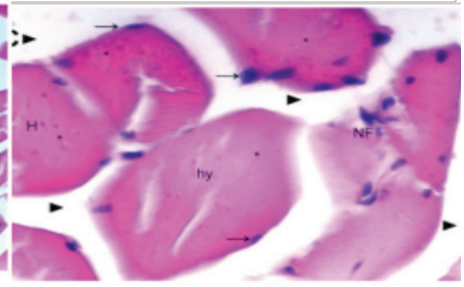

FIGURE 18: I/R+Car Group Muscle
Transverse Section
*= Muscle Fibers
►= Interfibrillar Space
NF=Necrotic Fibrils
hy= Hyalinization
H= Hypertrophy
→ = Peripheric Squamous Nucleus

Figure 13-18. Lower extremity I/R + Car group muscle tissue.

I/R: Ischemia-reperfusion, Car: Carvacrol.

medication is not suitable for cardiac or vascular surgery, and is not recommended at least. These major discrepancies might help to understand the reasons for the considerable research on various pharmacological agents that can be used for I/R injury. Previous studies have examined the impact of Car on I/R damage in various tissues, including myocardial tissue (22), cerebral tissue (23), gastric tissue (24), and liver tissue (25). They demonstrated that Car administration might have favorable outcomes in mitigating I/R injury. However, to the best of our knowledge, more studies are needed on this subject, specifically skeletal muscle.

CONCLUSION

In summary, after further research, particularly in humans, confirms the findings of our study, a detailed demonstration of Car's protective effects against I-R injury will be evident, leading to an expansion of its indications for use.

Ethics

Ethics Committee Approval: The experimental methods were appropriately implemented with the approval granted by our institution, the Gazi University Local Animal Care and Use Committee (approval number: E.184884, date: 27.12.2017).

Informed Consent: Patient approval has not been obtained as it is performed on animals.

Author Contributions

Concept: B.M., A.Ö., B.K., A.K., M.H.Z., Ş.C.S., M.K., M.A., G.L.O., Design: B.M., A.Ö., B.K., A.K., M.H.Z., Ş.C.S., M.K., M.A., G.L.O., Data Collection or Processing: B.M., A.Ö., B.K., A.K., M.H.Z., Ş.C.S., M.K., M.A., G.L.O., Analysis or Interpretation: B.M., A.Ö., B.K., A.K., M.H.Z., Ş.C.S., M.K., M.A., G.L.O., Literature Search: B.M., A.Ö., B.K., A.K., M.H.Z., Ş.C.S., M.K., M.A., G.L.O., Writing: B.M., A.Ö., B.K., A.K., M.H.Z., Ş.C.S., M.K., M.A., G.L.O.

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