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Biochemical, Histopathological, Immunohistochemical Evaluation of Ischemic Preconditioning and Krill Oil Effects in Ischemia / Reperfusion Model

The aim of the present study was to evaluate the protective effects of krill oil (KO) administration and ischemic preconditioning (IPC) protocol using biochemical, histopathological and immunohistochemical parameters in a rat model. Limb ischemia was achieved by clamping the femoral arteries. Fifty rats were divided into five groups (n=50). Effect of IPC strategy and drug-reinforcement protocol was evaluated together or separately in the experimental groups. Malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) activities were measured. Light microscopic images were supported by histological damage score findings and semiquantitative analysis findings of β -actin protein. Biochemical markers of oxidative stress were improved in KO and IPC groups compared with I/R group. The administration of IPC and KO resulted in a significant decrease in the damage score, 40% reduction of damage levels. Semiquantitative H-score analysis revealed that immunoreactivity increased in the treatment groups compared with I/R and IPC groups. It is concluded that ischemic-preconditioning protocol and pre-treatment with KO provides potent protection ischemia-reperfusion injury.

Key Words: Skeletal muscle, femoral artery, krill oil, ischemic preconditioning

İskemi/Reperfüzyon Modelinde İskemik Önkoşullama ve Krill Yağı Etkisinin Biyokimyasal, Histopatolojik, İmmünohistokimyasal Değerlendirmesi

Bu deneysel çalışmada, bir sıçan modelinde oluşturulan iskelet kası iskelemler-reperfüzyon hasarında krill yağı ve iskemik önkoşullama (İÖK) etkinliği, biyokimyasal, histopatolojik ve immünohistokimyasal parametreler yardımıyla araştırıldı. Ekstremitelerde iskemiyi, femoral artere klempe konularak sağlandı. Ratlar (n=50) beş eşit gruba ayrıldı. Gruplarda ise iskemik önkoşullama protokolü ve krill yağı uygulamasının etkinliği birlikte ve ayrı olarak değerlendirildi. Malondialdehit (MDA), nitrik oksit (NO) seviyeleri ve süperoksit dismutaz (SOD), glutatyon peroksidaz (GSH-Px), katalaz (CAT) aktiviteleri ölçüldü. Işık mikroskop görüntüleri, histolojik hasar bulguları ve semikantitatif analiz bulguları ile desteklendi. KO ve İÖK uygulanan sıçanlarda I/R grubuna göre oksidatif stresin biyokimyasal belirteçlerin iyileştiği görüldü. I/R ve İÖK uygulanan grupların hasar seviyelerinde ise önemli oranda azalma tespit edildi. Semikantitatif HSCORE analizi ile I/R ve İÖK gruplarına kıyasla tedavi gruplarında immünreaktivitenin arttığı ortaya konuldu. İskemik önkoşullama protokolünün ve krill yağı verilmesinin iskelemler-reperfüzyon hasarına karşı güçlü bir koruma sağladığı sonucuna varılmıştır.

Anahtar Kelimeler: İskelet Kası, femoral arter, krill yağı, iskemik önkoşullama

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Introduction

Ischemia-reperfusion injury is an important predictor in affecting the outcome in a wide variety of clinical conditions including organ transplantation, cerebrovascular diseases, myocardial infarction, major surgical procedures, thrombolytic therapy, and hemorrhagic (hypovolemic) shock with resuscitation (1, 2). Ischemia is an insufficient supply of blood to an organ or tissue, usually due to blockage of blood artery, which can lead to cell death as a result of the depletion of energy stores and the accumulation of toxic metabolites (1, 3). The tissues subjected to prolonged ischemia are exposed to damage (2). This ischemic damage can mediate growing tissue injury by reoxygenation during reperfusion (3). Although several mechanisms play roles in this event, free oxygen radical derivatives rapidly occurring as a result of molecular oxygen entry into the cell is understood at the level (4). Basic molecules such as superoxide anion (O_2^-), hydroxyl radical (OH^\cdot) and hydrogen peroxide (H_2O_2) are released under physiological conditions during biochemical reactions (5). Antioxidant defense system includes antioxidant enzymes (SOD, GSH-Px and CAT) which act as a scavenger of free radicals, and nutrient-derived antioxidant molecules (6). Oxidative stress is known as a disturbance in the oxidant/antioxidant balance in favor of oxidants between the

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production of free radicals (oxidizing agents) and antioxidant defence system in the body (7). Disturbances, occurring from the overproduction of peroxides and free radicals can cause damage to biomolecules (lipids, proteins and DNA) (8).

In contrast to standard fish oil, purified Antarctic KO supplements consist of a mix of around 40% phospholipids (primarily phosphatidylcholine), 30% total n-3 PUFAs (omega-3 polyunsaturated fatty acids), the most abundant being DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid). KO contains the natural antioxidant astaxanthin, vitamin A, vitamin E and other fatty acids. High intake of KO has been suggested to protect against chronic inflammation and arthritis symptoms with reduction of C-reactive protein levels. The phospholipid form of omega-3 fatty acids is found in high concentrations in certain marine animal species adapted for life in extremely cold water, such as Antarctic krill. It can be used directly without any conversion. That is, the phospholipid forms of the omega-3 fatty acids can be considered to be acting like a natural version of a cell membrane. Astaxanthin prevents the oxidation of long-chain polyunsaturated fatty acids (LC-PUFAs) (9, 10).

IPC is an alternative conservation strategy induced periodically by brief period of ischemia and reperfusion prior to a more prolonged ischemic period (11). The phenomenon of IPC was first described in 1986 by Murry et al (12). In this study, Murry et al. (12) showed that myocardial ischemic episodes protected the myocardium against this potentially cell injury. IPC period has a protective effect on muscle tissue and researchers showed possible mechanisms of action for its this protective effect against injury (13). Although many formation mechanism dealing with IPC is mentioned, yet neither the effect of reactive oxygen species (ROS) on IPC process nor the effect of IPC on ROS is clear. The potential mechanisms underlying the protective effect in muscle tissue have not been fully clarified. In this study, we investigated the effects of KO and IPC on skeletal muscle I/R injury in a rat model. The aim of this study was to answer questions such as whether these treatment combinations reduce injury in skeletal muscle tissue when they are applied alone or together. The present work may allow identification of a new method and conservation strategy that will serve as future therapies. For this purpose, the effects of KO and IPC were evaluated by biochemical, histopathological and immunohistochemical analysis.

Materials and Methods

Animals: Experiments were performed on the 50 adult male rats. Wistar rats weighing between 200 and 250 g. Animals were placed in a neutral temperature environment (22±2 °C) on a 12/12-h photoperiod and were fed with a standard diet. The study was approved

by the Institutional Review Board and the animal care complied with the guide for the care and use of laboratory animals.

Experimental Groups and Surgical Procedures:

All surgical procedures were performed while the rats were under anesthesia achieved by intraperitoneal injection of ketamine and xylazine cocktail (0.5 mL/kg + 1 mg/kg, i.p.). An additional dose of anesthesia cocktail was repeated at 30±45 min for maintenance. After induction, rats were placed with a preselected 37 °C temperature maintained during the whole surgery protocol.

Rats were randomized into five groups, each of which contained 10 rats. Sham operation, only an inguinal incision without femoral artery occlusion, was performed in sham-operated group. All rats in group 2 were exposed to 2 h ischemia period followed by 1 h reperfusion. After incision of the right inguinal region, the right femoral artery was dissected to free from the femoral vein and nerve. Then the femoral artery was occluded with microvascular clamp. After 2 h ischemia, the microvascular clamp was removed. All rats in group 3 were preconditioned with one-cycle of 10 min ischemia/10 min reperfusion before the long I/R protocol. The rats were subjected to 2 h ischemia and 1 h reperfusion, but 1 h after placing an occlusive vascular clamp across the right femoral artery. As indicated in the literature (14), KO (200 mg/kg) was injected into the peritoneum in group 4. The last group included not only IPC strategy, but also drug-reinforcement protocol. The rats were subjected to one-cycle of 10 min I/R episodes. After this, the specific occlusion and reperfusion periods (2 h/1 h) were performed.

Collection of Blood and Tissue Samples: At the end of the experiments, rats were sacrificed by taking blood from intracardiac area. Blood samples were collected into polyethylene tubes and centrifuged (3000 x g for 15 min at 4 °C). The right gastrocnemius muscle was also harvested and immediately stored at -80 °C until further biochemical analysis. The remaining muscle samples were firstly fixed in a 10% formaldehyde solution for histopathological analysis and reached the histology laboratory for processing.

Biochemical Analysis

Preparation of Tissue Sample: The extracted gastrocnemius muscle was washed three times in cold isotonic saline. After drying the gastrocnemius muscle, tissues were then weighed and homogenized in a four volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4) using a homogenizer for 3 min at 5000 g.

Level of MDA and NO were measured from a part of the homogenate. The other part of the homogenate was centrifuged at 3500 x g for 30 min to obtain

supernatant. The supernatant was used to determine CAT and GSH-Px activity. SOD activity was assessed in the ethanol phase of the supernatant after ethanol/chloroform mixture.

Tissue MDA concentration was measured by a method based on the ability of reaction with thiobarbituric acid (TBA) at 90 °C-100 °C (15).

Tissue nitrite (NO_2^-) and nitrate (NO_3^-) were assessed as an indicator of tissue NO level. The NO levels were measured using the Griess reaction assay (16), which is based on the spontaneous oxidation of NO to nitrite (NO_2^-), and subsequently to nitrate (NO_3^-).

Tissue CAT activity was measured according to the method of Aebi (17). The principle of the method was that the rate constant k of hydrogen peroxide decomposition determined at 240 nm. The absorbance changes per minute was measured, and then the rate constant (k) of the enzyme was detected.

Total SOD activity was determined in the tissue homogenates using the method described by Sun et al (18). This method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by a superoxide generator system (xanthine-xanthine oxidase).

Tissue GSH-Px activity was measured spectrophotometrically by the method of Paglia and Valentine (19). The amount of protein in the samples was measured by the Lowry method (20).

Statistical Analysis: Statistical analysis of biochemical data was performed using commercial software (IBM SPSS Statistics 19, SPSS inc., an IBM Co., Somers, NY). Kolmogorov-Smirnov test was used to analyze the distribution of the parameters. When the data were not normally distributed, Kruskal Wallis analysis of variance was used. Mann-Whitney U-test with Bonferroni adjustment was used for interval data. When the continuous variables followed a normal distribution, parametric test was used to analyze the data. One-way analysis of variance (ANOVA) was performed. In the ANOVA test, for post hoc multiple comparison, the Least Significant Difference test was used. Results were expressed as mean \pm SD. P value of <0.05 was considered to be statistically significant.

Histopathological Analysis: The fixed gastrocnemius muscle samples were passed through an increasing alcohol series. They were embedded in paraffin following treatment with xylene and paraffin. The paraffin-embedded blocks were cut in 5- μ m-thick sections, and the sections were stained with hematoxylin and eosin (H&E). The photomicrographs of the relevant stained sections were taken with the aid of a light microscope (Nikon Eclipse 200 Serial No:T1a1 944909, Japan).

The following scores were used to grade the degree of histological damage observed in the gastrocnemius muscle tissue: no any structural damage (0), mild damage (1), moderate damage (2) and severe damage and degeneration (3). These scores were computed as the average damage scores for each group by finding out the average of scoring coefficients. All groups were evaluated by muscle fiber disorganization and inflammatory cell infiltration.

Immunohistochemical Analysis

Immunohistochemistry Staining Protocol: The principal aim of this analysis was to immunohistochemically investigate the detection of beta-actin protein (β -actin protein) in the muscle tissue. Sections from representative regions of the gastrocnemius muscle tissue were immunohistochemically labeled for β -actin protein, and were microscopically examined to determine the distribution of specific immunoreactivities generated by these antibodies. An immunohistochemistry staining protocol was used for detecting the presence of β -actin proteins. Sections of 5 μ m were cut from paraffin blocks and were placed onto polylysine-covered slides and incubated overnight at 56 °C. The tissue sections were dehydrated with a series of alcohol washes, placed in distilled water. Following this the sections were microwaved in 360 W microwave oven in citrate buffer, for 5 minutes twice and allowed to cool. The sections were then incubated for 10 min in 3% H_2O_2 , washed (3x5 min) in phosphate-buffered saline (PBS). Sections were treated with a blocking serum and incubated overnight in a cold room with primary antibodies. The Mouse monoclonal beta Actin antibody (ab8226; Abcam) was used at a dilution 1:500. The sections were washed off (3x5 min; PBS), incubated overnight in biotinylated secondary antibodies for 30 min at room temperature. The slides were reacted with horse-radish peroxidase for 30 min, and treated with 3-amino-9-ethyl. The slides were reacted with horse-radish peroxidase (HRP) for 30 min, and treated with 3-amino-9-ethylcarbazole (AEC) chromogen. They were counter-stained with Mayer's hematoxylin solution for 3-5 min. The tissues were washed thoroughly with PBS, rinsed with distilled water and covered using a water-based sealing solution. After the all protocol, photomicrographs were taken with a research microscope.

Scoring of Immunoreactivity: The results of immunohistochemical staining were assessed by semiquantitative scoring. The most common method used in the analysis of images is the semiquantitative scoring system in mouse models (21). For semiquantitative analysis of immunoreactivity of β -actin protein, the histological score (H-SCORE) (22) was used in this study. The intensity of immunoreactivity was evaluated using four intensity categories for each

component: 0 (not present), 1+ (weak but detectable), 2+ (middle or distinct), and 3+ (intense). The H-SCORE is obtained by the formula: $\sum P_i (i+1)$, where "i" represents the intensity of the stained cells. The score was obtained by 2 investigators after determining the areas of immunostained slides using a light microscope (x40 objective). The H-SCORE value for each tissue sample and the mean H-SCORE value for each group was calculated and tabulated.

Results

Biochemical Findings: The results were summarized in Figure 1. In these figures, the levels of MDA, NO and the activity of antioxidant enzymes such as CAT, SOD, GSH-Px in experimental groups were shown. Results were expressed as the mean concentration and standard deviation of the mean, respectively. The increase in MDA concentration observed in I/R was significantly different from the MDA concentration in sham ($P < 0.001$; Figure 1a).

In the I/R group, the NO levels were higher compared with those in the sham group. The treatment of KO and IPC significantly reduced the NO levels compared with those in the I/R group ($P < 0.001$, Figure 1b).

There was a decrease in the CAT activity after I/R and IPC compared with the sham-operated animals. Treatment with KO and IPC prior to reperfusion resulted in an increase in CAT activity of I/R+KO, IPC+KO, respectively ($P < 0.05$, Figure 1c).

A slight increase was observed between the SOD and GSH-Px concentrations ($P > 0.05$; Figure 1d, e).

Histopathological Findings: Histopathological changes in muscle tissue samples are shown in Figure 2. The images in sham group showed the normal microscopic appearance of histologically muscle fibers (Figure 2A). Conversely, in the I/R group, muscle fiber degeneration was clearly observed. A stronger atrophy was detected with loss of A and I band, changes of diameter on muscle fiber. An extensive inflammatory cell infiltration of the damaged muscle tissue were markedly detected (Figure 2B). Muscle fiber damage in the I/R group was a small increase compared to those in the IPC group (Figure 2B, C). Positive histopathologic changes were observed in treatment groups (I/R + KO and IPC+KO) compared with I/R and IPC groups. Although the positioning of nuclei in skeletal muscle fibers and cell morphologies were not in normal appearance as in the control group, there was no significant deformation in treatment groups. KO and IPC groups had significant reduction in degradation and inflammation (Figure 2D, E). Light microscopic images were supported by histological damage score findings.

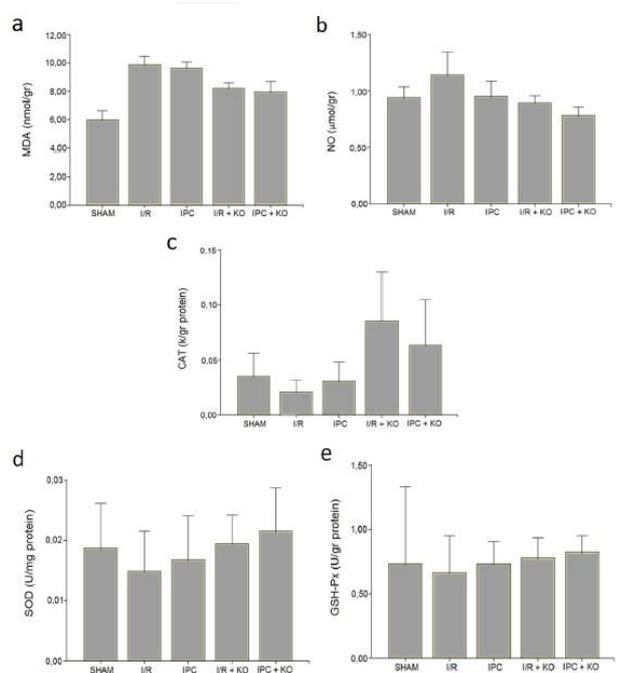


Figure 1. Levels of malondialdehyde (MDA), nitric oxide (NO), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in the muscle tissue of rats from the different groups. Values are given as mean \pm SD; $P < 0.05$ is significant

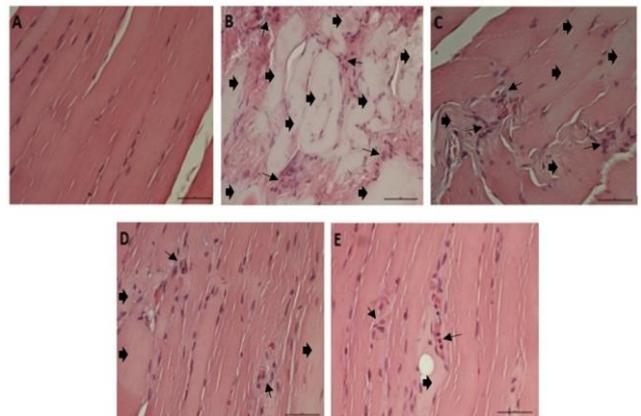


Figure 2. Hematoxylin and eosin staining of skeletal muscle fibers in the study groups. (A) Normal muscle tissue from a healthy sham group. (B) The degenerated muscle tissue from a rat with I/R, obtained prior to treatment. (C) The inflammatory and degenerative changes of muscle fibers in the IPC group as in I/R group. (D) Muscle tissue from a rat with I/R+KO, obtained after krill oil treatment. (E) Muscle tissue from a rat with IPC+KO, obtained after krill oil and ischemic preconditioning treatment. Scale bars= 50.0µm. (The thin arrow indicates focal polymorphonuclear leukocyte infiltration in interstitial area. The thick arrow indicates muscle fibers showing degenerative changes)

Histopathological examination of muscle tissues from the rats I/R- administered revealed that there were significant histopathological changes such as markedly muscle fiber degenerations and, inflammatory cell infiltrations. I/R group and IPC group had high-grade tissue damage scores compared to the sham group. The average score was nearly three or four times higher in the I/R group and IPC group compared to the control group. The administration of IPC and KO supplement resulted in a significant decrease in the damage score, 40% reduction of damage levels. The damage score was slightly lower in the IPC groups compared to the KO groups. Degenerative scores for each of these cases and group mean score for each group are demonstrated in Table 1.

Immunohistochemical Findings: Tissue sections were evaluated for β -actin protein by immunohistochemistry. Immunohistochemical changes in muscle tissue samples are shown in Figure 3. The immunostaining was located predominantly in connective tissue components (mainly fibroblasts) and blood vessel cells but was also found in muscle tissue. In sham samples, an intense immunoreaction was detected in both muscle fiber cells and connective tissue cells, endothelial and smooth muscle cells of the vessel wall (Figure 3A). Staining intensity was scored as strong and

the hscore percentage of staining intensity were highest for sham group. Staining intensity of I/R group was similar to that of IPC group and appeared to decrease in score value compared with those in sham group (Figure 3B, C). The stained cells, where there was no staining were observed to be stained in average 1(+) intensity. The staining intensity for these groups was seen to be higher in IPC group compared with those in I/R group (Figure 3C). Both treatment groups had a higher intensity than that of I/R group and IPC group (Figure 3D, E). Semiquantitative H-score analysis revealed that immunoreactivity increased in the treatment groups compared with I/R and IPC groups. The H-score results are presented in Table 2.

Table 1. Histopathologic damage score findings for each group

Groups	n	Group Mean Score	
		Disorganization of muscle fiber	Inflammatory cell infiltration
Sham	6	0.51	0.71
I/R	7	2.35	1.98
IPC	7	2.13	2.01
I/R+KO	7	1.37	1.44
IPC+I/R	7	1.32	1.49

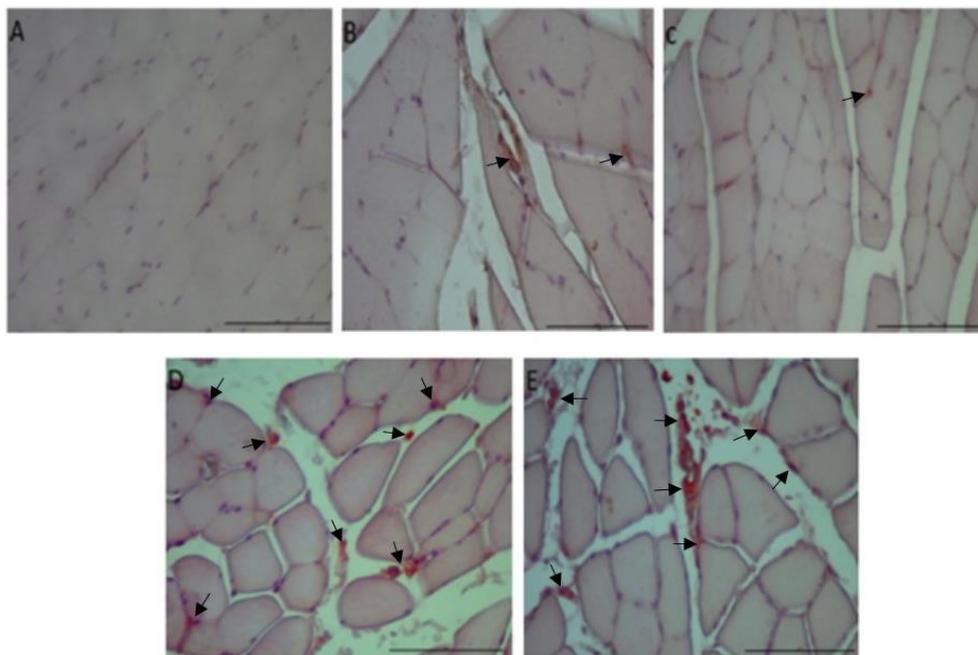


Figure 3. Immunohistochemistry staining of skeletal muscle fibers in the study groups. (A) An intense immunoreaction from a healthy sham group. (B) The decreased immunoreaction from a rat with I/R, obtained prior to treatment. (C) The higher staining intensity in the IPC group compared with those in I/R group (D) The higher intensity from a rat with I/R+KO, obtained after krill oil treatment. (E) The higher intensity from a rat with IPC+KO, obtained after krill oil and ischemic preconditioning treatment. Scale bars= 100.0µm. (Arrows indicate immunohistochemical reactivity for β -actin protein)

Table 2. Semiquantitative H-score analysis findings for some cases

Groups	Cases	Staining Intensities				Individual Staining Intensity Score	Group Staining Intensity Score	The percentage of staining intensity (%)	Individual Hscore	Group Hscore
		0 (-)	1 (+)	2 (++)	3 (+++)					
Sham	1	20	30	43	45	1.82	1.55	45.47	241.03	205.01
	2	10	17	15	20	1.73		43.15	228.62	
	3	35	41	27	10	1.11		27.65	145.38	
I/R	1	59	10	7	1	0.35	0.26	8.77	31.57	25.68
	2	60	12	5	0	0.29		7.14	28.39	
	3	63	11	0	0	0.15		3.72	17.07	
IPC	1	18	7	2	2	0.59	0.49	14.66	60.17	47.43
	2	25	4	5	3	0.62		15.54	52.59	
	3	25	7	4	0	0.42		10.42	43.29	
	4	35	10	1	1	0.32		7.98	33.68	
I/R + KO	1	32	10	11	4	0.77	0.64	19.30	77.72	69.06
	2	25	19	11	2	0.82		20.61	102.43	
	3	43	16	9	4	0.64		15.97	66.01	
	4	52	7	8	0	0.34		8.58	30.07	
IPC + I/R	1	20	15	4	3	0.76	0.78	19.05	92.29	90.31
	2	24	10	11	7	1.02		25.48	108.73	
	3	18	11	6	3	0.84		21.05	96.95	
	4	25	14	4	0	0.51		12.79	63.28	

Discussion

Experimental data demonstrated that IPC exerts protective effects on several organ system or tissues. One scientific approach in the experimental setting is IPC, whereby improved protection is observed after a number of very brief episodes or a single cycle of ischemia and reperfusion (12). Accordingly, cycle length and number of cycles are important factors that lead to success in IPC studies. Zahir et al. (23) found that three to five cycles of 10-min occlusion achieved a statistically superior outcome to one or two cycles. Similar results were observed in a study using musculocutaneous flap subjected to three cycles of 10 min ischemia and 10 min reperfusion at the onset of prolonged ischemi-reperfusion. In contrast to the requirement for multiple trigger episodes in these studies (24). Lepora et al. (25) was unable to observe a protective effect with one cycle or two cycles of 5-10 min preconditioning in both the ipsilateral and contralateral rat hind limb. In other study, isolated cremaster muscle models were preconditioned by one cycle 45 min occlusion, interspersed by 15 min of reperfusion prior to a sustained 40 min occlusion followed by 60 min reperfusion (26). Multiple trigger episodes of IPC have been assessed by many researchers, but the effects of a single cycle and length of this cycle have not yet been clarified. The preconditioning protocol used in this study was repeated for one cycle with a total preconditioning time of 20 min. We found that one cycle of 10 min preconditioning provided a protective effect.

The protective effect of KO also has previously been shown in many investigations. Additionally, in a recent study, it has been shown that KO administration had therapeutic effect on oxidative stress and DNA damage by reducing several lipid peroxidation components and the main marker of DNA oxidation in obese rats. Exposure to dietary KO resulted in increase in the metabolic response to radical attack (10). Kidd et al. (27) reported that KO contains omega-3 FAs bound to phospholipids-provide the building blocks for healthy cell membranes. It is essential to build up stores of DHA and EPA in the body tissue for release of these fatty acids and their lipid mediators (28). In KO, DHA and EPA is found in a double-chain phospholipid structure (27). Gamoh et al. (29) found that the level of lipid peroxide measured by estimating MDA concentration significantly decreased after KPL (krill-derived phospholipid) administration. Another important aspect of their work was that lower levels of ROS was observed in both the cerebral cortex and hippocampus of KPL-administered rats. The present study clearly showed that the level of MDA in association with KO, indicating a lipid peroxidation was significantly decreased compared with healthy samples and these findings are in accordance with the previously mentioned studies. In a previous study, six-week-old female mice were fed an American Institute of Nutrition (AIN-76) semipurified diet containing KO. A comparison of phospholipids from each group revealed significantly lower peroxidation indices and

thiobarbituric acid reactive substances generation. The total antioxidant capacities were measured (30).

In the study of the effects of ischemia cycle length, which involves ischemic episode of 5, 10, 15, 20 minutes in skeletal muscle, MDA level of IPC-15 (fifteen minutes of ischemic preconditioning) was decreased, leading to a decreased level of lipid peroxidation; but, IPC-20 did not decrease it (31). Kocman et al. (32) demonstrated a significant decrease ($p < 0.05$) in MDA, toxic molecule and biological marker, after IPC administration.

Three distinct isoforms of SOD, are present. The copper-zinc SOD (Cu/ZnSOD) is predominantly expressed in the cytosol and the manganese SOD (Mn-SOD) is located in the mitochondria of the cells (33). It is also known that Mn-SOD expression is inducible by factors such as hypoxia and ROS, which has been demonstrated in several experimental system (34, 35). The promoter of Cu/Zn SOD has been found to be inducible by oxidants and metal ions in hepatoma cells (36). It can be said that ROS lead to increased expression of antioxidant enzymes such as SOD, CAT and GSH-Px by inducing antioxidant defence system. To annihilate or reduce excessive amounts of free radicals, occurring in skeletal muscle after I/R, it has realized the production of more antioxidant enzyme with the body's natural defense mechanism. This approach have been supported by previous studies related to skeletal muscle I/R injury (37). Their study showed an increase in the activities of enzymes. In contrast, the antioxidant enzymes (SOD, CAT and GSH-Px) activities were decreased in the skeletal muscle tissue of IR rats (38). A study by Kocman et al. (32), provided evidence for decreased levels of CAT activity in rats subjected to IR and IPC administration. SOD, GSH-Px and CAT activities of skeletal muscle tissue were decreased in rats with IR with healthy rats in the present study. This result has also been attributed to various causes. The production of intracellular and extracellular ROS is regulated by various pro-oxidant and antioxidant enzymes (39). A contributing factor to the increase of oxidative stress after I/R is the increased use of antioxidant enzymes. It has been reported that activity of

antioxidant enzymes is decreased after I/R (40, 41). Teruya et al. (42) showed that I/R plays a significant role in the expression of genes related to generation and modulation of ROS. In our opinion, another possible contributing factor to the increase of oxidative stress after ischemia/reperfusion is the reduction of antioxidant enzyme activity.

Researchs are being conducted into the inhibitory effects and toxicity of NO. Hernandez et al. (43) and Laurinda et al. (44) stated that NO attenuated intracellular oxidative stress in cells as a result of many stimuli such as reperfusion. Egr-1 (Early growth response-1) contributes to accelerated oxidative stress (45). Henderson et al. (46) reported that subsequent NO production reduced Egr-1 gene expression in macrophages. In contrast, Beckman et al. (47) stated that a major mechanism of injury associated with the production of NO in vivo. Additionally, the reaction of NO and superoxide radicals at diffusion-controlled rates was shown to enhance direct toxicity of NO, producing toxic peroxynitrite. The generation of excess peroxynitrite (ONOO-) has led to oxidative stress. In a study, I/R procedure was associated with significantly higher concentrations of NO in rat skeletal muscle. IPC procedure provided better improvement in skeletal muscle function before ischemia and reperfusion (32). Our study reported a marked increase in NO level in the I/R group. KO treatment and IPC administration significantly attenuated this increment.

In conclusions, KO and IPC treatment led to a significant increase in enzyme activities as a result of direct or indirect mechanism in pathological tissue. I/R produced a significant increase in the skeletal muscle tissue MDA and NO contents, this was prevented by KO and IPC treatment. These biochemical results were supported by experiments, in which determination of damage scores and scoring of immunoreactivity in skeletal muscle tissue were performed histopathologically and immunohistochemically. These findings suggest that administration of KO and IPC have protective effects against skeletal muscle damage induced by I/R. Further studies are necessary to investigate the molecular mechanism of KO and IPC.

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