Effects of resveratrol on skeletal muscle in ischemia-reperfusion injury

İskemi reperfüzyon yaralanmasında resveratrol'ün iskelet kası üzerine etkileri

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BACKGROUND

Resveratrol, a polyphenol found in grape and red wine, was previously shown to have free radical scavenging and antioxidant properties in various tissues. In this study, the effects of resveratrol were investigated in muscle tissue concerning the ischemia reperfusion (I/R) injury of rat hindlimb.

METHODS

Arterial circulation of right hindlimbs of 24 Sprague-Dawley rats was ceased by a tourniquet applied for four hours (h). The tourniquet was released at the end of 4th hours and rats were divided into four groups of six rats. Then, extremity was reperfused for 4h in group I and for 8h in group II. Resveratrol in 0.5% ethyl alcohol was administered with a dose of 10 mg/kg in the treatment groups (group I and group II) intraperitoneally. Only 0.5% ethyl alcohol were administered in the control groups (group III and group IV) intraperitoneally. Gastrocnemius muscle was used for histological assessments and the anterior tibial muscle was used for measurement of malondialdehyde (MDA) levels.

RESULTS

MN infiltration, edema, changes in diameters of muscle fibers and segmental necrosis were less prominent in rats treated with resveratrol compared with control groups ($p<0.05$). The MDA levels was significantly lower in treatment groups ($p<0.05$).

CONCLUSION

The results suggest that resveratrol may protect the skeletal muscles against I/R injury with its potent antioxidant properties.

Key Words: Antioxidants; free radicals; ischemia; rats, Sprague-Dawley; reperfusion; resveratrol; skeletal muscle.

AMAÇ

Üzümde ve kırmızı şarapta bulunan polifenol yapıdaki resveratrol'ün çeşitli dokularda serbest radikal temizleyicisi ve antioksidan özelliklere sahip olduğu önceki çalışmalarda gösterilmiştir. Bu yazıda sıçanların arka ekstremitelerinde oluşturulan iskemi reperfüzyon (I/R) yaralanmasında resveratrol'ün kas dokusundaki etkileri araştırıldı.

GEREÇ VE YÖNTEM

Yirmi dört adet Sprague-Dawley cinsi sıçanın sağ arka ekstremitelerine turnikenin uygulanması ile arteryel kan dolaşımı 4 saat süreyle durduruldu. Dördüncü saatin sonunda turnike serbestleştirilerek sıçanlar her biri 6 adetten oluşan 4 gruba ayrıldı. Ekstremitenin grup 1'de 4 saat, grup 2'de 8 saat süreyle yeniden perfüzyonuna izin verildi. Tedavi gruplarına (grup 1 ve grup 2) %0,5 etil alkol içinde 10 mg/kg resveratrol intraperitoneal (i.p.) uygulandı. Aynı sürelerle reperfüzyonuna izin verilen kontrol gruplarına (grup 3 ve grup 4) ise sadece %0,5 etil alkol i.p. uygulandı. Histolojik değerlendirme için gastroknemius kası ve malondialdehit (MDA) seviyelerinin ölçümü için tibialis anterior kası çıkarıldı.

BULGULAR

Resveratrol ile tedavi edilen gruplarda kas dokusunda polimorf çekirdekli lökosit infiltrasyonu, ödem, kas liflerinin boyutlarında değişiklik ve segmental nekroz kontrollerle karşılaştırıldığında belirgin azalmıştı ($p<0.05$). MDA seviyeleri de aynı şekilde tedavi grubunda düşüktü ($p<0.05$).

SONUÇ

Bu sonuçlar resveratrolün güçlü antioksidan etkisi ile iskelet kasını I/R yaralanmasına karşı koruyabileceğini düşündürmektedir.

Anahtar Sözcükler: Antioksidanlar; serbest radikaller; iskemi; sıçan, Sprague-Dawley cinsi; reperfüzyon; resveratrol; iskelet kası.

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Reperfusion after critical limb ischemia is an essential prerequisite to tissue survival; however, paradoxically, readmission of oxygen may contribute to further tissue damage and this phenomenon has been termed as "reperfusion injury".[1] Ischemia reperfusion (I/R) injury constitutes a serious problem in traumatology and reconstructive surgery, often spoiling successful outcomes from surgical interventions for management of amputated or severely traumatized extremities.[2] If the mass of the ischemic tissue is large, such as legs, reperfusion results not only in local damage but also remote organ injury. In some cases, acute renal and respiratory failure, cardiac dysfunction and even death can occur as a result of systemic toxic effects of reperfusion products.[3] Reperfusion induces an inflammatory reaction with leukocyte infiltration and it is histologically shown that this reaction starts approximately in the first hour and reaches at a maximum level following 8 to 24 h of reperfusion.[4] Influx of activated neutrophyls at the site of I/R injury results in release of oxygen free radicals. The generation of oxygen free radicals such as hydroxyl radical (OH^-), superoxide radical ($O2^-$) and hydrogen peroxide (H_2O_2) has been clearly established as a fundamental process in the development of reperfusion injury in the skeletal muscle.[5,6]

Several antioxidants protecting the tissues against the damage of free radicals such as melatonin,[7] α-tocopherol,[8] ascorbic acid and carnitine[9] and N-acetylcysteine[10] have proven to be efficient in attenuating the changes of I/R injury. Resveratrol (3.4', 5 trihydroxytransstilbene), a polyphenolic, antifungal natural phytoalexin found in various food products, with particularly high levels in grape skin (50-100 µg/g) and red wine (1.5-2 mg/L) has been shown to regulate many biological activities.[11] Resveratrol has been reported to be protective against atherosclerosis by exerting antioxidant activity and to protect rat heart from I/R injury.[12] Moreover, resveratrol was considered to have anticancer and anti-inflammatory actions.[13,14] Resveratrol was found to be an effective scavenger of hydroxyl and superoxide, and to exhibit a protective effect against lipid peroxidation in cell membranes and DNA damage caused by free radicals.[15] Although it has been shown that free radical scavenging and antioxidant properties of resveratrol lead to diminish I/R injury in many tissues, the damage reducing effects in skeletal muscle tissue has not been addressed yet.

In this study, the effects of resveratrol were investigated histologically and malondialdehyde (MDA), the end-product of lipid peroxidation was measured as an indicator of free radicals biochemically in muscle an *in vivo* model of ischemia-reperfusion injury in rat hindlimb.

MATERIALS AND METHODS

This study was conducted according to the guidelines of the animal care review board of İnönü University Medical Faculty with adherence to the guide for care and use of laboratory animals.

Twenty-four mature male Sprague-Dawley rats weighing 250 to 350 g were divided into four groups consisting of six rats. All animals had free access to standard rat chow and water during reperfusion. At the room temperature (25°C) anesthesia was administered by intramuscular (IM) injection of ketamine hydrochloride (Ketalar) of 30 mg/kg and xylasine hydrochloride (Rompun) of 2 mg/kg to the anterior foot. Ten milligrams of IM ketamine was repeated at every 30±45 min for maintenance. Purified resveratrol was only commercially available as the trans-isomer, the most stable and pharmacologically active form of resveratrol was purchased from Sigma (St. Louis, MO, 500 mg) and prepared according to the manufacturer's protocol. After induction of anesthesia under aseptic conditions, the method proposed before by Hardy et al.[15] was employed to induce limb ischemia, in which a latex tourniquet was applied proximally on the thigh of the rat. Total ischemia was characterized by the absence of an arterial pulse distal to the tourniquet and cyanosis with coldness of the corresponding limb. The tourniquet was released at the end of fourth hour and extremity was reperfused according to groups for four and eight hours. In the treatment groups (group I and group II), 10 mg/kg resveratrol in 0.5% ethyl alcohol was administered intraperitoneally before the tourniquet was released. In control groups (group III and group IV), only 0.5% ethyl alcohol was administered with the same protocol (Table 1). At the end of experiment, rats were sacrificed under deep anesthesia with high doses of pentobarbital. Lipid peroxidation was assessed by measuring malondialdehyde (MDA) (an end product of fatty acid peroxidation) level in tibialis anterior muscle. Shortly, tissues were homogenized in 0.15 mMKCI for MDA determination. After the homogenate was

Table 1. Treatment and control groups

Groups	Timing of ischemia and reperfusion (hours=h)
Group 1 *(treatment group)*	10 mg/kg resveratrol in a 0.5% ethyl alcohol after 4 h of ischemia and 4 h of reperfusion
Group 2 *(treatment group)*	10 mg/kg resveratrol in a 0.5% ethyl alcohol after 4 h of ischemia and 8 h of reperfusion
Group 3 *(control group)*	only 0.5% ethyl alcohol after 4 h of ischemia and 4 h of reperfusion
Group 4 *(control group)*	only 0.5% ethyl alcohol after 4 h of ischemia and 8 h of reperfusion

centrifuged at 3000 r.p.m., the MDA levels in the supernatant and sera were determined by the thiobarbituric acid (TBARS) reaction.[16]

In each experiment, isolated gastrocnemius muscle was prepared and cut into slices along its longitudinal axis. Tissue samples were fixed in 10% formalin neutralized with phosphate buffers for 24 h. Overnight schedule was done for tissue processing using an automatic tissue processor.[17] Using standard techniques, samples were embedded in paraffin wax. Sections were prepared in 4 µm thickness, stained with Hematoxylin-Eosine (HE) in routine fashion and studied under light microscopy by a pathologist in a blinded manner. Polymorphonuclear leukocyte (PMN) infiltration was evaluated to determine the severity of inflammation that resulted from ischemia-reperfusion.

The total number of leukocytes per high power field (magnification 400) was counted in each section using the following scale: 0, no leukocyte infiltration; 1, <10 leukocytes; 2, 10-45 leukocytes; 3, >45 leukocytes.[18] Additionally histological changes in striation and diameters of the muscle fibers, nuclear centralization, segmental necrosis, edema and erythrocyte extravasation of skeletal muscle tissue were investigated.[7] Histological changes were scored in a semiquantitative manner on a scale from 0 to 3 corresponding to normal, mild (<25% of sample area showing injury), moderate (25-50% of sample area showing injury) and severe (>50% of sample area showing injury).

Statistical analysis

SPSS 10.0 software package was used for statistical analyses (SPSS Inc, Chicago, IL, USA). Results were expressed as mean (s.e.m.). Apparent differences between control and treatment groups were analyzed for their statistical significance by the use of Kruskall-Wallis test. Post-Hoc Tamhane test was used for multiple comparisons. p values less than 0.05 were considered as significant.

RESULTS

All animals have completed the study without any mortality. Soon after the tourniquet was applied on the right posterior extremities of the rats, tetanic contractions were observed lasting five minutes. In the following minutes cyanosis, coldness and edema developed and loss of capillary circulation was observed. When the tourniquet was discontinued capillary circulation turned back with severe edema, hyperemia and paralysis. Abnormalities in muscle fibres in control groups were clearly apparent at fourth h or eighth h of reperfusion. Edema (p=0.021), change in diameter of the muscle fibers (p=0.025), PMN infiltration (p=0.024) and segmental necrosis (p=0.043) were found to be significantly lower in group I as compared with group III. There was no statistical significance for loss of striation (p=0.125) erythrocyte extravasations (p=0.713) and nuclear centralization (p=0.99). In group II, edema (p=0.000), dimensional change of the muscle fibers (p=0.021), PMN infiltration (p=0.006) and segmental necrosis (p=0.009) were significantly lower as compared with group IV. There was no statistical significance for loss of striation (p=0.684), nuclear centralization (p=0.447) and erythrocyte extravasation (p=0.262) (Fig. 1-5).

As for the effects of resveratrol on histopathological changes due to reperfusion durations, changes of diameter and loss of striation on muscle tissue were much more remarkable in four hour reperfusion than eight hours reperfusion (p<0.05). It was seen that the effects of resveratrol on edema, PMN infiltration, change in diameter of the muscle fibers and segmental necrosis also maintained after eight hours reperfusion (p>0.05). Resveratrol was not effective on nuclear centralization and erythrocyte extravasation in both four hours and eight hours reperfusion (Table 2).

The MDA levels in tibialis anterior muscle were significantly lower in resveratrol treatment groups as compared with the controls (Fig. 6).

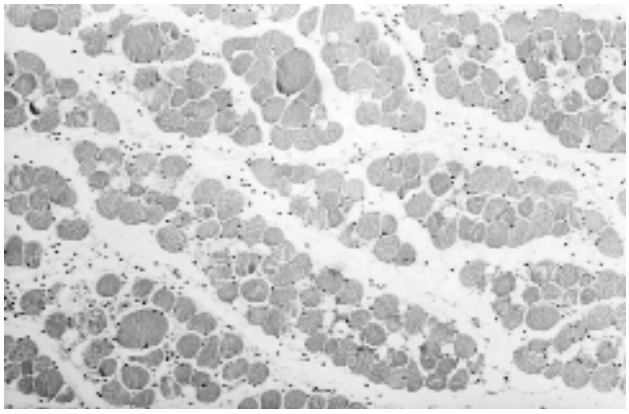

Fig. 1. Muscles in control group (group III) which received only 0.5% ethyl alcohol solution after 4 hours of ischemia and 4 hours of reperfusion.

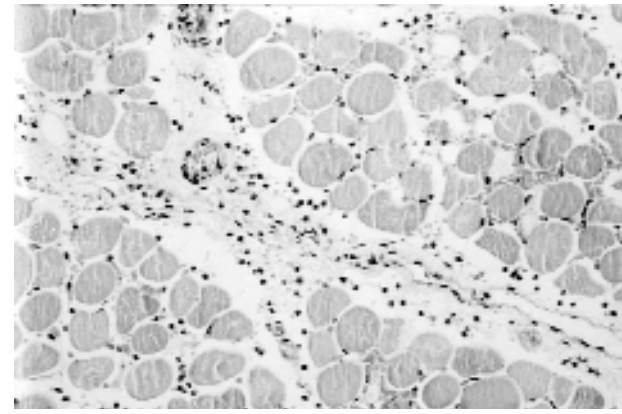

Fig. 2. Muscles in control group (group IV) which received only 0.5% ethyl alcohol solution after 4 hours of ischemia and 8 hours of reperfusion.

In both of control groups there were PMN infiltration, erythrocyte extravasation, segmental necrosis and changing on diameters of muscle fibers (H-E x 200).

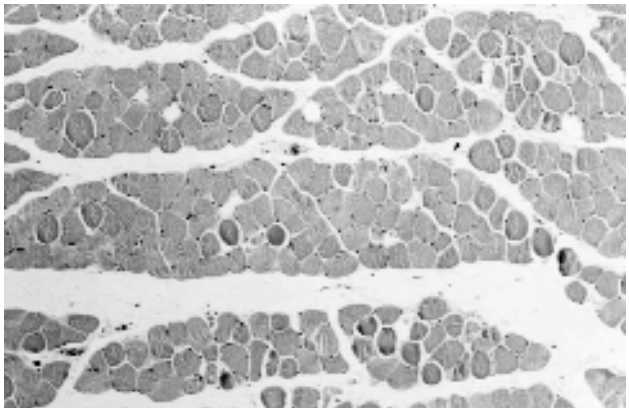

Fig. 3. Muscles in treatment group (group I) which received resveratrol in a 0.5% ethyl alcohol solution after four hours of reperfusions following a four hours of ischemia.

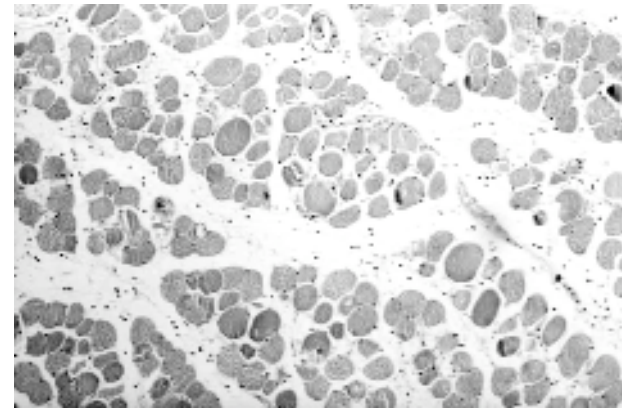

Fig. 4. Muscles in treatment group (group II) which received resveratrol in a 0.5% ethyl alcohol solution after eight hours of reperfusions following a four hours of ischemia.

In both of the treatment groups reduced edema, change in diameter of the muscle fibers, PMN infiltration, and segmental necrosis were found (H-E x 200).

DISCUSSION

In this study, we evaluated the results of resveratrol which had been in a 0.5% ethyl alcohol solution after four and eight hours of reperfusions following a four hours of ischemia period. Results were compared with controls of 0.5% ethyl alcohol administration with the same experimental procedure. To our knowledge, through a comprehensive literature review, this is the first study reporting the effects of resveratrol in skeletal muscle in an I/R injury model. Our results revealed that diminished findings of PMN infiltration, edema, diameter changes of muscle fibers, segmental necrosis in muscle, in conjunction with lower MDA levels when resveratrol treated groups were compared with controls.

In comparison with other organs, skeletal muscle is relatively resistant to ischemia although, irreversible damage has been shown following 3 to 4 hours of complete ischemia.[19] At the same time oxygen free radicals cause cellular injury by inducing

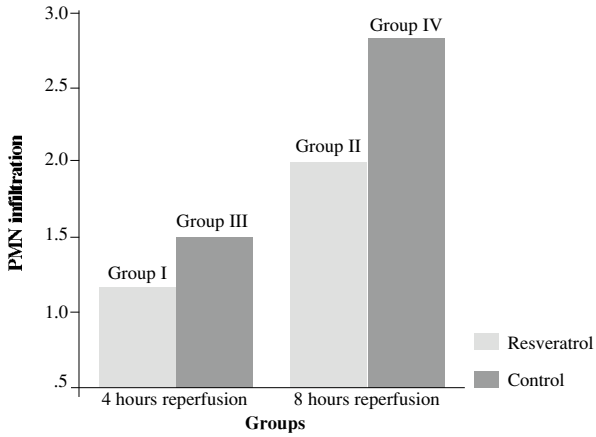

Fig 5. The PMN infiltration in the gastrocnemius muscle was significantly lower in resveratrol treatment groups as compared with the controls.

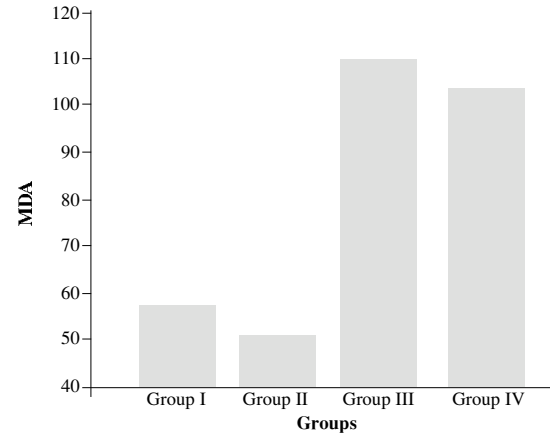

Fig 6. The MDA levels in the tibialis anterior muscle was significantly lower in resveratrol treatment groups as compared with the controls.

lipid peroxidation which results in functional and structural cell alterations.[20] The available literature on I/R indicates that it is characterized by a sequence of events that closely resembles an acute inflammatory process.[21,22] In an I/R injury model, Köksal et al.[10] reported increased neutrophyl infiltration on histopathological examination of the gastrocnemius muscle. In their I/R injury model, Akar et al.[7] have reported an increase in neutrophyl infiltration, segmental necrosis, loss of striation, nuclear centralization and dimensional changes in the soleus muscles of the rats. Resveratrol is considered to be one of the major constituent of natural antioxidants in red wine and grape skin.[23] The role of resveratrol in preventing I/R injury may also be related to its antioxidant properties or its capacity to modulate the function of PMN, because resveratrol interferes with the release of inflammatory mediators by activated PMN.[24] In our study, reperfusion induced an inflammatory reaction with leukocyte infiltration and it was histologically shown that this reaction continued increasingly following 8 h of reperfusion and this effect was greatly reduced by resveratrol. Addition to the effects on PMN infiltration of resveratrol, it was seen that effects on the edema, changing in diameters of muscle fibers and segmental necrosis also maintained after eight hours of reperfusion.

Resveratrol was found to be an effective scavenger of hydroxyl and superoxide, as well as exhibits a protective effect against lipid peroxidation in cell membranes and DNA damage caused by free radicals.[25] Malondialdehyde (MDA) is a well-known product that indicates the presence of lipid peroxidation. In the present study, the MDA levels in the tibialis anterior muscle were significantly lower in resveratrol treatment groups as compared with the controls.

Table 2. Comparison of groups as regard histological parameters

Groups	Edema	Diameter change	Loss of striation	Erythr. extrav.	Neutrophyl infiltration	Segm. necrosis	Nuclear centr.
I	0.50±0.55	0.33±0.52	0.67±.052	0.83±0.41	1.17±0.41	0.67±0.52	0.67±0.52
II	1.17±0.41	1.50±0.55	2.00±0.63	1.67±0.52	1.50±0.55	1.00±0.00	1.17±0.41
III	1.67±0.52	2.00±0.89	1.50±0.55	1.17±0.41	2.00±0.00	1.67±0.52	0.83±0.41
IV	2.83±0.41	2.67±0.52	2.50±0.55	2.33±0.52	2.83±0.41	2.33±0.52	1.67±0.52
I-III	p=0.021	p=0.025	p=0.125	p=0.713	p=0.024	p=0.043	p=0.992
II-IV	p=0.000	p=0.021	p=0.684	p=0.262	p=0.006	p=0.009	p=0.447
I-II	p=0.216	p=0.021	p=0.016	p=0.277	p=0.838	p=0.684	p=0.447

Although numerous effects have been described for resveratrol, the molecular mechanisms responsible for its biological effects and informations about the pharmacokinetics are not yet clear. Resveratrol is pharmacologically active both *in vitro* and *in vivo*.[26] In most ischemia reperfusion injury studies, resveratrol was used at concentrations ranging from 5 to 10 mg/kg.[14,27,28] In this study, we also injected 10 mg/kg resveratrol. But trying to test the effects of resveratrol in different doses will be more reasonable.

The results of the present study supports that resveratrol can exert a protective effect against skeletal muscle injury caused by I/R in the rats. Resveratrol is not solely an antioxidant, but also inhibits the neutrophyl infiltration. Following clarification of the underlying protective mechanism, optimal dose and timing, resveratrol may be used in humans as an adjunct to prevent the I/R injury of the skeletal muscle.

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