Brain Research Bulletin 92 (2013) 84–88

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

Effects of crush and axotomy on oxidative stress and some trace element levels in phrenic nerve of rats☆

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ARTICLE INFO

Article history:
Received 25 March 2011
Received in revised form 21 June 2011
Accepted 6 July 2011
Available online 23 July 2011

Keywords:
Phrenic nerve degenerations
Oxidative stress
Trace element levels
Rat

ABSTRACT

This study was designed to investigate the effect of crush and axotomy on oxidative stress and some trace element levels in phrenic nerve of rats. Eighteen male Wistar-albino rats 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 control. Phrenic nerves of the animals in the second and third groups were crushed and axotomized, respectively. Animals in all groups were sacrificed one week after the crush or axotomy, and degenerated phrenic nerves were harvested for the determination of tissue oxidative stress and trace element levels. Lipid peroxidation product malondialdehyde and antioxidant glutathione levels increased in both crushed and axotomized phrenic nerves. The activities of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase were lower in crushed and axotomized phrenic nerves than in controls. The levels of Fe, Pb, Mn, Cd and Co increased, and Mg and Cu levels decreased in crushed phrenic nerves. The levels of Fe and Mg decreased, Pb and Co levels increased in axotomized phrenic nerves. It was concluded that crushing or axotomizing the phrenic nerves may produce oxidative stress by increasing lipid peroxidation and decreasing antioxidant enzyme activities. It was also concluded that while crush to phrenic nerves causes accumulation of minerals, axotomizing phrenic nerves causes depletion of minerals in the tissues.

1. Introduction

Crush to 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]. The first week after injury, Wallerian (anterograde) degeneration occurs whereby distal axons and their myelin sheaths are phagocytosed by invading macrophages [2]. Schwann cells, deprived of axonal contact, play a key role in regulating macrophage recruitment [3].

Aerobic organisms are protected from oxygen toxicity by a natural antioxidant defense system involving enzymatic and non-enzymatic mechanisms [4]. The increased formation of reactive oxygen species (ROS) and decreased antioxidant defense is defined as oxidative stress, which is widely recognized as an important feature of many diseases. Superoxide dismutase (SOD) and catalase (CAT) are cellular antioxidants, which 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 [5].

Oxidative radicals have been implicated in a broad range of neuropathological conditions such as ischemia, edema, infarction, seizure disorders, aging, Alzheimer's disease and Parkinson's disease for many years. 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 [6].

Trace elements are essential components of biological structures, but at the same time they can be toxic at concentrations beyond those necessary for their biological functions. In addition, the toxicity can be extended to other non-essential elements of very similar atomic characteristics that can mimic the reactivity of a trace element. To deal with this essentiality/toxicity duality, biological systems have developed the ability to recognize a metal, and deliver it to the target without allowing the metal to participate in toxic reactions [7]. Proteins are primarily responsible for such recognition and transport, and most of the associations of

☆ This article is part of a Special Issue entitled 'Animal Models'.

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doi:10.1016/j.brainresbull.2011.07.013

trace element with other biomolecules lead to undesirable chemical modifications of these molecules.

Co is an essential trace element being an integral part of vitamin B_{12}, which is essential for folate and fatty acid metabolism [8]. Fe is essential for many biological processes and its deficiency or excess is involved in pathological conditions. Cd is one of the most dangerous occupational and environmental toxins. It is found in drinking water, atmospheric air and even in food. Products of vegetable origin are the main carrier of Cd compounds in food [9].

Some surgeons deliberately crush the phrenic nerve, allowing the diaphragm to rise up to minimize the postpneumonectomy and postlobectomic spaces in the thoracic cavity. This allows a pulmonary expansion and a residual postpneumonectomic pleural cavity obliteration. In the cases of phrenic paralysis and crush, diaphragm muscle may be seriously injured leading to the respiratory disorders [10]. Therefore, this study was designed to investigate the effect of crush and axotomy on oxidative stress and some trace element levels in phrenic nerve of rats.

2. Materials and methods

2.1. Treatment of animals

Eighteen male Wistar–albino rats, aging approximately 6 months, weighing between 180 and 200 g were obtained from the Animal Laboratory of Yuzuncu Yil University. Rats were housed in specific cages. A 12-h light/dark cycle was maintained and the rats were feed ad libitum. The study was approved by the local ethics committee of Yuzuncu Yil 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 C4–C6 root [11].

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 in the site of the insertion (5–8 mm length) of the main phrenic nerve into the thoracic cavity, which receives fibers from C4–C6 root. After recovery from the surgery, rats were caged individually and isolated for the duration of the experiment [12].

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 control. Phrenic nerves of the animals in the second and third groups were crushed and axotomized, respectively. Animals in all groups were sacrificed one week after the crush or axotomy, and degenerated phrenic nerves were harvested for the determination of tissue oxidant/antioxidant status and trace element levels.

2.2. Biochemical analysis

2.2.1. 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 Kavak et al. [13]. 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 mg/dL.

2.2.2. Measurements of SOD and glutathione peroxidase (GSH-Px) enzyme activities

The tissues were homogenized in physiological saline (1 g in 5 mL) using a homogenizer (B. Braun MelsungenAG 853202, Germany) and then, centrifuged at 4000 × *g* for 20 min (Heraus Labofur 200, Germany). GSH-Px activity was measured by following changes in NADPH absorbance at 340 nm [14], by measuring decrease of H_2O_2 absorbance at 240 nm [15]. SOD activity was measured by the method based on nitroblue tetrazolium (NBT) reduction rate. One unit for SOD activity was expressed as the enzyme protein amount causing 50% inhibition in NBT reduction rate [16].

2.2.3. Measurement of glutathione (GSH) level

The GSH levels of tissues were measured at 412 nm using the method of Sedlak and Lindsay [17]. The samples were precipitated with 50% TCA and then centrifuged at 1000 × *g* force for 5 min. The reaction mixture contained 0.5 mL of supernatant, 2.0 mL of Tris–EDTA buffer (0.2 M; pH 8.9) and 0.1 mL of 0.01 M DTNB. The solution was kept at room temperature for 5 min, and then read at 412 nm on the spectrophotometer.

2.2.4. Measurement of CAT level

Biochemical analysis of erythrocyte CAT activity was performed with a method described by Aebi [18]. 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 (Shimadzu UV-1201, Japan).

2.2.5. Measurements of mineral levels

Exactly 2.0 mL of the mixture of HNO_3/H_2O_2 (2/1) was added to 0.7 g of the tissue samples. The mixture was placed into the water bath at 70 °C for 30 min and stirred occasionally. Then, 1.0 mL of the same acid mixture was added, and the mixture was transferred into a Teflon vessel bomb for the microwave oven. The bomb was closed, and the solution was placed inside the microwave oven. Radiation was applied for 3 min at 450 W. After addition of 0.5 mL of the same acid mixture, radiation was repeated for 3 min. After cooling for 5 min, 2.0 mL of 0.1 mol/l HNO_3 was added, and the solution was transferred into a Pyrex tube. After centrifugation, the clear solution was used for the determination of Cu, Zn, Mg, Mn, Pb, Cd, Ni and Fe [19,20]. They were measured by Atomic Absorption Spectrophotometer measurements using a UNICAM-929 spectrophotometer (Unicam Ltd., York Street, Cambridge, UK).

2.3. Statistical analysis

The results were expressed as mean ± standard deviation (SD). Kruskal–Wallis (which is non-parametric) test was used for the comparison of groups. When significant differences were observed ($P < 0.05$), Tukey multiple comparison test was used to determine the difference between groups. Statistical analyses were carried out using the SPSS® statistical software package (SPSS for Windows version 13.0, SPSS Inc., Chicago, IL, USA).

3. Results

Lipid peroxidation product MDA and antioxidant GSH levels increased significantly ($P < 0.001$) in both crushed and axotomized phrenic nerves (Figs. 1 and 2, respectively). The activities of antioxidant enzymes such as SOD, CAT and GSH-Px were significantly ($P < 0.001$) lower in crushed and axotomized phrenic nerves than in controls (Figs. 3–5, respectively). The levels of Fe, Pb, Mn, Cd and Co increased, and Mg and Cu levels decreased in crushed phrenic nerves. The levels of Fe and Mg decreased, Pb and Co levels increased in axotomized phrenic nerves (Table 1).

4. Discussion

This study was designed to investigate the effect of crush and axotomy on oxidative stress and some trace element levels in phrenic nerve of rats. Generation of reactive oxygen species is a part of normal life, and their interaction with host antioxidant defense systems appears to exert a significant influence on cellular chemistry in health and disease. This study is one of the few

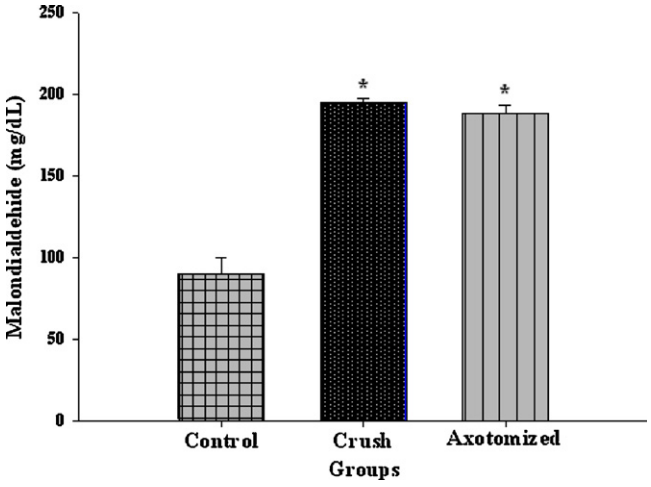

Fig. 1. MDA levels in control, crushed and axotomized phrenic nerves of rats. *A significant difference ($P < 0.001$) between treatment and control groups.

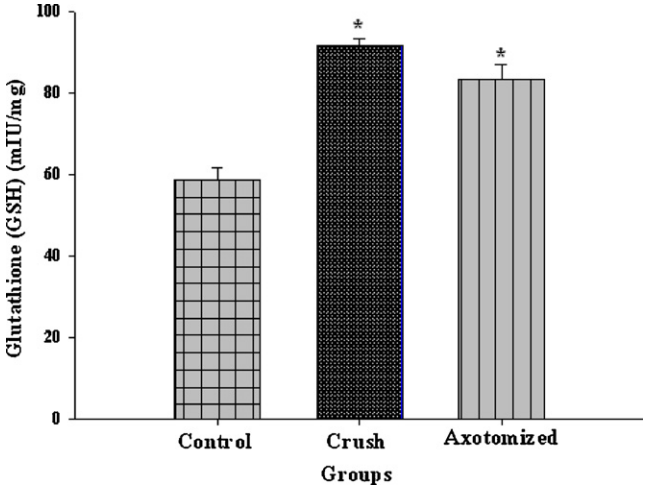

Fig. 2. GSH levels in control, crushed and axotomized phrenic nerves of rats. *A significant difference ($P < 0.001$) between treatment and control groups.

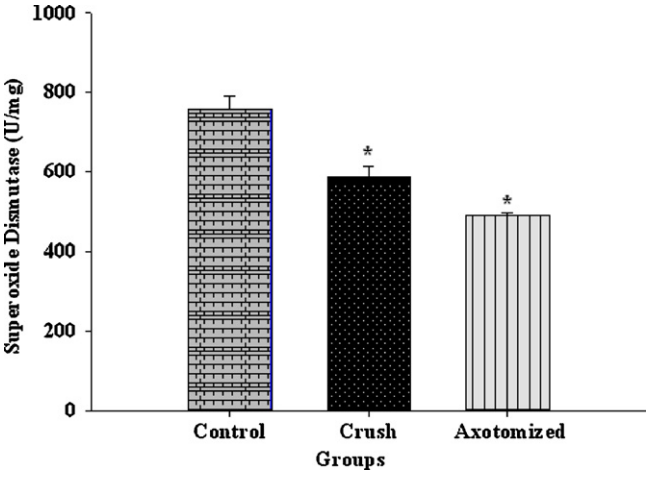

Fig. 3. SOD levels in control, crushed and axotomized phrenic nerves of rats. *A significant difference ($P < 0.001$) between treatment and control groups.

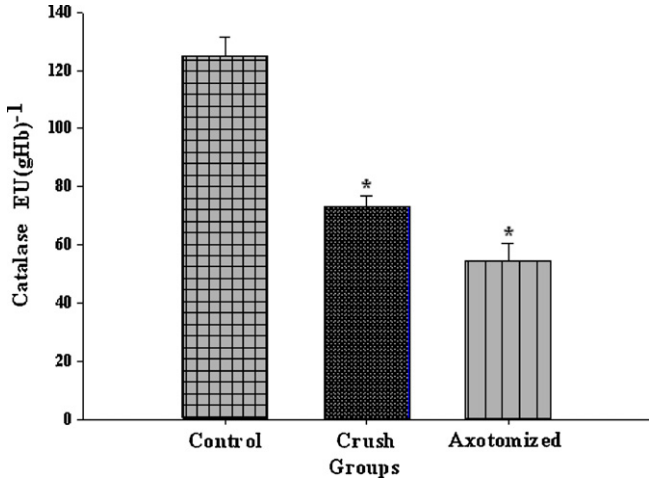

Fig. 4. CAT levels in control, crushed and axotomized phrenic nerves of rats. *A significant difference ($P < 0.001$) between treatment and control groups.

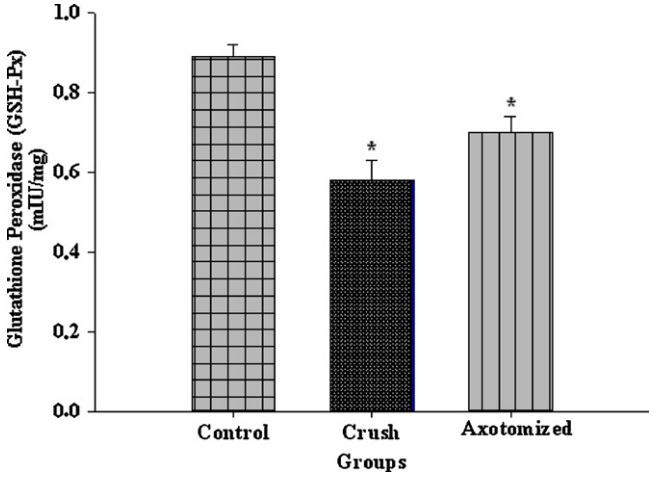

Fig. 5. GSH-Px levels in control, crushed and axotomized phrenic nerves of rats. *A significant difference ($P < 0.001$) between treatment and control groups.

studies in the literature investigating the changes in SOD, GSH-Px, GSH and CAT enzyme activities and Fe, Mg Cu, Pb, Mn and Cd levels in the injured phrenic nerve. It was found that the lipid peroxidation product MDA increased in phrenic nerve after the crush or axotomy. Normal phrenic nerve cell functions and integrity of cell structures may be broken via considerable reactivity of ROS. The organism has enzymatic and non-enzymatic antioxidant mechanisms that work as scavengers for the harmful ROS [21,22]. 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 [6].

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, other free radical scavengers, such as GSH. In this study, SOD, GSH-Px and CAT enzyme activities were significantly lower in crushed and axotomized phrenic nerves than in controls. 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

Table 1
Some mineral and heavy metal concentrations in control, crushed and axotomized phrenic nerves of rats ($n = 6$).

Minerals	Control	Crushed-phrenic nerve	Axotomized phrenic nerve
Co (μg/dL)	0.19 ± 0.02	0.43 ± 0.02*	0.40 ± 0.03*
Pb (μg/dL)	0.65 ± 0.04	0.74 ± 0.05*	0.91 ± 0.05*
Cd (μg/dL)	0.07 ± 0.00	0.35 ± 0.08*	0.07 ± 0.01
Mg (μg/dL)	44.55 ± 15.86	20.04 ± 0.84*	12.51 ± 1.98*
Mn (μg/dL)	0.02 ± 0.00	0.04 ± 0.00*	0.01 ± 0.00
Fe (μg/dL)	3.09 ± 0.46	4.29 ± 0.28*	2.34 ± 0.38*
Cu (μg/dL)	0.92 ± 0.03	0.29 ± 0.10*	0.82 ± 0.09

The results were expressed as mean ± standard deviation (SD).
* A significant difference ($P < 0.001$) between treatment and control groups.

is observed when an exogen antioxidant is applied [23]. Decreased tissue GSH-Px activity in this study indicated that oxidation of GSH to GSSG is likely to be lowered by crushing or axotomizing of phrenic nerves. Decreased tissue SOD and GSH-Px enzyme activities in this study suggested that crush or axotomy damage cell membrane leading to an increase in MDA level, and also cause damage to cell components such as mitochondria, which contains SOD and GSH-Px [24].

GSH level increased in crushed and axotomized phrenic nerves. GSH is one of the most important water-soluble antioxidants, because not only of its action as a scavenger, but also as an indispensable factor in proper catalytic action of some antioxidative enzymes [25]. Because the glucose-6-phosphate dehydrogenase (G-6-PD) catalyzes the first step of the pentose phosphate pathway, which provides the nicotinamide adenine dinucleotide phosphate (NADPH) necessary for the conversion of oxidized glutathione (GSSG) to GSH, increased tissue GSH level was probably due to the increased G-6-PD activity that caused the increased production of the GSH.

In biological systems metals are mostly bound to proteins, forming metalloproteins. Many of the metals in metalloproteins are part of enzymatic systems, have structural and storage functions, or use the protein to be transported to their target site in the organism. In humans, Mn, Fe, Cu, Zn, and Se accomplish decisive functions to maintain human health. Deficiency in any of these trace elements leads to undesirable pathological conditions that can be prevented or reversed by adequate supplementation [26]. In sufficiently nourished persons, supplementation should be carefully controlled, given the toxic effects ascribed to trace elements when present in quantities exceeding those required for accomplishing their biological functions [26].

The levels of Fe, Pb, Mn, Cd and Co increased, and Mg and Cu levels decreased in crushed phrenic nerves. The levels of Fe and Mg decreased, Pb and Co levels increased in axotomized phrenic nerves. Mn is associated with bone development, and with amino acid, lipid, and carbohydrate metabolism. Mn is found in different enzymes, e.g. mitochondrial Mn superoxide dismutase, glutamine synthetase, arginase, and activates several hydrolases, transferases and carboxylases. Mn is transported in the body by transferrin and by macroglobulins and albumin [27,28]. Sources of dietary Mn include grain, rice, tea, and nuts. It has been suggested that Mn is toxic in excess; and in brain it can cause a Parkinson-type syndrome [29].

Fe is found in four classes of proteins: Fe-heme proteins (e.g. hemoglobin (2/3 body iron), myoglobin, catalase, cytochromes); Fe-sulfur enzymes (e.g. aconitase, fumarate reductase); proteins for Fe storage and transport (transferrin, lactoferrin, ferritin, hemosiderin), and other Fe-containing or Fe-activated enzymes (e.g. NADH dehydrogenase, succinate dehydrogenase, alcohol dehydrogenase, cyclooxygenases) [30]. The accumulation of Fe in the central nervous system has been linked to variety of neurodegenerative disorders including Alzheimer's disease, Huntington's disease and Hallervorden-Spatz syndrome [31]. The hypothesis that Fe contributes to the development of neurological disorders has largely been based on the participation of Fe in the Fenton reaction and the production of ROS [30]. In addition, Fe deficiency alone produces a significant impairment of motor behavior [32].

Cu is necessary for the development of connective tissue, nerve coverings, and bone. Cu also participates in both Fe and energy metabolism. Cu acts as a reductant in the enzymes superoxide dismutase, cytochrome oxidase, lysil oxidase, dopamine hydroxylase, and several other oxidases that reduce molecular oxygen. It is transported in the organism by the protein ceruloplasmin. Cu deficiency in humans is rare, but when it occurs it leads to normocytic, hypochromic anemia, leucopenia and neuropenia, and inclusive osteoporosis in children [33]. It is essential for normal central nervous system development and function. Cu is also a highly toxic metal that has been associated with several neurodegenerative disorders [31]. It has been suggested that Cu-mediated oxidation coverts the dopamine metabolite salsolinal into a neurotoxic compound that in part mediates neuronal death due to apoptosis [31]. There is also evidence of Cu mediated necrosis [34]. This is accompanied by a striking elevation in transcription of the gene that codes for the heat shock protein.

Recent observations in nonneuronal systems suggest that Mg deficiency may promote cellular oxidative injury. A low-Mg diet enhanced spontaneous lipid peroxidation in rat liver, heart, skeletal muscle, lipoproteins, and testis, as assessed by MDA production and α-tocopherol content [35,36]. A low Mg medium reduces fatty acid double-bond content and chain length in vascular smooth muscle cells, consistent with lipid peroxidation [37]. Consistently, in the present study MDA level was higher but Mg level was lower in crushed or axotomized phrenic nerves than controls.

5. Conclusion

It was concluded that crushing or axotomizing the phrenic nerves may produce oxidative stress by increasing lipid peroxidation and decreasing antioxidant enzyme activities. It was also concluded that while crush to phrenic nerves causes accumulation of mineral, axotomy of phrenic nerves causes depletion of minerals in the nerve tissues which both are undesirable conditions for the normal physiological functions of nerves.

Conflict of interest

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Acknowledgement

We thank Dr. Sıdık Keskin for his helps in statistical evaluation of our data.

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