Acute Neuroprotective Effects of Alpha-lipoic asit in Experimental Spinal Cord Injury In Rats

Emre DURDAĞ1, Memduh KAYMAZ2, Alp Özgün BÖRCEK2, Hakan EMMEZ2, Özlem GÜLBAHAR3, Figen KAYMAZ4, Emrah ÇELTIKÇI2, Şükrü AYKOL2

1Elazığ Research and Education Hospital, Neurosurgery, Elazığ, Türkiye 2Gazi University Faculty of Medicine, Neurosurgery, Ankara, Türkiye 3Gazi University Faculty of Medicine, Biochemistry, Ankara, Türkiye 4Hacettepe University Faculty of Medicine, Histology and Embryology, Ankara, Türkiye

Summary

Aim: Extensive research has focused on neuroprotection after spinal cord trauma to evaluate the effects of secondary injury. This study aims to investigate the acute neuroprotective effects of alpha-lipoic asit (LA) in experimental spinal cord injury.

Materials and Methods: Thirty-two adult, male Wistar rats were made spinal cord injury using the clip compression method. Animals were divided into four groups. First group is the sham group that only received laminectomy, second group is control group that received laminectomy and clip compression, third group is administered (30mg/kg) methylprednisolone e (MP) after laminectomy and treatment group is administered (100 mg/kg) LA. Ultrastructural findings of spinal cord were investigated, and brain stem, spinal cord, sciatic nerve malonildialdehyde (MDA) levels were compared.

Results: Regarding tissue MDA levels after trauma, animals in alpha-lipoic asit group demonstrated better results than the trauma group. These results were similar with the methylprednisolone e group. The results regarding the ultrastructural findings were favourable in MP group. Alpha-lipoic asit group demonstrated similar ultrastructural findings with control group.

Conclusions: According to the MDA levels, LA demonstrated similar neuroprotective effects as methylprednisolon e in acute phase of spinal cord injury. But ultrastructural findings has not differed between control and LA group, LA has worse effects on acute phase of spinal trauma.

Key words: Alpha lipoic asit, spinal ischemia, neuroprotection, ultrastructure

Özet


Yöntem ve Gereç: 32 erişkin erkek Wistar rat'a klip kompresyon modeli ile spinal kord hasarı olusturuldu. İlk grubu (sham) sadece laminektomi uygulandı, ikinci grubu (kontrol) laminektomi ardından klip kompresyon uygulandı. Üçüncü grubu laminektomi ardından 30 mg/kg metilprednisolon uygulandı, tedavi grubunda ise laminektomi ardından 100 mg/kg alfa-lipoik asit uygulandı. Spinal kord'un gruplar arası ultrastruktürel farklılıklarla ve beyin sapı, siyatik sinir, beyin sapının Malonildialdehit (MDA) değerleri karşılaştırıldı.
Bulgular: Travma sonrası doku MDA degerlerine bakıldığında travma grubuna kıyasla alfa-lipoik asit grubunda daha iyi sonuçlar elde edildiği görüldü. Ultrastrüktürel çalışmadı MP grubunda olumlu etkiler saptanırken, alfa-lipoik asit grubunda kontrol grubuna benzer etkiler saptandı.
Sonuç: MDA degerlerine bakıldığında alfa-lipoik asit grubunda MP grubuna benzer nöroprotektif etkinlik olmasına rağmen ultrastrüktürel incelmede benzer etkinlik saptanamamıştır. Alfa lipoik asitin spinal kord hasari akut evresinde biyokimyasal olarak etkin olsa da, histopatolojik olarak kötü olabilecek etkiler saptanmıştır.
Anahtar Kelimeler: Alfa-lipoik asit, nöroproteksiyon, ultrastrüktürel

INTRODUCTION
Spinal cord injury (SCI) often leads to catastrophic dysfunction and disability. In the past two decades experimental research has focused on acute SCI to find methods to conserve and restore neurological function(13,35). It is well known that acute neuronal injury has two different mechanisms: The primary mechanical injury and a subsequent secondary injury due to an additional damaging process after the initial injury(5,9,10). The primary process results in activation of membrane phospholipases, excitatory neurotransmitters, and initiation of lipid peroxidation. It triggers a cascade of neurotoxic events in the spinal cord including energy failure, glutamate release and activation of N-methyl-D-aspartate (NMDA) receptors, formation of nitric oxide, calcium influx, and a local inflammatory response(25). Lipid peroxidation and free oxygen radicals have importance on the secondary damage(19).
Various studies have investigated pharmacologic agents such as methylprednisolone e, melatonin, erythropoietin, magnesium, mexiletine, naloxone, infliximab, clotrimazole, lamotrigine and hyperbaric oxygen, gabapentin, pregabalin that protect against or reduce the secondary injury after experimental SCI(7,14,15,16,17,19,20,21,22,40). Among these only methylprednisolone e has shown to provide benefit in large clinical trials(5). This objective has inspired the clinician to find a new agent against spinal injury for many years.

LA is a neuroprotective metabolic antioxidant that has well known immunomodulatory effects(1). LA is both hydrophilic and lipophilic, which is a unique feature among other antioxidants. For this reason, it is referred as "antioxidant of antioxidants"(33). LA potently suppresses clinical and pathological diseases in the animal model of multiple sclerosis and autoimmune encephalomyelitis. It reduces ischemia–reperfusion (I/R) injury of heart(30,31), peripheral nerves and brain(13,6,23,27,38) LA has proven favorable effects on experimental diabetic neuropathy(4,24) and on prevention of peritoneal adhesions(26). LA has also immunomodulatory effect in CNS by inhibiting T cell migration and increasing cyclic adenosine monophosphate(6,29). The neuroprotective effects of LA on subacute phase of spinal trauma were well defined(8,32,34,37). The aim of this study is to investigate the effect of LA on acute phase of experimental SCI and to evaluate the effect on the oxidative stress of forward and backward neuronal tissue.

MATERIAL AND METHODS
Thirty-two male, adult Wistar rats weighing between 250 and 300 g were used in this study. Animals were kept under stable laboratory conditions of 18° to 21°C room temperature, a 12-hour light–dark cycle and were allowed free access to food and water. All experiments were approved by our Institutional Review Board and performed in accordance with
the local guidelines to minimize animal discomfort.

**Anesthesia and Surgical Procedure**

Anesthesia was induced by intramuscular administration of 50 mg/kg ketamine hydrochloride (Ketalar, Pfizer; Istanbul, Turkey) and 10 mg/kg xylazine (Rompun, Bayer; Istanbul, Turkey). The rats were numbered with ear tags. Their mid-backs were shaved and cleaned with 10% polyvinylpyrrolidone/iodine. Using aseptic technique and a surgical microscope a midline incision was made along the spinal processes of T5 and T12. Fascia was opened sharply and paravertebral muscles along the T7 and T10 vertebrae were dissected bilaterally. After exposing the vertebral column between T7 and T10, total laminectomy was performed with the assistance of a high speed drill and a surgical microscope. The dura was left intact. SCI was performed on all rats using an aneurysm clip of 70 g closing force (Yasargil FE 721, Aesculap; Tuttlingen, Germany) for 1 minute (Figure 1a,b,c,d). The wounds were closed in layers after the operation. Paraplegia was observed in all rats. Until the end of the experiment protocol (24 hours) the animals' bladders were manually voided twice a day and they were housed in a temperature-controlled room where food and water were provided ad libitum.

**Description of Groups**

The rats were randomly allocated into four groups: 1, sham operation group: after performing laminectomy, no further intervention was applied (n = 8); 2, Control group: animals underwent the trauma procedure after laminectomy (n = 8); 3, Methylprednisolone group: animals in this group received 30 mg/kg of methylprednisolone intraperitoneally immediately after the trauma procedure (n = 8); 4, Trauma and high dose LA group: animals in this group received 100 mg/kg LA intraperitoneally immediately after the trauma procedure (n = 8).

**Preparation**

Secondary damage is prominent in the acute phase of SCI. To detect early changes in spinal cord after injury the rats were killed with an overdose of pentobarbital after 24 hours. Several studies examined the spinal cord changes at the 24th hour. Spinal cords at the injury site were excised 2 cm long —1 cm rostrally and 1 cm caudally to the injury site (Figure 1e,f). For electron microscopic examination, 1-mm³ tissue samples were obtained from the epicenter of the trauma. Brain stem and sciatic nerve were also excised. Along with spinal cord, they were immediately stored in a -20°C freezer for assays of malondialdehyde (MDA).

![Figure 1: Surgical Procedure and Tissue Sampling](image_url)
Determination of Lipid Peroxidation in Traumatized Spinal Cord Tissue, Brain stem, Sciatic Nerve

The level of MDA was determined by spectrophotometric assay using a commercial kit (OxisResearch; Foster City, CA, USA). This method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA. Tissues were homogenized at a ratio of 1:5. First, probucol was added to each assay tube. Then, samples and standards were added to assay tubes. The N-methyl-2-phenylindole was added to each assay tube. Each tube was mixed by vortexing and incubated at 45°C for 60 minutes. Turbid samples were centrifuged at 10,000 g for 10 minutes to obtain a clear supernatant. Absorbance values of supernatants were measured at 586 nm and calculated. Minimum detectable concentration for MDA was 0.0801 _M. Intra-assay coefficient of variation for MDA was 1.2%(21).

Sample Preparation for Electron Microscopy

The tissues used for transmission electron microscopy were obtained from the trauma site in samples of 1 mm³, as described by Kaptanoglu et al.(8,20) They were kept in the phosphate-buffered 2.5% glutaraldehyde, 2% paraformaldehyde solution for 24 hours, postfixed with phosphate-buffered 2% osmium tetroxide for 1 hour. After fixation, tissues are dehydrated in a graded series of alcohol and infiltrated with epon 812 in electron microscope tissue processor (Leica EM TP; Vienna, Austria). After epon embedding, 1- to 2-µm semithin sections were obtained with ultramicrotome (Leica EM UC7) and stained with toluidine blue.

The same ultramicrotome was used to obtain 70-nm-thick sections, which were contrasted with uranyl acetate and lead citrate (Leica EM AC20), examined with JOEL JEM 1400 (Tokyo, Japan) electron microscope, and pictured with a Gatan Orius SC 1000 CCD (Munchen, Germany) camera. Samples were evaluated blindly by same co-author according to the ultrastructural scoring system performed by Emmez et al(7).

Ultrastructural Scoring System of the Spinal Cord Injury

Table 1 demonstrates the ultrastructural scoring system performed by Emmez et al.(7) for quantitative tissue evaluation of the spinal cord samples. In this method we evaluated 20 neurons per sample for intracellular edema and nuclear changes, 20 axons per sample for axonal changes, 20 mitochondrion per sample for mitochondrial changes, and 20 endothelial cells for endothelial edema. A mean value was calculated and recorded after examination of 20 axons, 20 neurons, 20 mitochondria, and 20 endothelial cells in each sample as the ultrastructural score (general neuronal score [GNS]) of that rat. Recorded parameters were intracellular edema, nuclear damage, axonal degeneration, axonal myelin splitting, mitochondrial damage, and vascular endothelial edema. The calculated values were expressed as mean scores with standard deviations (SD). Higher values are associated with increased severity of SCI.

Statistical Evaluation

Statistical analysis were carried out by SPSS for Windows Version 15.0 statistical package. Continuous variables were presented by mean±standard deviation, median and min-max. Categorical variables were given by frequencies and percents. Differences between the groups were evaluated by Kruskal Wallis and chi square test. Where analysis of variance showed significant differences, Mann-Whitney U test was applied to determine the source of the difference. Data were expressed as mean _ SD. Significance value was considered as p<0.05.
Table 1: Ultrastructural Scoring

<table>
<thead>
<tr>
<th>Category</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuronal edema</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Light</td>
<td>1</td>
</tr>
<tr>
<td>Mild</td>
<td>2</td>
</tr>
<tr>
<td>Severe (cell membrane defect)</td>
<td>3</td>
</tr>
<tr>
<td>Nucleus</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Clumping</td>
<td>1</td>
</tr>
<tr>
<td>Sparse chromatin</td>
<td>2</td>
</tr>
<tr>
<td>Severe damage</td>
<td>3</td>
</tr>
<tr>
<td>Axonal myelin</td>
<td></td>
</tr>
<tr>
<td>Normal myelin layers</td>
<td>0</td>
</tr>
<tr>
<td>Vesiculated myelin</td>
<td>1</td>
</tr>
<tr>
<td>Cracked myelin layers</td>
<td>2</td>
</tr>
<tr>
<td>Honeycomb and extruded vesicles</td>
<td>3</td>
</tr>
<tr>
<td>Axonal edema</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Light edema</td>
<td>1</td>
</tr>
<tr>
<td>Mild edema</td>
<td>2</td>
</tr>
<tr>
<td>Severe edema and loss of structure</td>
<td>3</td>
</tr>
<tr>
<td>Mitochondrion</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Light edema</td>
<td>1</td>
</tr>
<tr>
<td>Mild edema</td>
<td>2</td>
</tr>
<tr>
<td>Severe edema and loss of structure</td>
<td>3</td>
</tr>
<tr>
<td>Vascular endothelium</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Light edema</td>
<td>1</td>
</tr>
<tr>
<td>Mild edema</td>
<td>2</td>
</tr>
<tr>
<td>Severe edema</td>
<td>3</td>
</tr>
</tbody>
</table>

RESULTS

Spinal Cord MDA Levels

Bar graph figure 2 represented the measured MDA values from the tissues of spinal cord as mean and standard deviation. The Kruskal wallis confirmed the significant difference between groups. Further tests with Mann-Whitney U demonstrated that MDA levels of control group are significantly higher from all other groups (p=0.007). The difference between LA and methylprednisolone groups was not statistically significant. MDA values of treatment groups were similar as of the sham group. Intergroup significance values are listed on Table 2.

Anterograde and retrograde oxidative stress due to the clip compression of spinal cord was analyzed. MDA values revealed no significant differences in groups of brainstem and sciatic nerve samples (p=0.570, p=0.202 respectively). Figure 3 and 4 demonstrates MDA values of brain stem and sciatic nerve.

Electron Microscopic Evaluation Results

Ultrastructural scoring rates revealed neuroprotective effect of methylprednisolone e. When compared with control and LA groups, methylprednisolone e group had less neuronal edema (p<0.001), less nucleus damage (p<0.001), less axonal damage (p<0.001), less axonal edema (p<0.001), less mitochondrial edema (p<0.001), less vascular endothelial edema (p<0.003). Although there was no significant difference between LA group and control group. Ultrastructural features showed worse effects of LA according to electron microscopic evaluation on acute phase (Figure 5,6).
Table 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham- Control</td>
<td>0.005</td>
</tr>
<tr>
<td>Methylprednisolone - Control</td>
<td>0.003</td>
</tr>
<tr>
<td>Alpha lipoic asit - Control</td>
<td>0.005</td>
</tr>
<tr>
<td>Sham- Methylprednisolone</td>
<td>0.505</td>
</tr>
<tr>
<td>Sham- Alpha lipoic asit</td>
<td>0.878</td>
</tr>
<tr>
<td>Methylprednisolone - Alpha lipoic asit</td>
<td>0.959</td>
</tr>
</tbody>
</table>

Figure 2: Spinal cord MDA values
Figure 3: Brain and nerve MDA values

Figure 4: Brain and nerve MDA values
DISCUSSION

SCI is still an important medical and social problem with high rates of mortality and morbidity. It is often seen at younger population at second or third decades of their lives. Advancements in medicine on the last 20 years has improved outcomes and survival rates of SCI. Despite ongoing improvements on the issue, no certain treatment has been founded yet. Recent studies are commonly focused on secondary damage and negative effects of the SCI. And it is well known that oxidative stress is one of the important mechanism involving secondary injury of spinal cord. The findings that antioxidants improve neurological function after traumatic CNS injury in animals provide further evidence that oxidative stress has a devastating effect on neuronal cells.

The central nervous system (CNS) contains high levels of polyunsaturated lipids with low antioxidative enzyme capacity when compared with other tissues. This make CNS susceptible to oxidative stress. In spinal cord injury models, oxidative stress counteracts antioxidant activity and is responsible for neurodegeneration in
neurons, whereas constitutive antioxidants protect the neural tissue from oxidative stress. Reperfusion occurs in the first few days following SCI, aggravates the oxidative stress and secondary damage of neuronal tissue\(^{(28)}\).

Secondary damage involves excessive release of glutamate and aspartate resulting excitotoxicity. This is resulted with intracellular Ca influx and activation of arachidonic acid cascade and free radical induced lipid peroxidation. Lipid peroxidation is strongly responsible from posttraumatic neuronal degradation\(^{(34)}\).

LA is an essential substance of mitochondrial complex of glycine. Itself and its reduced form dihydrolipoic acid (DHA) are potent antioxidants. It scavenges superoxide radicals and peroxyl radicals consisted after lipid peroxidation. Hydroxyl radicals, singlet oxygen, peroxynitrite, and hypochlorous acid are scavenged by LA, and hydroxyl radicals, superoxide, peroxyl radical, peroxynitrite, nitric oxide, and hypochlorous acid are scavenged by DHA\(^{(8,34)}\).

There are researches that examine the neuroprotective effect of LA. Smith et al. pointed that CNS damage after a stroke, cardiac arrest or head injury is strongly related to the reactive oxygen substances (ROS). They showed in their study that in LA group, level of ROS in brain cells decreased, extent of damage was reduced, and survival time of animals was longer in comparison with the control group\(^{(33)}\). Pehn et al. demonstrated that, in focal ischemia model in mice, dihydrolipoate reduced the volumetric size of the infarct zone\(^{(28)}\). Cao and Phillips also showed a protective effect of LA against ischemia–reperfusion injury\(^{(3)}\). LA pretreatment also almost completely abolished ischemia–reperfusion-induced losses of glutathione in the brain and dramatically decreased lipid peroxidation in the brain\(^{(28,34)}\). LA was shown to protect C6 glial cells from glutamate-induced cytotoxicity. LA was demonstrated to bypass the adverse effects of elevated extracellular glutamate\(^{(12)}\). LA potently suppressed clinical and pathological disease in the animal model of multiple sclerosis and autoimmune encephalomyelitis, by inhibiting the migration of pathogenic T cells to the spinal cord\(^{(29)}\).

In most of the studies 100mg/kg LA was shown to be the sufficient dose for these neuroprotective effects\(^{(8,32,36)}\). Consequently, we used the same dose regimen to assess the effect of MDA levels and ultrastructural findings.

In our study when biochemical results are checked, we examined MDA levels after SCI as a reliable and important index to determine the extent of lipid peroxidation and membrane degretation. The result of our study presented significant effects of LA in lowering MDA levels in traumatized spinal cord. These effects were similar with MP group. This supports the hypothesis that LA has neuroprotectional effects on traumatic SCI.

Additionally we also examined forward and backward effect of spinal trauma on brain stem and sciatic nerve by evaluating MDA levels. No significant traumatic impact was found on neuronal tissue except the site that clip was applied. This preliminary report is important to show that trauma effects only the trauma site, no transmission can be demonstrated back and front from the injured site of spinal cord.

Emmez et al. demonstrated the neuroprotective effects of LA at 48th hour on spinal ischemia\(^{(8)}\). Toklu et al. examined the subacute effects of LA on spinal trauma in rats and proved favourable effects by both histopathologically and biochemically\(^{(37)}\). Tas et al. found no significant protective effect of LA and MP on spinal injury at hyperacute and subacute phases histopathologically\(^{(34)}\).

In this study, we examined the effect of LA on the 24th hour of trauma as we focused on the acute phase. Despite our biochemical findings demonstrating
neuroprotective effects of LA, our ultrastructural features revealed some worse effects of LA on SCI on acute phase. Authors suggest that the histopathological effects of LA may appear on subacute phase after administration.

CONCLUSION
The results of the present study demonstrate the favourable effects of LA biochemically. Ultrastructural findings did not support any favourable result. Further studies are needed to demonstrate the behaviour of histopathological effects of LA on phases of traumatic SCI.

Correspondence to:
Emre Durdağ
E-mail: emredurdag@yahoo.com

Received by: 22 April 2013
Revised by: 24 October 2013
Accepted: 29 October 2013

REFERENCES


