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Protective Effect of Alpha Lipoic Acid on Rat Sciatic Nerve Ischemia Reperfusion Damage

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Background: Alpha lipoic acid is a potent antioxidant that plays numerous roles in human health. This study examined the effect of ALA on rat sciatic nerve ischemia reperfusion damage.

Aims: Protective effect of alpha lipoic acid (ALA) on sciatic nerve following ischemia-reperfusion in rats was investigated by using light microscopy and biochemical methods. Provided that the protective effect of ALA on sciatic nerve is proven, we think the damage to the sciatic nerve that has already occurred or might occur in patients for various reasons maybe prevented or stopped by giving ALA in convenient doses.

Study Design: Animal experiment.

Methods: Forty-two adult male Sprague-Dawley rats (250-300 grams) were used in this study. Rats were randomly divided into six groups including one control (Group 1), one sham (Group 2), two ischemia-reperfusion (Groups 3 and 4) and two treatment groups (Groups5 and 6). Doses of 60 and 100 mg/kg ALA were given (Group 5 and 6) intra peritoneally twice, 1 and 24 hours before the ischemia to each treatment group. Ischemia was carried out the abdominal aorta starting from the distal part of the renal vein for two hours followed by reperfusion for three hours. In immunohistochemical methods, fibronectin immunoreactivity was analyzed. For biochemical analyses, the tissues were taken in eppendorf microtubes and superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) enzyme activities as well as malondialdehyde (MDA) and nitricoxide (NO) levels were measured.

Results: Fibronectin was observed to have increased significantly in the ischemia group; on the other hand, it was observed to have decreased in parallel to the doses in the ALA groups. Biochemical studies showed that SOD and GSHPx declined with ischemia-reperfusion, but the activities of these enzymes were increased in the treatment groups in parallel with the dose. It was found that increased MDA levels with ischemia-reperfusion were decreased in parallel with ALA dose. There were no statistically significant changes in NO.

Conclusion: Increased fibronectin observed after ischemia/reperfusion of rat sciatic nerve is reduced after the administration of ALA. This indicates that the function of fibronectin, to reconnect cut nerve segments and regenerate nerves, is more prominent than its function in tissue healing after ischemia. ALA administered before ischemia decreases MDA and increases SOD and GSHPx. We think that ALA may protect against the pathological changes in ischemic nerve and may be used to devise more efficient treatments.

Keywords: Alpha lipoic acid, ischemia-reperfusion, sciatic nerve, rat

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Alpha-Lipoic acid (α-LA, ALA) is a compound which is found in many prokaryotic and eukaryotic cell types and is formed naturally (1). α-LAhaspositive effectsonsomatic and autonomic neuropathies in diabetes, normalizes the endoneural blood flow, lowers oxidative stress and improves vascular dysfunction (2). Therefore, it has been used for the treatment of the following conditions: alcohol-dependent liver damage, fungal intoxications, diabetes, glaucoma, damage by radiation, chagas disease, neurodegenerative disorders, ischemia-reperfusion (I/R) damage, heavy metal intoxications and Human Immunodeficiency Virus (HIV) infections for a long time (3).

Reperfusion aggravates ischemic injury to the peripheral nerve even more. It is thought that the main mechanism of reperfusion injury forms reduced oxygen species (4). In animal studies, it has been shown that ALA reduces oxidative stress and cell damage in organs caused by I/R(5).

We determined very few studies in the literature about the protective effect of ALA on I/R of the sciatic nerve, one of the peripheral nerves. However, we did not encounter any previous study that used the same materials and methods as the current paper. Therefore, we believe that our results will shed light on other studies related to this topic.

In this study, the protective effect of ALA on sciatic nerve after I/R in rats was investigated by using light microscopy and biochemical methods. Provided that the protective effect of ALA on the sciatic nerve is proven, we believe that damage to the sciatic nerve that has already occurred or might occur in patients for various reasons may be prevented or stopped by giving ALA in convenient doses.

MATERIALS AND METHODS

Animals and surgery

Forty-two adult male Sprague-Dawley rats (250-300 grams) were randomly divided into six groups, namely one control (Group I), one sham (Group II), two I/R (Group III and IV) and two treatment groups (Group V and VI).Each rat was anesthetized with 90 mg/kg ketamine hydrochloride i.m. (Ketalar Flacon; Pfizer Pharmaceutical Co, Istanbul, Turkey) and 10 mg/kg xylazine i.m. (Rompun; Bayer, Istanbul, Turkey) which were re-administered to maintain the required anesthesia level. Groups 5 and 6 received 50 mg/ml ethanol plus ALA (60 and 100 mg/kg, 0.5 ml, intraperitoneally (ALA; Sigma-Aldrich, St. Louis, MO, USA) at 1 h and 24 h before the ischemia.

A part of the sciatic nerve was kept in formalin for histological study. The other part was washed with 0.9% NaCl, then stored at -30°C until the biochemical analysis was performed to determine the tissue levels of malondialdehyde (MDA) and nitric oxide (NO), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activity.

Histological examination

Following blood collection from rats, sciatic nerves were excised and weighed. Then, sciatic nerve tissue specimens were fixed in 10% formaldehyde solution with PBS and embedded in paraffin, sliced at a thickness of 5 μmand stained with immunohistochemistry techniques. Slices were examined under a light microscope (CX31; Olympus, Tokyo, Japan).

Immunohistochemical staining

Biopsies from the sciatic nerve tissues of the rats were harvested and tissue fragments were fixed in 10% neutral buffered formalin solution, embedded in paraffin and sectioned at a thickness of 5 μm. Immunocytochemical reactions were performed according to the ABC technique described by Hsu et al. (6).

The positive immunostaining of fibronectin was scored semi-quantitatively in order to determine the differences between the control group and the experimental groups. The intensity of the positive staining was recorded as weak (±), mild (+), moderate (++), strong (+++), and very strong (++++).

Biochemical analysis

After weighing, sciatic nerve samples were homogenized in five volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4) which contains 0.50 ml/l Triton X-100 with a homogenizer (Ultra-Turrax T 25 Basic; IKA-Werke GmbH & Co. KG, Staufen, Germany) for 3 min at 13,500 rpm. NO and MDA were measured at this stage of homogenization. Debris was removed by centrifugation of homogenate (3200 x g for 40 min). Supernatant was used to determine GSH-Px and protein activity. Then, the supernatant was extracted in ethanol/chloroform mixture (5/3, v/v). After second centrifugation at 3200 x g for 40 min, the upper ethanol phase was used to determine SOD activity. All procedures were done at +4°C.

The method of Lowry et al. (7) was used for spectrophotometric measurement of tissue protein. All samples were assayed in duplicate. Measurement of SOD activity was performed as described by Durak et al., which is a modified form of the method of Sun et al. (8,9). Glutathione peroxidase activity was measured by the method of Paglia and Valentine (10). MDA levels show generation of free radicals that increase at the end of the lipid peroxidation. The double-heating method of Draper and Hadley was used to estimate MDA levels (11). Due to the rapid degradation of NO, it is difficult to measure it directly, so tissue nitrite (NO2-) and nitrate (NO3-) were accepted as an index of NO production (12). The method for determination of sciatic nerve nitrite levels was based on the Griess reaction (13).

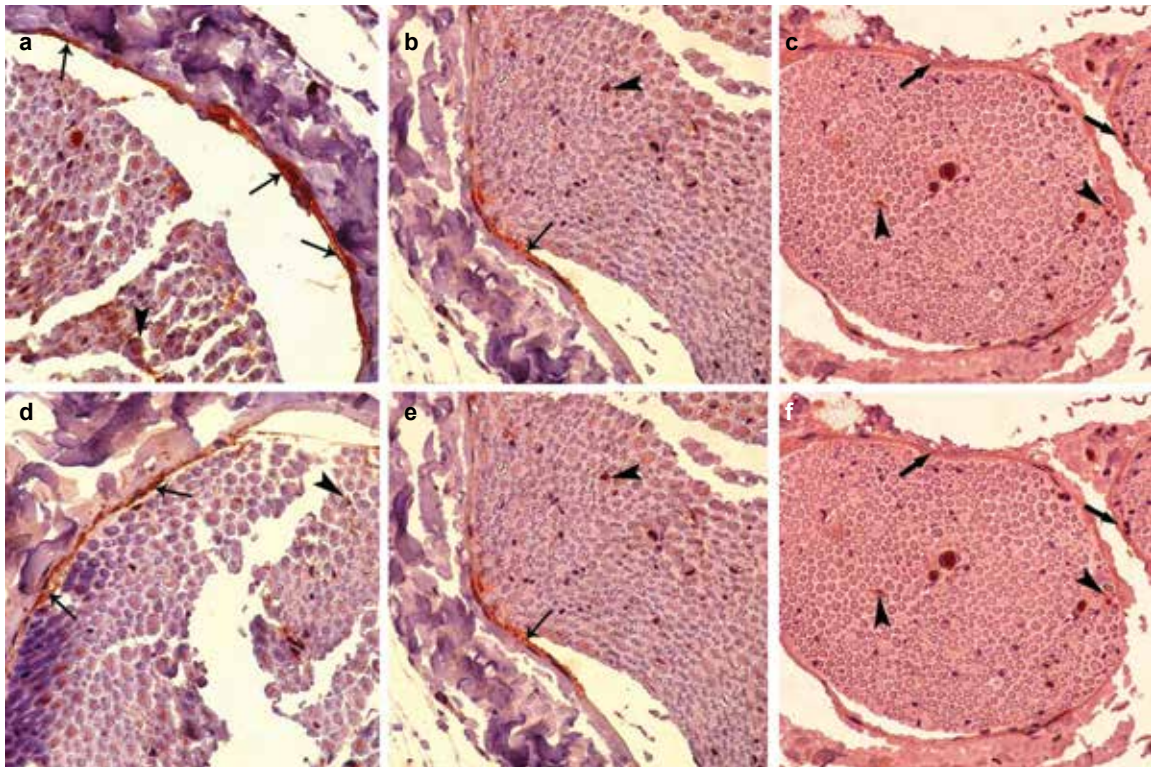

FIG. 1. a-f. Light microscopic images of showing fibronectin immunoreactivity in nerve tissue belonging to Group I (a), Group II (b), Group III (c), Group IV (d), Group V (e), and Group VI (f).Thin arrow: Epineurium, Thick arrow: Perineurium, Head arrow: Endoneurium (Immunoperoxidase, hematoxylin staining x400)

Statistical analysis

Statistical analyses for histological and biochemical data were performed using Shapiro-Wilk, Kruskal-Wallis H and Mann-Whitney U and Pearson correlation tests in the SPSS 16.0 computer program (SPSS Inc.; Chicago, IL, USA). All variables were expressed as the median, mean ± standard deviation with the range. Differences were accepted to be significant when the probability was less than 0.05.

RESULTS

The evaluation of cross-sections of sciatic nerve marked with immunohistochemically stained anti-fibronectin antibody showed fibronectin immunoreactivity in the epineurium, perineurium and endoneurium (Figure 1).

A weak positive fibronectin activity was noted in the analysis of neural tissues of the control (Group 1) and sham (Group2) groups. The evaluation of neural tissues from (Group 3) and (Group 4) showed diffuse strong fibronectin immunoreactivity in each of three nerve sheaths; on the other hand, the fibronectin reactivity of the groups which received ALA (Group 5 and 6) was weaker than those of Groups 3 and 4 (Figure 1, Table 1).

TABLE 1. Semi quantitative assessment of intensity of fibronectin immunoreactivity between control, sham and experimental groups

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Fibronectin	±	±	+++	++++	+++	++

The comparison of the treatment groups demonstrated that fibronectin reactivity of Group 6 was weaker than that of Group 5. Fibronectin reactivity was significantly decreased in each of three nerve sheaths in the groups given ALA (Groups 5 and 6) compared to I/R groups (Groups 3 and 4) (Figure 1, Table 1).

The parameters in this evaluation were the activities of SOD and GSH-Px enzymes and the levels of MDA and NO (Table 2).

It was noted that activity of the SOD enzyme was decreased in Group 3; the increase in enzyme level in Group 5 was not statistically significant and there was a marked and significant increase in Group 6 (Table 2, Figure 2).

The level of MDA, which showed a significant increase in Group3, diminished in parallel to the ALA dosage. Despite significant differences between Group 3 and the groups receiving ALA, no significant differences among ALA groups was found (Table 2, Figure 3).

Even though GSH-Px enzyme activity was diminished with Group 3, it was demonstrated that it was increased proportion-

TABLE 2. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) enzyme activities and malondialdehyde (MDA), nitric oxide (NO) levels

Groups	SOD (U/mg protein)	MDA (nmol/g protein)	GSH-Px (U/mg protein)	NO (μmol/g protein)
I	0.0460±0.0133	75.4831±9.1936	1.5900±0.1057	8.2504±0.9611
III	0.0112±0.0076	85.1650±7.5948	1.0397±0.1413	6.7877±0.6862
V	0.0135±0.0100	74.8688±7.2847	1.3824±0.1478	7.4167±0.6895
VI	0.0382±0.0210	66.7438±8.4046	1.5305±0.1567	7.5517±0.7095
p-value				
I - III	0.001	0.026	0.001	0.007
I - V	0.002	NS	0.026	NS
I - VI	NS	NS	NS	NS
III - V	NS	0.038	0.004	NS
III - VI	0.017	0.007	0.001	NS
V - VI	0.026	NS	NS	NS

NS: Non-significant

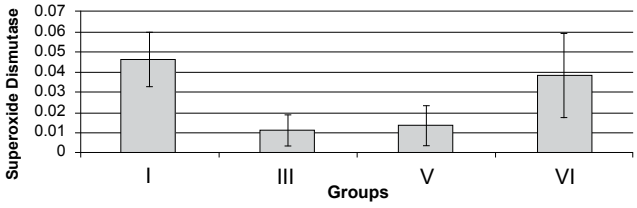

FIG. 2. The changes in superoxide dismutase enzyme activity between groups

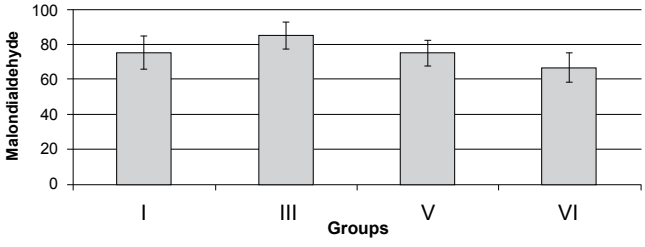

FIG. 3. The changes in malondialdehyde levels between groups

ally to the ALA dosage and this was statistically significant (Table 2, Figure 4).

The level of NO that diminished with ischemia and increased proportionally to the dosage in the treatment groups showed no statistically significant differences between the groups (except the control and ischemia groups) (Table 2, Figure 5).

Rat sciatic nerve model is used extensively in assessing the functional changes following nerve injury and in determining the effectiveness of various surgical and medical treatments. We assessed the effects of ALA on rat sciatic nerve following I/R.

Despite degenerative findings on our parameters due to I/R damage, we observed positive effects with ALA administration.

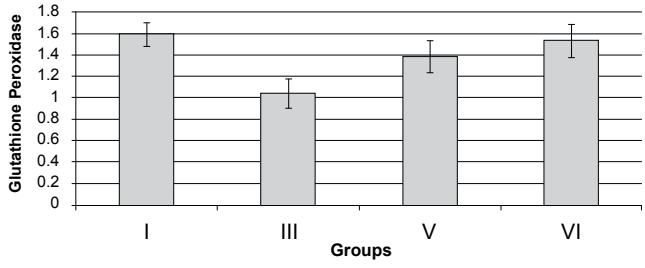

FIG. 4. The changes in glutathione peroxidase enzyme activity between groups

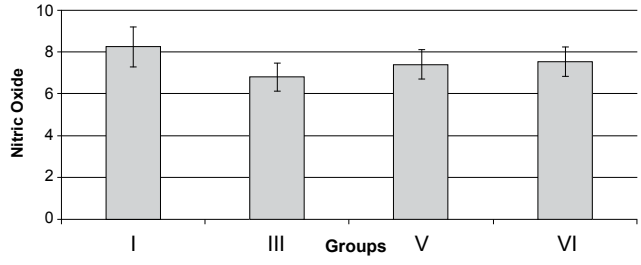

FIG. 5. The changes in nitric oxide levels between groups

DISCUSSION

Fibronectin, which is a main component of extracellular matrix of the developing peripheral nerve system, aids in nerve regeneration. Fibronectin has been proven to be a more appropriate material in the peripheral nerve repair compared to nerve and muscle grafts. Severe cardiovascular and neural system defects were observed in rats with fibronectin gene deletions. Fibronectin presence in the normal peripheral nerve is prominent in Schwann cell, blood vessels and basal lamina of perineural cells in all layers of the perineurium (14). In our study, the significant increase of fibronectin immunoreactivity

following I/R administration in the epineurium, perineurium and endoneurium of all sciatic neural tissues demonstrates the reaction of neural tissues to the injury. In order to compensate the injury, a severe fibronectin increase occurred in the neural tissues. In a study that investigated the effect of peripheral nerve injury on the fibronectin secretion and distribution, the distribution of fibronectin in regenerated nerves was evaluated in the chicks with the right medial-ulnar nerve cut and it was shown that there was a severe increase in fibronectin expression in the nerve regenerated within a week following injury compared to the contralateral control nerve. In conclusion, raised fibronectin expression is an element of injury site response due to cut nerves; this has a crucial role in the stimulation of axonal elongation and Schwann cell migration. The fibronectin increase observed in the sciatic nerve development is also seen in the regeneration of this nerve in a similar fashion (15). In our study, the fibronectin increase of the ischemia group was caused by peripheral nerve injury and the aim of this increase was to reconnect the nerve segments and to provide nerve regeneration. In the study of Nacar et al. (16), the significant rise of fibronectin immunoreactivity in the extracellular matrix of diabetic rats was consistent with our findings.

We found that the increase in fibronectin reactivity due to nerve injury following I/R was diminished with ALA administration; moreover, we noted that an increased dosage (100 mg/mL) resulted in a more significant effect. These findings suggested that ALA, as an antioxidant, might be effective in the regeneration of the sciatic nerve tissue following ischemia and can potentially protect the neural tissue.

Malondialdehyde is produced during lipid peroxidation and, since it reacts with thiobutyric acid, it is widely used. Bagdatoglu et al. (17) determined significantly increased MDA levels in the rat sciatic nerve following I/R compared to the control group and this was consistent with our study. Senoglu et al. (18) investigated the prevention of rat sciatic nerve damage following crush injury by ALA treatment and found that the tissue MDA levels were high in the groups that were given ALA instead of saline and low in the control groups. Therefore, they concluded that the administration of ALA prior to the crush injury of the sciatic nerve prevented injury due to a decrease in oxidative stress. The alteration of MDA levels in this study was concordant with our study. Likewise, Cosar et al. (19) showed that the high tissue and plasma MDA levels in ischemia groups were diminished with ALA treatment in their study assessing the effect of I/R exposure to the ovaries.

In our study, we demonstrated that the level of SOD enzyme decreased with ischemia and increased with ALA treatment. This increase was more pronounced with the administration of higher doses of ALA (100mg/kg). Senoglu et al. (18) demonstrated that the decrease in the tissue SOD activity following sciatic nerve injury was reversed with ALA treatment. Likewise, Cosar et al. (19) noted that the reduced level of SOD subsequent to ovarian ischemia was significantly increased with ALA treatment. Militao et al. (20) showed that LA administration to the paralytic rats induced by pilocarpin reduced the lipid peroxidation products while increasing the SOD enzyme level. Cui et al. (21) demonstrated the peripheral oxidative stress increase in rats by means of SOD enzyme level reduction and also showed that the addition of ALA raised the SOD level. The data from the literature are consistent with our findings. One of the most important antioxidant enzymes, SOD, catalyzes the conversion of superoxide radicals to hydrogen peroxide. The decrease in SOD enzyme levels following I/R was associated with the depletion of SOD. Since the SOD level increase was subsequent to ALA treatment, it can be concluded that the antioxidant mechanisms of ALA was related to SOD increase.

Superoxide dismutase converts superoxide radicals to hydrogen peroxide (22). Then, hydrogen peroxide is converted to water molecules by either GSH-Px or catalase enzymes (23). Due to the very low activity of catalase enzymes in the brain, GSH-Px is the main enzyme that causes the detoxification of hydrogen peroxide in the neural system (24).

A review of the literature revealed that GSH-Px enzyme levels decreased due to oxidative damage; on the other hand, ALA treatment, as an antioxidant, reversed this decrease. We also obtained similar findings concordant with these studies. Odabasoglu et al. (25) found that LA reduced the GSH-Px enzyme activity in rats with chronic inflammation. In the study of Sehirli et al. (26), despite a significant decrease in the GSH levels in cats with colitis, ALA treatment reversed this decrease. In our study, the high consumption rate of GSH-Px in the ischemia group may be responsible for the decrease in GSH-Px. The reason for the increased level of GSH-Px in the treatment groups with the addition of ALA can be the decreasing effect of ALA on the GSH consumption related to lipid peroxidation. This activity might be due to the thiol group content of ALA. The fact that the level of GSH increases as the dose of LA increases supports this hypothesis.

Cosar et al.(19) found that the tissue and plasma NO level of the ischemia group was greater than those of the treatment group in their study assessing ovarian ischemia. Abdel-Zaher et al. (27) investigated the effect of ALA on potassium cyanide-induced stroke and mortality in the rats and showed that NO level was high prior to treatment and a decrease was noted with the administration of ALA.

NO is a free radical oxygen member and is widely found in the nervous system. Its half-life is decreased with the formation of peroxynitrite after combination with superoxide radicals (28). The decreased NO level following ischemia can be a result of this mechanism in our study.

In conclusion, the administration of ALA is followed by a reduction in the fibronectin increase observed after I/R of the rat sciatic nerve. This indicates that the function of fibronectin to reconnect cut nerve segments and regenerate nerves is more prominent than its function in tissue healing after ischemia.

ALA administered before ischemia decreases MDA and increases SOD and GSH-Px. We believe that these positive changes show that ALA may protect against the pathological changes in ischemic nerve and may be used to develop more efficient treatments.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Afyon Kocatepe University.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - O.T., O.A.Ö.; Design - O.T., O.A.Ö., A.S.; Supervision - O.T., M.Y., S.A.; Resource - O.T., O.A.Ö.; Materials - O.T., O.A.Ö., A.S.; Data Collection &/or Processing - O.T., A.S., H.M.; Analysis &/or Interpretation - O.T., O.A.Ö., H.M., M.Y.; Literature Search - O.T., O.A.Ö., S.A.; Writing - O.T., O.A.Ö.; Critical Reviews - O.T., O.A.Ö., A.S., M.Y.

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