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Effects of Cu/Zn Superoxide Dismutase on Strain Injury-Induced Oxidative Damage to Skeletal Muscle in Rats

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Summary

This study was designed to determine whether the supplement of superoxide dismutase (SOD) could attenuate strain-induced oxidative damage to skeletal muscle in rats. Experimental animals were injured in right gastrocnemius muscles by a strain injury model. SOD-treated groups were given Cu/Zn SOD 10 000 U/kg body weight per day since injured, while control groups were given normal saline. Parameters of antioxidant and muscle damage were detected in plasma 3 and 7 days postinjury. The injured muscles were removed and fixed for histology observation and immunohistochemistry assay of desmin. The results showed that plasma levels of SOD, glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC) in SOD group were significantly higher than in the saline group on day 3 or 7, while the plasma creatine kinase (CK) and malondialdehyde (MDA) were lower in the SOD group than in the saline group. The histological examination of muscle sections revealed a lower degree of damage in the SOD group in which the expression level of desmin was higher than in the saline group. It is suggested that SOD supplement may attenuate strain-induced muscle damage and facilitate its regeneration.

Key words
Superoxide dismutase • Skeletal muscle • Injury

Introduction

Skeletal muscle injuries often occur in professional and recreational sports or daily activities. Most of them are strain injuries caused by an episode of sudden stretch. These injuries often cause significant morbidity and result in much time lost for training and competition. Eccentric actions can result in disruption to connective tissue (Stauber *et al.* 1990) and diverse myocellular components including sarcolemma, myofibrils and cytoskeleton (Friden *et al.* 1983, Tidball *et al.* 1993). After initial injury, oxidative stress could be increased by the presence of neutrophils and macrophages in muscles. The production of free radicals by such infiltrates can damage lipid membranes and produce cell necrosis (Halliwell and Chirico 1993). Lipid peroxidation appears to be an important mechanism underlying exercise-induced muscle damage. Although it has been demonstrated that antioxidant enzymes increase following oxidative stress and exercise training (Duthie *et al.* 1990, Gohil *et al.* 1988), this increase in antioxidant defenses might not be physiologically proportionate to the needs created by the increase in prooxidant events. Thus exogenous enzymatic antioxidant may play a

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protective role in muscle cells by reducing associated oxidative damage to lipids, nucleic acids, and protein.

Superoxide dismutases (SODs) are important enzymes that are implicated in the regulation of cellular antioxidant defenses. SOD induction may be important for cell defense against oxidative stresses such as inflammation and the action of redox-active compounds (Hassan 1988). This study was conducted to investigate whether exogenous Cu/Zn SOD (Cu/Zn superoxide dismutase) can attenuate the strain-induced damage to skeletal muscle and facilitate its repair in rats.

Methods

A total of 40 adult male Sprague-Dawley rats weighing 250 to 300 g were used in this study. All animals were housed in cages and allowed free access to standard rat food and water. They were divided randomly into three groups. One group (n=8) served as the uninjured group. The other two groups (n=16) were designed separately as injured+saline group (saline group) or injured+SOD group (SOD group).

The experimental procedures were reviewed and approved by the Peking University Health Science Center for Animal Care and Use Committee.

Development of an animal model for skeletal muscle strain injury

The model used was developed in rats according to the method described by Almeskinders and Gilbert (1986). A controlled strain injury of the gastrocnemius muscle was produced on the right hindlimb of saline and SOD group while a sham operation was performed on the right hindlimb of uninjured group. The animals were anesthetized with chloral hydrate (400 mg/kg) injected intraperitoneally. The right hindlimb was shaved and the distal tendon of the gastrocnemius muscle was exposed and separated from other tendons and tissues through a 0.5 cm incision, which was kept moist by 37 ^{o}C normal saline. The rat lay on the back on a specially adapted platform attached to the materials testing machine (Bei Hang University, Peking). A needle was inserted transversely through the distal femur of the right leg and the needle was secured to a frame on the machine. The distal tendon of gastrocnemius was connected to the load cell by a surgical silk and the muscle was pulled at a speed of 6 cm/min under the control of a computer until the horizontal plateau of recorded load-elongation curve was reached. Then the needle and the surgical silk were immediately removed and the gastrocnemius muscle was gently laid back into its bed. Subsequently, the incision was closed after desinfection. Gastrocnemius muscles of the uninjured group were treated by the same procedure but without the strain injury. Each animal was given benzylpenicillin for 3 days to avoid infection and allowed to feed and move freely. In this model of muscle strain injury, experimental injury was limited in degree to the plastic region of muscle deformation. This simulated the clinical situation where most common injuries to the musculotendinous region are partial tears but not complete ruptures (Obremsky *et al.* 1994).

Experimental protocol

In the SOD group, the animals were given Cu/Zn SOD (Peking Chemical Reagents Company, Peking) 10 000 U/kg body weight per day by intraperitoneal injection since the day of injury. The saline group received an equal volume of normal saline as the SOD group. Eight randomly selected animals in the saline and SOD group were sacrificed 3 or 7 days after the injury, while the uninjured group was sacrificed 3 days after the sham operation. Blood obtained from the descending aorta of rats on day 3 and 7 was centrifuged immediately and the plasma fraction was frozen at –80 °C until analysis. We measured the superoxide dismutase (SOD) activity in the plasma to observe the changes of its blood concentration. The antioxidant enzyme, glutathione peroxidase (GSH-Px) and total antioxidant capacity (T-AOC) values were measured to detect the antioxidant activity. Plasma creatine kinase (CK) and malondialdehyde (MDA) were determined as indicators of oxidative damage. The right gastrocnemius muscles of each group were removed and fixed on day 3 and 7 for histology examination or immunohistochemistry assay.

Chemical analysis

Plasma SOD was assayed by the method of McCord and Fridovich (1969) Briefly, SOD was detected on the basis of its ability to inhibit the superoxide-mediated reduction of ferricytochrome c by xanthine oxidase and xanthine. GSH-Px activity was determined by a modified version of Flohe and Gunzler (1984) at 37 ^{o}C. One unit of activity is equal to the mM of NADPH oxidized/min/mg protein. To determine the T-AOC, the luminal-enhanced chemiluminescence was used to follow the peroxyl radical reaction (Whitehead *et al.* 1992). Antioxidant capacity was determined relative to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic

acid (Trolox), a soluble vitamin E analogue, and expressed as Trolox equivalents (Trolox Eq.). Plasma CK levels were analyzed using a detecting kit obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, PR China). The level of plasma MDA was measured by thiobarbutiric method modified by Yagi (1984) Peroxidation was measured as the production of MDA which in combination with thiobarbituric acid forms a pink chromogen compound whose absorbance was measured at 532 nm.

Immunohistochemistry assay

After anesthesia and perfusion of phosphate-buffered 4 % paraformaldehyde through the aorta, the medial head of right gastrocnemius muscles was removed and immersed in freshly prepared buffered formalin. The fixed muscle samples were embedded routinely in paraffin and cut into 6 μm sections. The expression of desmin was monitored by immunohisto-chemistry using specific antibodies (Bornemann and Schmalbruch 1992). The first antibodies used to detect desmin were rabbit antidesmin antibodies (Sigma Immunochemicals, St. Louis, MO) at a dilution of 1/100 overnight. Following several rinses in phosphate-buffered serum saline (PBS), a goat antirabbit was used at a dilution of 1/200 for 2 h. The visualization of the immunohisto-chemistry was done by microscopy.

The amount of desmin immunoreactivity was computerized as index of regeneration. To measure the area of desmin expression/field under microscope, 33-36 random fields (equal size area 331647.57 μm^2) were selected for each group. Images were rendered to monochrome, the features extracted, and the absolute area of desmin-positive staining collected in each image field. We collected the positive area and the mean optical density of each selected field (LEICA Q550CM, LEICA Germany), and the product of their multiplication, called integral optical density, was used to indicate the expression level of desmin.

Data analysis

All data were expressed as mean ± S.D. Statistical analysis was performed by SPSS (Version 11.0) statistical software, and comparisons were made using one-way ANOVA. Significance was indicated by $P<0.05$ values.

Table 1. The values of plasma SOD, GSH-Px, T-AOC, CK, MDA of each group on days 3 and day 7 after the injury.

		Number of days after the injury			
	Uninjured (n=8) [e]	**3**		**7**	
		Saline (n=8)	**SOD (n=8)**	**Saline (n=8)**	**SOD (n=8)**
SOD (U/ml)	93.98±12.56	71.71±13.82 [a]	211.25±31.62 [a b]	89.49±22.12	229.07±33.52 [a b]
GSH-Px [c]	166.11±8.68	188.86±7.79 [a]	196.58±7.44 [a]	173.69±10.77	198.58±5.51 [a b]
T-AOC [d]	310.70±41.14	273.47±61.15	387.35±55.51 [a b]	328.83±45.73	477.81±50.45 [a b]
CK (U/l)	85.78±5.61	433.36±31.53 [a]	285.67±23.74 [a b]	207.19±32.95 [a]	153.30±21.91 [a b]
MDA (μmol/l)	3.11±0.74	16.29±2.04 [a]	9.17±2.77 [a b]	9.03±2.90 [a]	4.52±1.33 [b]

[a] Statistically significant differences vs. uninjured group ($P<0.05$). [b] Statistically significant differences vs. saline group at the same time points ($P<0.05$). [c] The results of GSH-Px was expressed as μmol NADPH oxidized/min/mg protein. [d] The results of T-AOC was expressed as μmol Trolox Eq/l. [e] n indicate the number of animals in each group.

Results

Table 1 shows the values of plasma SOD, GSH-Px, T-AOC, CK, and MDA of each group 3 and 7 days after the injury.

Plasma SOD

The plasma SOD level decreased significantly in the saline group compared to the uninjured group on day 3, while no difference versus the uninjured group was found on day 7. Plasma SOD in the SOD-treated group was markedly higher than in the uninjured and saline group on days 3 and 7, being more than double of the uninjured level (Table 1).

Effects of SOD supplementation on plasma GSH-Px and T-AOC

The GSH-Px values were significantly higher on

day 3 in both the saline and SOD group compared with the uninjured group. On day 7, the plasma GSH-Px in SOD group was significantly higher than in the uninjured and saline group. It is suggested that CuZnSOD administration raised the GSH-Px level in the plasma after the strain injury. The plasma T-AOC showed no significant changes after injury on days 3 and 7 in the saline groups, but the T-AOC levels in the SOD groups were notably higher than the sham and saline groups on days 3 and 7 (Table 1).

Effects of SOD supplementation on plasma CK and MDA

Plasma CK and MDA levels were found to be significantly higher in the saline and SOD group than in the uninjured group on day 3 after the injury. Both of them decreased on day 7, but were still higher than the uninjured group except for the plasma MDA in SOD group, which decreased to the uninjured level. In the SOD groups, CK and MDA levels were lower than in the saline group on days 3 and 7. This indicates that to some extent, the administration of SOD suppressed the increase of CK and MDA levels (Table 1).

Histological evaluation

Histological observation showed limited rupture of most distal fibers near the musculotendinous junction in the saline and SOD groups on day 3 after the injury. In sections from the saline group, there was intense inflammation with limited muscle fiber necrosis, infiltration of leukocytes, tissue edema, and hemorrhage. The SOD group also showed obvious inflammation, but the necrosis and infiltration of inflammatory cells were not as serious as in the saline group.

There were still manifestations of inflammation in sections from the saline group on day 7 after the injury. In some local fields, active phagocytosis of necrotic muscle cells could still be found. There was some muscle regeneration present, but most of the myotubes were small with little cytoplasm. In the sections from SOD, group, the area of inflammation was smaller than in the saline group and the regeneration was very active. Numerous myotubes were present with well-developed cytoplasm in several areas.

Effects of SOD supplementation on desmin expression

Table 2 shows integral optical density of desmin stained sections in each group. In sections from the uninjured group, desmin immunostaining was positive with a normal striated pattern. After injury, the desmin expression in both saline and SOD groups was notably decreased on day 3 compared to the sham-injured group, but it was enhanced significantly on day 7 versus sham-injured group. The SOD group exhibited significantly stronger expression of desmin than the saline group on days 3 and 7 (Table 2).

Table 2. Integral optical density (IOD x 10^3) of desmin-stained sections in each group.

Group	Number of days after the injury	
	3	7
Uninjured	10.31±4.89 (n=36)	
Saline	6.35±2.16 [a] (n=33)	23.33±6.02 [a] (n=34)
SOD-treated	8.52±3.02 [a b] (n=35)	28.28±6.54 [a b] (n=33)

[a] Statistically significant differences vs. uninjured group ($P<0.05$).
[b] Statistically significant differences vs. saline group at the same time points ($P<0.05$). n indicates the number of selected fields, data are means ± S.D.

Discussion

Effects of SOD supplementation on the antioxidant parameters after injury

SOD and GSH-Px are two major antioxidant enzymes *in vivo*, which belong to the enzymatic defense mechanisms for overcoming the oxidative challenge. The plasma SOD decreased significantly on day 3 after the injury. Most investigators have observed increases in superoxide dismutase (SOD) activity in oxidative fibers in response to endurance training (Criswell *et al.* 1993, Powers *et al.* 1994), but others have found little or no change in SOD activity in skeletal muscle (Laughlin *et al.* 1990). However, little is known about the changes of SOD in the blood after injury to skeletal muscles. The decreased level of plasma SOD in our experiment may be due to the inhibition or oxidative inactivation of enzyme proteins in the plasma caused by excess generation of reactive oxygen species. The rise of GSH-Px on day 3 may be a compensatory mechanism among the antioxidant enzymes in response to increased oxidative stress. Similarly, the GSH-containing enzymes are also found to increase in response to oxidative stress caused by acute exercise and exercise training (Tessier *et al.* 1995).

The measurement of total antioxidant capacity (T-AOC) seems to be a convenient approach to disclose the combined action of the body's antioxidants

(Whitehead *et al.* 1992). This parameter can constitute a useful index of the capacity of a given compound or fluid to modulate the damage associated with the enhanced production of free radicals. There was no significant change in the plasma T-AOC after injury, which may reflect the contrary effect of the consumption of antioxidants due to the oxidative stress and the reactive increase of antioxidant substances *in vivo*.

Our administration of SOD evidently raised its concentration in the plasma, with more than a twofold increase on days 3 and 7. As observed in our study, SOD supplementation significantly enhanced the total antioxidant capacity in the plasma of injured rats. The enhancement of T-AOC may increase the resistance to oxidative cellular injury or facilitate the biosynthesis in cells which undergo sublethal injury (Rajguru *et al.* 1994). It might thus reduce undesired oxidation by infiltrating cells and/or could facilitate tissue repair.

Effects of SOD supplementation on the oxidative damage caused by strain injury

Elevations of plasma CK are characteristic responses to strenuous exercise and are often used as indicators of muscle damage. Many investigators suggest that this enzyme is a basic diagnostic indicator useful in early detection of changes caused by pathological damage of the muscle (Osame *et al.* 1994). In our study, we observed a major elevation of CK level in saline groups on day 3, and then it decreased on day 7. This suggests that our strain injury model had caused pathological damage to the gastrocnemius muscle. Moreover, the delayed rise in CK level may be a manifestation of secondary muscle damage (Faulkner *et al.* 1993). MDA is one of the major secondary oxidation products derived from polyunsaturated fatty acids so that the measurement of MDA has been regarded as an index of structural oxidative injury of the cell membrane. The increase in MDA suggests increased lipid peroxidation initiated by free radical reactions (Fantini and Yoshioka 1993). The elevation of MDA in our experiment could be a direct reflection of an oxidative injury of the skeletal muscle after passive stretch. The correlation between MDA and CK was similar to that reported in exercise-induced damage, which showed that the increase in plasma MDA significantly correlated with the increase in serum CK after an 80-km running race (Ji 1993). This relationship between muscle-enzyme release and biomarkers of oxidative stress might result from an increase in membrane permeability due to lipid peroxidation.

The CK and MDA levels in the SOD groups were lower than in the saline groups. We can conclude that the administration of Cu/Zn SOD may attenuate the stretch-induced damage to the gastrocnemius muscles. This may be due to the protective effect of SOD on the membranous structure in skeletal muscle tissue. We considered that the protective role of SOD in our experiment was related to its antioxidant property. The enzyme CuZn SOD, which catalyzes the conversion of superoxide radicals (O_2^-) into H_2O_2, plays an important role in the metabolism of oxygen free radicals. It catalyzes the first reaction in the removal of oxygen radicals, and thus prevents oxygen-induced cytotoxicity (Turrens *et al.* 1984). As observed in our study, the antioxidant capacity *in vivo* was enhanced by SOD supplementation. SOD may decrease the inflammatory reaction in tissue injury by reducing extracellular oxidative stress induced by infiltrating cells so that the damage to the muscle tissue is attenuated.

Effects of SOD supplementation on the regeneration

From the histology observation, we can see that the administration of SOD attenuates the degree of inflammation occurring in skeletal muscles after injury and facilitates the regeneration process. After injury, muscle undergoes a distinct set of healing phases, consisting of degeneration, inflammation, regeneration and fibrosis. Active muscle degeneration and inflammation occur in the first few days post-injury, whereas muscle regeneration usually occurs 7-10 days after the injury.

Desmin is a 52-kDa peptide synthesized in skeletal, smooth and cardiac muscles. It is anchored at the Z disc in striated muscle and links the fibers to each other and to the sarcolemma (Small and Sobieszek 1977). Many findings indicate that desmin is important for the integrity of myofibrils and tissue cohesion. It has been proposed that desmin forms a cytoplasmic lattice of filaments linking individual myofibrils laterally, linking myofibrils to organelles such as mitochondria, and linking myofibrils to the sarcolemma (Gard and Lazarides 1980). Because it has been found to be uniformly expressed in regenerating myofibers (Bornemann and Schmalbruch 1992). the characterization of desmin expression in the injured muscle was used as an approach to evaluate the level of muscle regeneration following injury.

Based on our results of the immuno-histochemical study, we can see that administration of

SOD after injury may enhance the expression of desmin on days 3 and 7. The decrease level of desmin expression on day 3 may reflect the destruction of myocytes (Barash *et al.* 2002). SOD appeared to attenuate such damage so that a higher level of desmin was observed in SOD group than in the sham group. The rise of desmin expression on day 7 after the injury in saline groups revealed its participation in muscle regeneration. The expression level of desmin seemed to be enhanced by the SOD supplement. This result indicates that early SOD treatment after injury dy may be beneficial for the regeneration of injured muscles. We suppose that based on its antioxidant nature, SOD may reduce the peroxidant content in the cytosol and interstitial space in muscle tissue. Such an effect may facilitate the regeneration process including protein synthesis.

Tissue damage induces directional migration of polynuclear leukocytes and macrophages into the extravascular parenchymal tissue, and initiates fibroblast recruitment and proliferation. Extravascular neutrophils and macrophages are stimulated by contact with degraded collagen to release free radicals and proteases. Previous studies have linked the anti-inflammatory effects of SOD to its ability to impair leukocyte migration *in vitro* (Michelson 1987, Vaille *et al.* 1990). By such an effect, SOD may interfer with the infiltration of inflammatory cells and attenuate the damage in injured muscle.

In conclusion, the supplement of SOD after strain-induced injury may reduce the degree of damage in the skeletal muscle and facilitate its healing. It is suggested that SOD may serve as an auxiliary drug in the treatment of strain-induced injury to skeletal muscles.

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