Comparison of Bencyclane Hydrogen Fumarate, Pentoxifylline and Cilostazol's Protective Effects in the Treatment of Peripheral Occlusive Artery on a Rat Model of Ischemia-Reperfusion Injury

İskemi Perfüzyon Hasarı Oluşturulan Rat Modelinde Periferal Tıkayıcı Arter Tedavisinde Bensiklan Hidrojen Fumarat, Pentoksifilin ve Silostazolun Koruyucu Etkilerinin Karşılaştırılması

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ABSTRACT Objective: Acute limb ischemia is a clinical event that may result by arterial thrombosis, peripheral arterial occlusive disease or arterial clamping during arterial reconstructive surgery. We investigated the effects of Bencyclane hydrogen fumarate (BF), Pentoxifylline (PTX) and Cilostazol (CZ) in the attenuation of ischemia reperfusion (I/R) injury on skeletal muscle in a rat model. **Material and Methods:** Twenty-eight adult male Wistar rats were used in a model of hind limb ischemia. The animals were randomized into four equal groups (N= 7) submitted to 2 hours of ischemia followed by 4 hour of reperfusion. Group 1: control group; received IR injury to both hind limbs. Group 2 (BF + IR) had received BF, Group 3 (PTX + IR) had received PTX, Group 4 (CZ + IR) had received CZ before they underwent the same model of IR injury. Blood samples and muscle biopsies were taken for histological and biochemical evaluation. **Results:** In the treated groups, tissue MDA and serum/tissue CAT levels were found to be significant compared to the control group. In groups 3 and 4, SOD levels were significantly increased compared to the control group. SOD values were found to be significant between the groups 2 and 4. In groups 3 and 4, Na-K ATPase activity and GSH levels were significantly different when compared to groups 1 and 2. Histopathological scoring and degree of injury failed to demonstrate any statistical difference. **Conclusion:** We suggest that CZ and PTX treatment in the ischemic and early reperfusion periods can improve muscle viability and antioxidant capacity.

Key Words: Reperfusion injury; pentoxifylline; cilostazol; bencyclane

ÖZET Amaç: Akut bacak iskemisi; arteriyel trombozis, tıkayıcı periferik arter hastalığı veya arteriyel rekonstrüktif cerrahi sırasında arteriyel klempleme ile ortaya çıkabilen klinik bir durumdur. Biz bu çalışmada, sıçan iskelet kası modelinde Bensiklan hidrojen fumarat (BF), Pentoksifilin (PTX) ve Silostazolun(CZ) iskemi reperfüzyon (I/R) hasarını azaltması üzerine olan etkilerini araştırdık. **Gereç ve Yöntemler:** Arka bacak I/R hasarı modelinde 28 yetişkin erkek Wistar sıçan kullanıldı. Hayvanlar seçici olmaksızın eşit sayıda (7) dört gruba ayrılarak 2 saat iskemi sonrası 4 saat reperfüzyona maruz bırakıldı. Grup 1: her iki arka bacağa iskemi-reperfüzyon uygulanan kontrol grubu; Grup 2: BF + IR, BF uygulanan I/R grubu; Grup 3: PTX + IR, PTX uygulanan I/R grubu; Grup 4: CZ + IR, CZ uygulanan I/R grubu. Tüm gruplarda ilaçlar verildikten sonra I/R uygulandı. Biyokimyasal ve histolojik değerlendirmeler için kan örnekleri ve doku biyopsileri alındı. **Bulgular:** Tedavi edilen gruplarda, doku MDA ve serum/doku katalaz düzeyleri kontrol gru-buna göre anlamlı bulundu. Grup 3 ve 4'teki SOD düzeylerinde kontrol gurubuna göre anlamlı bir artış vardı. Grup 2 ve 4 arasındaki SOD düzeyleri arasındaki fark anlamlıydı. Grup 3 ve 4'teki Na-K ATPaz aktivitesi ve GSH düzeyleri Grup 1 ve 2'ye göre anlamlı farklıydı. Histopatolojik skorlama ve hasar derecesinde gruplar arasında istatistiksel anlamlı bir fark yoktu. **Sonuç:** CZ ve PTX'in iskemi ve erken reperfüzyon tedavisinde kas canlılığını ve antioksidan kapasiteyi düzelttiğini söyleyebiliriz.

Anahtar Kelimeler: Reperfüzyon hasarı; pentoksifilin; silostazol; bensiklan

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Peripheral arterial occlusive disease (PAOD) is a common manifestation of atherosclerosis. The prevalence of PAOD continues to increase, with recent data suggesting that almost 30% of patients are under risk.[1] The alternatives for the treatment of PAOD are rapidly expanding. These new therapeutic options include pharmacotherapy, improved surgical techniques, and endovascular interventions. The appropriate initial therapy for patients having claudication is the medical therapy which will be sufficient for the majority of them. Many agents have been tried but few drugs have demonstrated efficacy in adequately designed, placebo-controlled trials.[2] Generally, all these classes of drugs have been thought to increase oxygen delivery to skeletal muscles or increase the efficiency of oxygen utilization. Bencyclane hydrogen fumarate (BF) has been used as a vasodilator drug for the treatment of peripheral arterial occlusive disease.[3] Pentoxifylline (PTX) has been widely used to treat intermittent claudication but had conflicting clinical results.[4,5] Cilostazol (CZ) has improved absolute claudication distances in randomized, double-blind, placebo controlled trials.[6] Acute limb ischemia is a common clinical event that may be caused by arterial thrombosis, PAOD or arterial clamping during arterial reconstructive surgery. Reperfusion of ischemic muscle increases the hazardous effect of early ischemic injury by the release of reactive oxygen species (ROS). ROS sources include the electron transport chain, oxidant enzymes such as xanthine oxidase, and myeloperoxidase (MPO). ROS may cause cellular damage by peroxidation of membrane lipids, sulfhydryl enzyme inactivation, protein cross-linking and DNA breakdown.[7] In order to counter this potential damage, organisms have enzymatic such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) and nonenzymatic (tocopherols, carotenes, ubiquinol, glutathione, ascorbic acid) antioxidant defence mechanisms.[8] Oxidative stress occurs when there is either an over production of ROS or a decrease in the antioxidant status.

In the current experimental study, we investigated the effects of BF, PTX and CZ in the attenuation of ischemia reperfusion (I/R) injury on skeletal muscle in a rat model.

MATERIAL AND METHODS

STUDY GROUPS

The study was conducted on 28 male adult Wistar rats weighing 300-350 g. The protocol for this study was approved by the Ethics Committee of Mersin University School of Medicine, which follows the National Institutes of Health Guidelines of Care and Use of Experimental Animals.

The rats were randomly divided into four equal groups. Group 1 (I/R control group) rats were subjected to ischemia for 2 h following a 4 h reperfusion period, while group 2 (BF-treated group) animals were pre-treated with 50 mg/kg per day of oral BF *(*Angiodel®, Organon, Turkey), group 3 were pre-treated with 50 mg/kg per day of oral PTX (Trental® Aventis, Istanbul, Turkey), group 4 were pre-treated with 50 mg/kg per day of oral CZ (Pletal® Abdi Ibrahim, Istanbul, Turkey) for 4 weeks via an orogastric tube twice a day prior to I/R.

RAT SKELETAL MUSCLE I/R MODEL

All of the rats were anaesthetized with ketamine hydrochloride 30 mg/kg intramuscular (Ketalar®, Pfizer, Istanbul,Turkey). Latex tourniquets were applied both hind-limbs, proximal to trochanter major in all animals. Following 120 min of ischemia, the tourniquets were removed allowing 240 min of reperfusion. At the end of the reperfusion period, the rats were sacrificed by a high intraperitoneal dose of thiopentone sodium injection. Blood samples were drawn from the left ventricles. Malondialdehit (MDA), GSH, SOD, CAT, MPO, Na-K ATPase and nitric oxide (NO) levels were determined in tissue biopsies and blood samples. Soleus muscle tissue samples were also taken for histological examination.

BIOCHEMICAL ASSAYS

Measurement of MPO: The MPO activity was measured as a simple quantitative method of detecting leucosequestration. Tissue (300 mg) was homogenized in 0.02 M EDTA (pH: 4.7) in a Teflon Potter homogenizer. Homogenates were centrifuged at 20 000 g for 15 min at + 4 ^{0}C. The pellet was re-homogenized in 1.5 ml 0.5% hexadecyltirimethylammo-

nium bromide (HETAAB) prepared in 0.05 M KPO4 (pH: 6) buffer, then re-centrifuged at 20 000 g for 15 min at + 4^{0}C. The determination of serum and supernatant tissue MPO activity depends on the fact that it reduces o-dianisidine which can be measured at 410 nm in a spectrophotometer.[9] Serum MPO values were expressed as U/mg protein while the tissue values were expressed as U/gram tissue.

Determination of Nitrate and Nitrite (NO_X): The levels of nitrite and nitrate in serum and tissue were analyzed by a photometric endpoint determination (Nitrite/Nitrate, colorimetric kit, Roche Diagnostics, Mannheim, Germany). Nitrate is reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate in the presence of the enzyme nitrate reductase. The nitrite which was formed reacts with sulphanilamide and N-(1-naphtyl)-ethylene-diamine dihydrochloride to give a red-violet diazo dye. The diazo dye is measured on the basis of its absorbance in the visible range at 540 nm. The level of NO_x was marked as μmol/l. Nitrite plus nitrate is known as NO_x. Therefore, serum and tissue nitrite+nitrate can be used as an indirect indicator for NOx.

Measurement of MDA: Tissues were homogenized in 0.15mM KCI for MDA determination. After the homogenate was centrifuged at 3000 rpm, the MDA levels in the supernatant and 50 uL serum were determined by the thiobarbituric acid reaction according to Hiroshi and Yagi. The principle of the method depends on the colorimetric measurement of the intensity of the pink color produced by the interaction of the thiobarbituric acid with MDA. The colored reaction 1,1,3,3 tetraetoxypropane was used as the primary standard.[10,11] Serum and tissue MDA values were expressed as nmol/ml.

Determination of serum and tissue CAT activity: Serum and tissue CAT activity was performed as described previously.[12] CAT catalyses the break down of hydrogen peroxides. This reaction is monitored in UV spectrum at 240 nm wavelength by a spectrophotometer. The decrease in absorbance is proportional to the activity of CAT. Serum and tissue CAT values were expressed as IU/ml while the tissue values were expressed as IU/mg total protein. Protein amount was measured by Lowrys method.[13]

Determination of SOD activity*:* Serum SOD assay was performed as described previously.[14] SOD enzyme activities were expressed as international units. One unit of SOD activity was defined as the amount of enzyme protein causing 50% inhibition in nitroblue tetrazolium reduction rate, and results were expressed as unit/mg protein. Protein amount was measured by Lowrys method.[13]

Determination of erythrocyte Na^+-K^+ ATPase activities: Measurement of ATPase specific activity is based on the principal of the inorganic phosphate released in 1 h for each milligram protein in the presence of 3mM disodium ATP, added to the incubation medium. The inorganic phosphate released from the ATP to the incubation medium, was measured according to the method suggested by Reading and Isbir.[15,16] The protein quantity contained in samples was determined according to the method of Lowry et al.[13] The results of the ATPase enzyme systems were expressed in nmol Pi mg $^{-}$1 protein/h.

Determination of Whole Blood reduced Glutathione: Venous blood was collected into tubes containing EDTA as an anticoagulant. GSH were measured by fluorometric high performance liquid chromatography (Hewlett Packard 1100) using Chromosystems calibrators and kits (Chromosystems Instruments and Chemicals GmbH, Munich, Germany). Blood GSH values were expressed as mmol/L.

Histopathological examination: Muscle tissue samples that were initially fixed in 2.5% gluteraldehyde were then postfixed in 2% (weight per volume) osmium tetroxide, dehydrated in ethanol, treated with propylene oxide, and embedded in Spurr's epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL JEM 1011 1200EX electron microscope (Jeol Corp, Tokyo, Japan). 70 nm sections were taken with ultramicrotome after twenty four hours polymerization at 70 ^{0}C. Sections were contrasted with uranyl acetate and lead citrate. Ultrastructural damage to the muscle tissue was recorded in a blind manner. The intracellular and ex-

tracellular edema, mitochondria, nuclei, capillaries and myofibrils were analyzed separately in each biopsy specimen by a semi-quantitative method with a scoring from 0 (unchanged) to 3 (severe alterations). A total score of all ultrastructural changes less than 5 were defined as slight damage, scores ranging from 5 to 10 were defined as moderate and scores exceeding 10 were defined as severe ultrastructural damage.

STATISTICAL ANALYSIS

Statistical analysis was performed with SPSS software package, version 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Biochemical results were controlled for normal distribution by Kolmogorov-Simirnov test and according to test result all data were normally distributed. For all biochemical data, statistical analysis was performed using repeated measurements of ANOVA followed by *post-hoc* analysis with the Tukey test to detect differences between the groups. Results were expressed as mean ± standard deviation (SD). A p-value < 0.05 was considered as significant.

RESULTS

BIOCHEMICAL ANALYSIS

Serum MDA levels in the treated groups were not significantly different compared to the control group. In serum MDA levels, no statistically significant differences were found among treated groups (p= 0.271). In the treated groups, tissue MDA levels were found to be decreased when compared with the control group and the difference between these groups was significant (p< 0.05, Figure 1). There was no statistically significant difference among the treated groups.

Serum and tissue MPO levels in the treated groups were not significantly different when compared to the control group and there was no statistically significant difference among the treated groups. With regard to the serum and tissue nitrite/nitrate levels, there were no statistically significant differences among any of the groups of rats (p> 0.05). In the treated groups, serum and tissue CAT levels were found to be increased when compared with the control group and the difference between

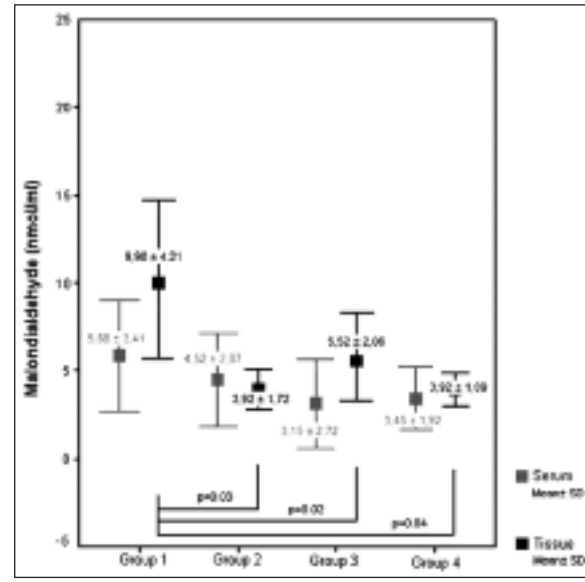

FIGURE 1: Serum and tissue Malondialdehyde levels in all groups. Data were expressed as mean ± SD.

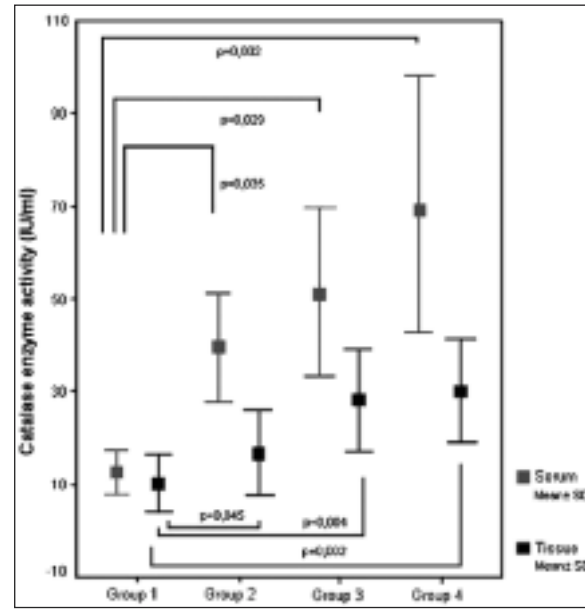

FIGURE 2: Serum and tissue Catalase enzyme activity in all groups. Data were expressed as mean ± SD.

these groups was significant (p< 0.05, Figure 2). In groups 3 and 4, serum SOD levels were significantly increased compared to control group (p< 0.05, Figure 3). Also, SOD values in the sera were found to be significant between the groups 2 and 4 (Figure 3). In groups 3 and 4, Na-K ATPase activity and erythrocyte GSH levels were found to be increased when compared to that of groups 1 and 2

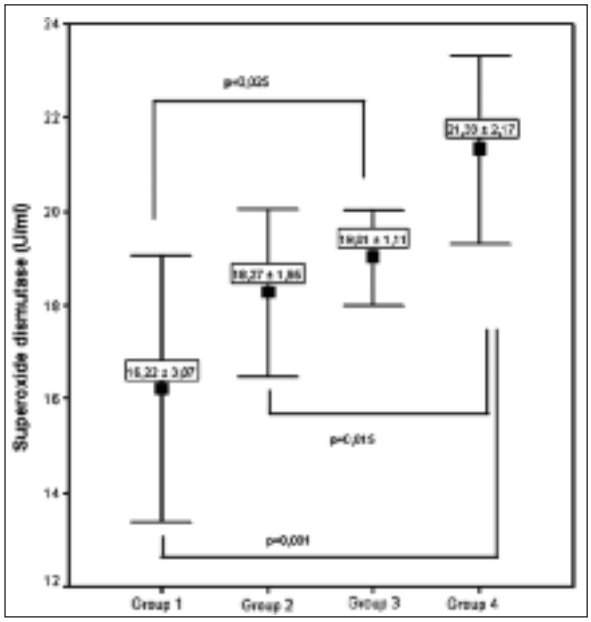

FIGURE 3: Serum superoxide dismutase levels in all groups. Data were expressed as mean ± SD.

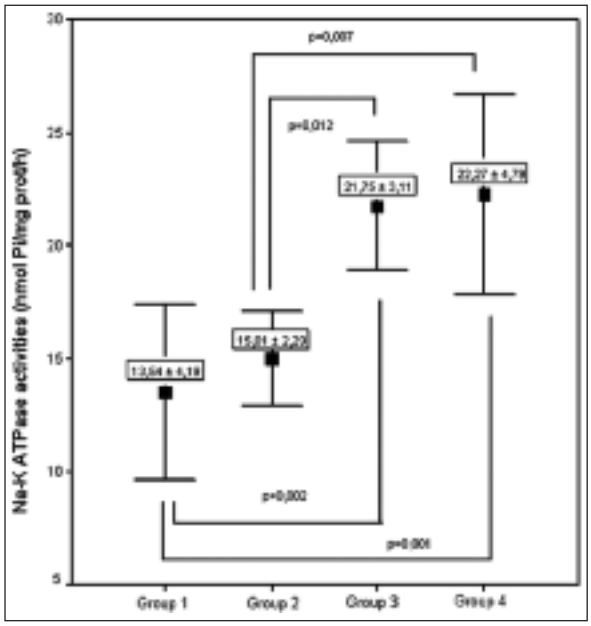

FIGURE 4: Serum Na-K ATPase activities in all groups. Data were expressed as mean ± SD.

and the differences between these groups were turned out to be significant ($p < 0.05$, Figures 4, 5).

HISTOPATHOLOGICAL RESULTS

The ultrastructural evaluations performed on the treated groups unveiled a similarity between the control group in terms of the mild and moderate degree injuries however group 3 and group 4 were affected less in terms of severe injuries. Table 1 demonstrates clearly that this is basically associated with the preservation of the mitochondrial and the myofibrillar structure and the edema. However this difference did not prove to be statistically significant.

Cisternal expansion of sarcoplasmic reticulum was found in all groups. There were many mitochondrial swelling. Expanded cisternal structures were found settled among the myofibrils and sarcomers (Figure 6). The cristae of mitochondria were dissolved and disappeared. Parts of myofibrils were observed to be relaxed. Histopathological scoring and degree of injury were shown in Table 2. Unfortunately we were unable to demonstrate any statistically significant difference.

DISCUSSION

Up to date, different pharmacological agents such as N-acetylcysteine, Vitamins C and E, melatonin, L-Arginine, carbenoxolone and ilioprost have been used in the treatment of I/R injury of skeletal muscle both clinically and experimentally.[17-21]

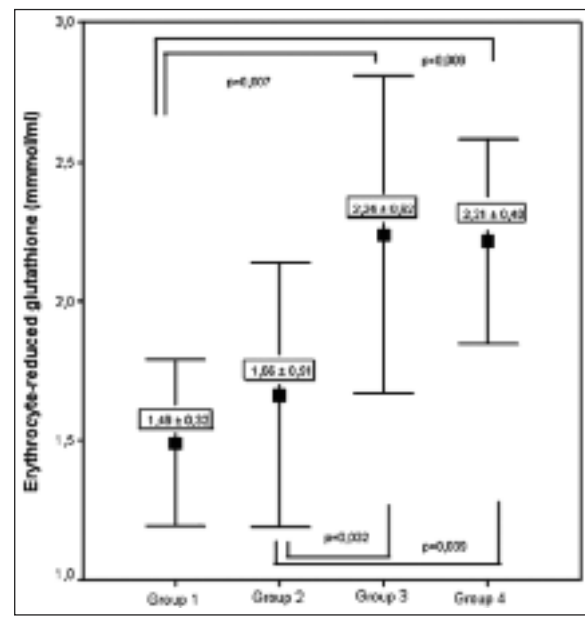

FIGURE 5: Serum eryhrocyte reduced glutathione levels in all groups. Data were expressed as mean ± SD.

TABLE 1: Histopathological evaluation of biopsy specimens.

	Group 1	Group 2	Group 3	Group 4	P
Edema	1.14 ± 0.89	1.71 ± 0.75	1.28 ± 0.95	1.14 ± 1.0	NS*
Nuclei	1.14 ± 1.0	1.42 ± 1.2	1.28 ± 0.95	1.57 ± 0.9	NS
Myofibrils	1.71 ± 0.7	1.57 ± 0.78	1.28 ± 0.95	0.85 ± 0.69	NS
Capillaries	0.85 ± 0.69	0.82 ± 0.69	0.57 ± 0.53	0.71 ± 0.48	NS
Mitochondria	1.57 ± 1.1	1.0 ± 1.1	1.14 ± 1.0	1.0 ± 1.1	NS

NS: Nonsignificant Statistical analysis was performed by using the ANOVA test followed by the Tukey post hoc test. Data were expressed as mean ± SD. *P< 0.05 control versus treated groups.
NS: Nonsignificant.

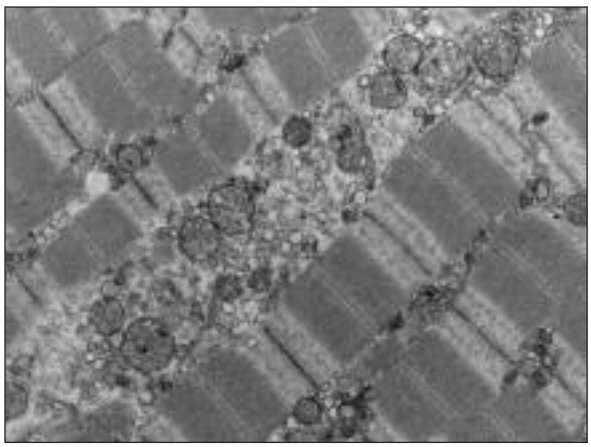

FIGURE 6: Electron microscopic view of myofibrils revealed expanded cisternal structures settled among the myofibrils, sarcomeres and Z band destructions. (uranyl acetate and lead citrate, X 12000).

TABLE 2: Histopathological scoring and degree of injury.

Ultra-structural damage	Group 1	Group 2	Group 3	Group 4	P
Slight	2	5	6	7	NS*
Moderate	14	12	13	13	NS
Severe	4	3	1	1	NS

Statistical analysis was performed by using the ANOVA test followed by the Tukey post hoc test. *P> 0.05 control versus treated groups. NS: Nonsignificant.

In our study, we investigated the effects of BF, PTX and CZ in the attenuation of I/R injury on skeletal muscle in a rat model.

BF, which is one of the drugs used in the treatment of PAOD, has been shown to protect intestinal ischemia reperfusion injury in a rat model but there is no published data for I/R of the skeletal muscle. Possible potential properties of BF are positive effects on free radical scavengers and inhibition of neutrophil accumulation.[22] Albeit we were unable to demonstrate the effect of BF on the activation of the neutrophils, the presence of lower MDA levels in the sera and the tissue samples; increased CAT and SOD in comparison with the control group and removal of a hydroxyl radical source, which is extremely oxidant, from the medium may be helpful in explaining this function. On the other hand in studies concerning PTX and CZ; PTX exhibits marked anti-inflammatory properties by inhibiting cytokine production. It inhibits lipopolysaccharide-induced production of tumor necrosis factor-α by monocytes and T cells and interleukin-2 induced adherence of leukocytes.[23]

CZ has been shown to have possible attenuating effects on the inflammatory cascade from review of in vitro, animal and case-controlled human studies. Hakaim et al.[24] demonstrated that a CZ infusion 10 min before re-vascularising an ischaemic leg prevented anterior compartment pressure increases. In addition, Otsuki et al.[25] reported an inhibition of tumor necrosis factor-alpha and vascular cell-adhesion molecule-1 mediated products with CZ. This inhibition may be via cAMP elevation in human vascular endothelial cells, thus preventing atherosclerosis. O' Donnell et al.[26] reported that CZ significantly attenuates an exercise-induced host-inflammatory response in patients with PAOD with significant improvements in levels of lipid hydroperoxides, β-carotene, P-selectin and cell-adhesion molecules. They also pointed out that the mechanism of action for CZ still unclear, free-radical generation and antioxidant consumption are key pathways for the modulation of host inflammatory response in patients with intermittent claudication. Afore mentioned studies suggest that these two drugs, as in BF, put forward their effects by blocking the oxido-inflammatory process. In our study, albeit the NOx and the MPO values both in the sera and the tissue samples did not reach to a significant difference in comparison with the control group, the lower sera MDA levels and statistically significant tissue MDA levels, that stands for the reflection of the oxidative injury, in the treated groups denotes that enzymes other than

the MPO and iNOS might be effective in the oxidative stress and/or the drugs are effective on the antioxidant system. In this study, increased CAT values in the sera and the tissue samples of the treated groups reflect the common properties of these drugs on the antioxidant system however increased levels of the serum SOD, erythrocyte GSH and the Na-K ATPase were only demonstrated in the CZ and the PTX groups. Moreover low rates of severe ultrastructural changes that have been observed during histopathological evaluation in the groups treated with CZ and PTX also supports this idea. The outcomes of the ultrastructural investigations that brought forward similar findings between the control and the treated groups in terms of mild and moderate injuries and the decreased presence of more severe injuries in groups 3 and 4 are strong supporters of these findings. Evaluation of table 1 may explain this difference by putting forward the preserved structure of the mitochondria and the myofibrils and edema however the difference did not reach to a statistical significance. These results might be related with the relatively less number of the experimental subjects.

CONCLUSION

In our study, BF, PTX and CZ agents have been studied for comparing their effects on acute skeletal muscle I/R injury. According to our results, we suggest that CZ and PTX treatment in the ischemic and early reperfusion periods can improve muscle viability and antioxidant capacity.

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