EFFECT OF CRUSH AND AXOTOMY OF PHRENIC NERVES ON OXIDATIVE STRESS IN DIAPHRAGM MUSCLE OF RATS

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ABSTRACT: *Introduction*: In this study we investigated the effect of crush and axotomy of phrenic nerves on oxidative stress and antioxidant enzyme activities in rat diaphragm muscle. *Methods*: The animals in the first group were not crushed or axotomized and served as controls. Phrenic nerves of the rats in the second and third groups were crushed or axotomized in the diaphragm muscle. *Results*: The malondialdehyde level increased in diaphragm muscles after both crush and axotomy. The antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, carbonic anhydrase, and catalase, decreased in diaphragm muscles after both crush and axotomy. *Conclusions*: Crushing or axotomizing the phrenic nerves may produce oxidative stress in the diaphragm muscle of the rat by increasing lipid peroxidation and decreasing antioxidant enzyme activities.

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Crush of a peripheral nerve induces cellular and molecular changes in the injured neurons and in the microenvironment of their axon stumps, which together support axonal regeneration.[1] Increased levels of oxidative stress have been shown to be involved in many different pathological conditions, such as chronic obstructive pulmonary disease,[2] atrophic muscles and sepsis-induced muscle dysfunction,[3] and protein diaphragm oxidation after prolonged mechanical ventilation.[4] Although reactive oxygen species (ROS) are normally required in multiple cell processes, such as in excitation–contraction coupling,[5] they may also overwhelm tissue antioxidant capacity when produced at high levels during inflammatory–immune conditions. Specifically, in skeletal muscle fibers, ROS are normally synthesized at low levels and are required for normal force production.[6] Investigators have shown that a major source of ROS in muscle is the motochondria and that oxidant flux in muscle is directly related to the rate of mitochondrial oxygen consumption.[7] If skeletal muscle production of ROS is enhanced by the maturational increase in mitochondrial capacity and is not matched by an increase in antioxidant enzyme defense, then oxidative stress may result and contribute to an increased susceptibility to fatigue in the adult.

Aerobic organisms are protected from oxygen toxicity by a natural antioxidant defense system involving enzymatic and non-enzymatic mechanisms.[8] The increased formation of ROS and decreased antioxidant defense is defined as oxidative stress, which is widely recognized as an important feature of many diseases. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), carbonic anhydrase (CA), and catalase (CAT) are the cellular antioxidant enzymes that protect cells from oxidative stress. Lipid peroxidation (LPO) is one of the most important expressions of oxidative stress induced by ROS. Malondialdehyde (MDA) is an indicator of lipid peroxidation and increases in various diseases.[9]

Oxidative radicals have been implicated in a broad range of neoropathological conditions, such as ischemia, edema, infarction, seizure disorders, aging, and Alzheimer and Parkinson diseases. Although there have been some new developments linking free radicals to a variety of central nervous system diseases, it is extremely difficult to establish whether they actually cause the disorders or represent byproducts of tissue destruction caused by other primary factors.[10]

Some surgeons deliberately crush the phrenic nerve to allow the diaphragm to rise up to minimize the post-pneumonectomy and post-lobectomic spaces in the thoracic cavity. This allows pulmonary expansion and residual post-pneumonectomy pleural cavity obliteration. In cases of phrenic paralysis and crush, the diaphragm muscle may be seriously injured, leading to respiratory disorders. As a part of this study we have demonstrated that crushing or axotomizing the phrenic nerves may produce oxidative stress by increasing lipid peroxidation and decreasing antioxidant enzyme activities in this nerve.[11] Therefore, our study was designed to investigate whether crushing or axotomizing phrenic nerves may also produce oxidative stress in the diaphragm muscle of rats.

Abbreviations: CA, carbonic anhydrase; CAT, catalase; GSH-Px, glutathione peroxidase; LPO, lipid peroxidation; MDA, malondialdehyde; NADPH, nicotinamide adenine dinucleotide phosphate; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid

Key words: antioxidant enzyme, diaphragm muscle, oxidative stress, phrenic nerve degeneration, rat

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METHODS

Treatment of Animals. Eighteen male Wistar albino rats, aged approximately 6 months and weighing 180–200 g, were obtained from the animal laboratory of Yuzuncu Yıl University. Rats were housed in individual cages. A 12-h light/dark cycle was maintained, and the rats were fed ad libitium. The study was approved by the local ethics committee of Yuzuncu Yıl University.

The cervical phrenic nerve was axotomized with a midline incision while the animals were in the supine position under ketamine (50 mg/kg) anesthesia. The unilateral (left) phrenic nerve denervation, the side of which was selected at random, was carried out by cutting the insertion (5–8 mm length) of the main phrenic nerve into the thoracic cavity, which receives fibers from the C4–C6 roots.[12]

The cervical phrenic nerve was crushed with a midline incision while the animals were in the supine position under ketamine (50 mg/kg) anesthesia. The unilateral (right) phrenic nerve crush was carried out by crushing the nerve for 15 s with fine (0.5-mm-wide) forceps at the site of the insertion (5–8 mm in length) of the main phrenic nerve into the thoracic cavity, which receives fibers from the C4–C6 roots. After recovery from surgery, rats were caged individually and isolated for the duration of the experiment.[13]

The animals were divided randomly into three groups, each consisting of 6 rats. The animals in the first group were not crushed or axotomized and served as controls. The phrenic nerves of the animals in the second and third groups were crushed or axotomized, respectively. Animals in all groups were euthanized 1 week after the crush or axotomy, and diaphragm muscles were harvested for the determination of tissue oxidative stress and antioxidant enzyme activities.

Biochemical Analysis. ***Measurement of MDA Level.*** A tissue specimen of 50 mg was homogenized in 0.15 mol/L KCl. After the homogenate had been centrifuged at 1600 × *g*, MDA levels in tissue homogenate supernatant were determined by the thiobarbituric acid (TBA) reaction according to Yagi.[14] The principle of the method is based on measuring absorbance of the pink color produced by the interaction of TBA with MDA at 530 nm. Values were expressed as milligrams per deciliter.

Measurements of SOD and GSH-Px Enzyme Activities. The tissues were homogenized in physiological saline (1 g in 5 ml) using a homogenizer (B. Braun Melsungen AG, Germany) and then centrifuged at 4000 × *g* for 20 min (Labofur 200; Heraeus Germany). GSH-Px activity was measured by following changes in nicotinamide adenine dinucleotide phosphate (NADPH) absorbance at 340 nm,[15] by measuring the decrease in H_2O_2 absorbance at 240 nm.[16] SOD activity was measured by the method based on the nitroblue tetrazolium (NBT) reduction rate. One unit for SOD activity was expressed as the enzyme protein amount that caused 50% inhibition in the NBT reduction rate.[17]

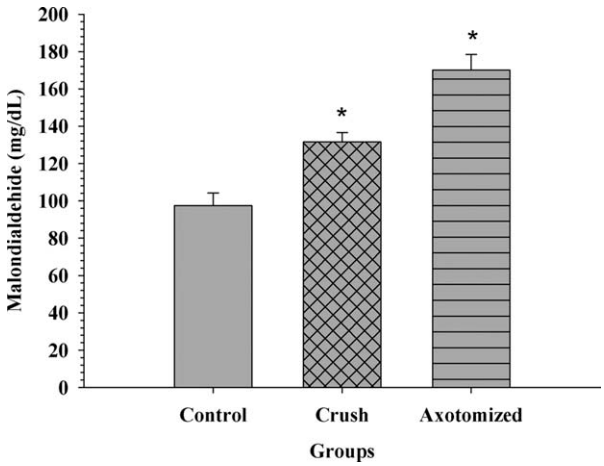

FIGURE 1. MDA levels in diaphragm muscles in the control, crushed, and axotomized groups. *Significant difference ($P < 0.001$) between treatment and control groups.

Measurement of CAT Level. Biochemical analysis of erythrocyte CAT activity was performed using a method described by Aebi.[16] Briefly, the supernatant (0.1 ml) was added to a quartz cuvette containing 2.95 ml of 19 mmol/L H_2O_2 solution prepared in potassium phosphate buffer (0.05 M, pH 7.00). The change in absorbance was monitored at 240 nm for 5 min using a spectrophotometer (UV-1201; Shimadzu, Japan).

Measurement of CA Enzyme Activity. CA activity was assayed by hydration of CO_2. The hydration of CO_2 was measured by the method of Rickli and Wilbur-Anderson with bromothymol blue as indicator.[16]

Statistical Analysis. The results are expressed as mean ± standard deviation (SD). The Kruskal–Wallis (non-parametric) test was used for comparison of groups. When significant differences were observed ($P < 0.05$), Tukey's multiple comparison test was used to determine differences between groups. Statistical analyses were carried out using SPSS for Windows, version 13.0 (SPSS, Inc., Chicago, Illinois).

RESULTS

Lipid peroxidation product MDA level (97.38 ± 6.83 in control, 131.62 ± 5.04 in crushed, and 170.08 ± 8.34 in axotomized groups) increased significantly ($P < 0.001$) in diaphragm muscles after both crush and axotomy of phrenic nerves (Fig. 1). The protective enzyme activities, including SOD (774.52 ± 27.76 in controls, 572.36 ± 28.69 in crushed group, and 442.23 ± 3.69 in axotomized group), GSH-Px (0.96 ± 0.04 in controls, 0.67

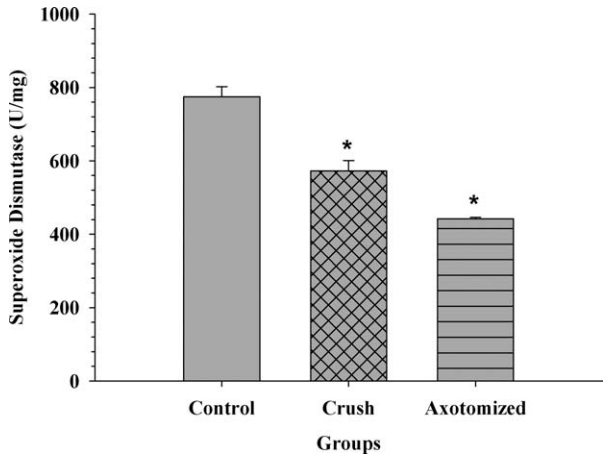

FIGURE 2. SOD levels in diaphragm muscles in the control, crushed, and axotomized groups. *Significant difference ($P < 0.001$) between treatment and control groups.

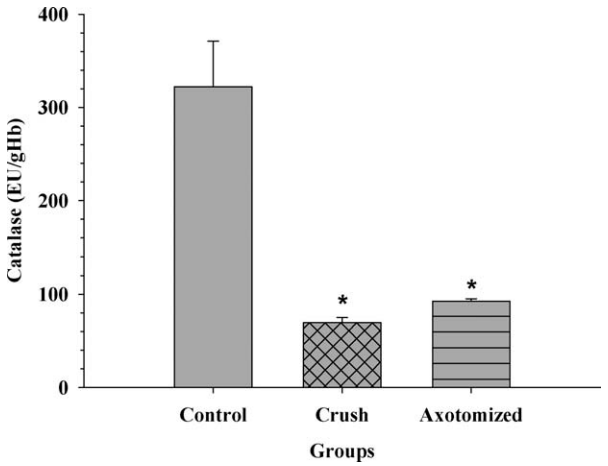

FIGURE 4. CAT levels in diaphragm muscles in the control, crushed, and axotomized groups. *Significant difference ($P < 0.001$) between treatment and control groups.

± 0.01 in crushed group, and 0.80 ± 0.01 in axotomized group), CAT (322.23 ± 48.90 in controls, 69.60 ± 5.42 in crushed group, and 92.47 ± 2.67 in axotomized group), and CA (0.09 ± 0.01 in controls, 0.06 ± 0.001 in crushed group, and 0.02 ± 0.01 in axotomized group) decreased significantly ($P < 0.001$) in diaphragm muscles after both crush and axotomy (Figs. 2–5).

DISCUSSION

This study was designed to investigate the effect of crush and axotomy of phrenic nerves on oxidative stress and antioxidant enzyme activities in rat diaphragm muscle. We found that the lipid peroxidation products increased in diaphragm muscle after either crush or axotomy. Normal phrenic nerve cell functions and the integrity of cell structures may be disrupted via the considerable reactivity of ROS. The total plasma lipid peroxidation level as an indicator of oxidative stress reflects the redox balance between oxidation and anti-oxidation.[18,19] Proteins, membrane lipids, and nucleic acids are targets of free radical–mediated injury. Free radicals may act by peroxidation of membrane lipids, inactivation of enzymes by oxidation of sulfhydryl groups, depolymerization of polysaccharides, and disruption of nucleic acids.[10]

Under normal circumstances, varieties of antioxidant defense systems exist to prevent or regulate formation of these highly reactive and toxic moieties. These include SOD, GSH-Px, CAT, and other free radical scavengers, such as GSH. In this study, the activities of antioxidant enzymes such as SOD, GSH-Px, CAT, and CA decreased significantly in diaphragm muscles after both crush and axotomy of the phrenic nerves. Most living organisms possess enzymatic and non-enzymatic defense systems against excess production of reactive oxygen species. However, different external factors, such as crush or axotomy to tissues, decrease the capability of such protective systems and result in

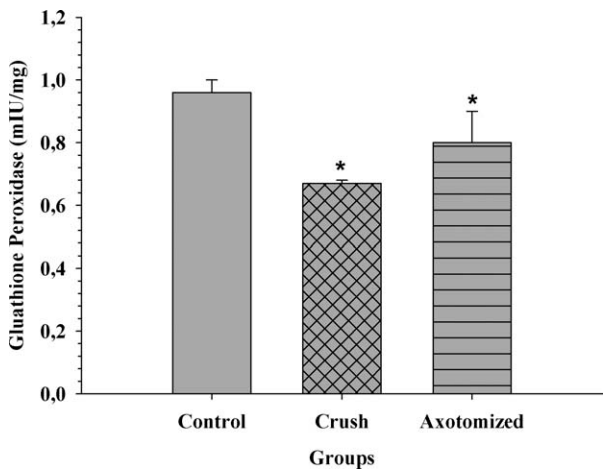

FIGURE 3. GSH-Px levels in diaphragm muscles in the control, crushed, and axotomized groups. *Significant difference ($P < 0.001$) between treatment and control groups.

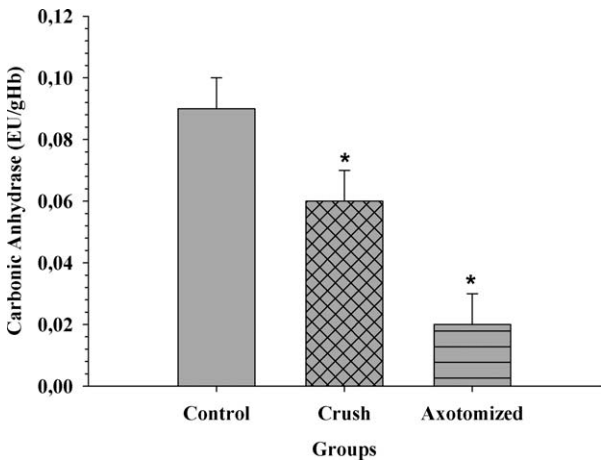

FIGURE 5. CA levels in diaphragm muscles in the control, crushed, and axotomized groups. *Significant difference ($P < 0.001$) between treatment and control groups.

disturbances of the redox equilibrium that is established in healthy conditions.[9]

The SOD–CAT system provides the first defense against oxygen toxicity. SOD catalyzes the dismutation of the superoxide anion radical to water and hydrogen peroxide, which is detoxified by the CAT activity. Usually, a simultaneous induction response in the activities of SOD and CAT is observed when an exogenous antioxidant is applied.[20,21] Decreased tissue GSH-Px activity in this study indicated that oxidation of GSH to GSSG is likely to be lowered by crush or axotomy of phrenic nerves. Decreased tissue SOD and GSH-Px enzyme activities in our study suggest that crush or axotomy damage cell membrane led to an increase in MDA level and also caused damage to cell components such as mitochondria, which contain SOD and GSH-Px.[22]

CAT is the major antioxidant enzyme involved in the degradation of hydrogen peroxide.[23] We found that CAT activity significantly decreased in the diaphragm muscles after both crush and axotomy of phrenic nerves. This indicates that crush and axotomy of phrenic nerves caused a depression of diaphragm muscle antioxidant systems due to increased lipid peroxidation and formation of free radicals.[24]

CA is a Zn-containing metalloenzyme that catalyzes the reversible hydration of carbon dioxide. It plays different roles in various tissues. Species can produce many different CA isozymes, some of which act in the cytosol, whereas others are membrane-bound. For instance, in humans, there are three cytosolic isozymes (I, II, and III), five membrane-bound isozymes (IV, VII, IX, XII, and XIV), a mitochondrial isozyme (V), and a secreted salivary isozyme (VI), as well as several related proteins that lack catalytic activity. CAs are key enzymes that regulate acid–base homeostasis in both normal and pathological conditions. CA isoenzyme II deficiency, formerly called the syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification, is an autosomal recessive "inborn error of metabolism" that has disclosed important insights concerning osteoclast function. It has been shown that there is a markedly reduced level of activity of this enzyme in the blood of infants with respiratory distress syndrome, and it was suggested that this might be related to the etiology of this disease.[25–27] Similarly, our study also showed that CA activity decreased significantly in the diaphragm muscles after both crush and axotomy of phrenic nerves. This finding may be considered evidence of an acid–base imbalance in these tissues.

We conclude that: (a) crushing or axotomizing the phrenic nerves may produce oxidative stress in diaphragm muscle by increasing lipid peroxidation and decreasing antioxidant enzyme activities; and (b) the decreased CA activity that was detected in diaphragm muscles might be considered evidence of acid–base imbalance in diaphragm tissue.

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