The Effects of Alpha Lipoic Acid on Cerebral Vasospasm Following Experimental Subarachnoid Hemorrhage in the Rabbit

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ABSTRACT

AIM: The aim of this study is to determine the effects of a strong dithiol antioxidant, alpha lipoic acid (ALA) on cerebral vasospasm following subarachnoid hemorrhage in a rabbit model.

MATERIAL and METHODS: Twenty-one New Zealand white rabbits were assigned to one of three groups: group 1 (control), group 2 (SAH only), group 3 (SAH+ALA). ALA was administered (100 mg/kg/day, single dose, intraperitoneally). The rabbits were sacrificed 72 hours after SAH. The basilar artery lumen areas, arterial wall thickness and endothelial apoptosis in a cross section of basilar artery were measured in all groups. The tissue MDA, SOD, GSH-Px levels were also determined.

RESULTS: The elevated tissue MDA levels after SAH were significantly reduced by ALA treatment. The reduced tissue SOD and GSH-Px levels after SAH were also elevated by ALA treatment. In the treatment group the average wall thickness and the mean percentages of apoptotic cells (apoptotic index) were reduced and the average cross-sectional areas of the basilar artery were increased statistically significantly.

CONCLUSION: ALA treatment attenuates the severity of cerebral vasospasm by its strong antioxidant, antivasospastic and antiapoptotic properties. ALA may potentially serve as agents in the prevention of cerebral vasospasm after SAH.

KEYWORDS: Alpha lipoic acid, Subarachnoid hemorrhage, Rabbit, Vasospasm

ÖZ

AMAÇ: Bu çalışmanın amacı, tavşanlarda oluşturulan deneysel subaraknoid kanama sonrası görülen serebral vazospazm üzerine etkilerini incelemektir.

YÖNTEM ve GERÇEKLER: 21 erkek, beyaz Yeni-Zelanda tavşanı tanı tani randomize olarak 3 gruba ayrılır: Grup 1 (Kontroll), Grup 2 (SAK), Grup 3 (SAK+ALA). ALA tedavisi (100 mg/kg/gün) dozundan intraperitoneal olarak uygulanır. Tavşanlar 72 saat sonra sakrifiye edilir. Baziller arter lümen alanı, baziller arter duvar kalınlığı ve kesit alanındaki endotelial apoptoz yüzdesi ölçüldü. Ayrıca biyokimyasal olarak doku MDA, SOD, GSH-Px enzim aktiviteleri ölçüldü.

BULGULAR: SAK sonrası anatik MDA düzeyleri ALA tedavisi ile istatistiksel olarak anlamlı şekilde azaldı. Azalan doku SOD ve GSH-Px enzim aktiviteleri ise tedavi ile arttı. Tedavi grubunda baziller arter duvar kalınlığı ve apoptoz yüzdesi azaldıken, lümen alanı ise SAK grubuna göre anlamlı şekilde arttı.

SONUÇ: ALA tedavisi serebral vazospazmin ciddiyetini güçlü antioksidan, antivasospastik, antiapoptotik etkileri ile azaltmıştır. ALA, SAK sonrası görülen serebral vazospazmin engellenmesinde önemli bir ajan olarak rol alabilir.

ANAHTAR SÖZCÜKLER: Alfa lipoik asit, Subaraknoid kanama, Tavşan, Vazospazm
INTRODUCTION

Cerebral vasospasm is a major leading factor for mortality and morbidity after aneurysmal subarachnoid hemorrhage (SAH) (7). The underlying pathogenic mechanisms of cerebral vasospasm (CV) remain poorly understood yet (7).

The breakdown products of blood in the subarachnoid space are involved in the development of CV by some direct or indirect pathways (7). Oxyhemoglobin (OxyHb) released from lysed erythrocytes in the subarachnoid clot is considered to play an important role in the pathogenesis of cerebral vasospasm (13). Oxidation of OxyHb to methemoglobin (MetHb) with concomitant release of the reactive oxygen species (ROS) plays some crucial roles in the development of CV (3,13). The beneficial effects of free radical scavengers have been demonstrated in some animal models of SAH (3,4,13) and some clinical trials (3) on ischemic neurological deficits due to SAH-induced CV. We have recently shown that n-acetylcysteine, which is a potent free radical scavenger, prevented SAH-induced histopathological and biochemical alterations (4).

Alpha lipoic acid (ALA) is a naturally occurring thiol antioxidant, a cofactor for mitochondrial alpha-ketoacid dehydrogenase complex and is one of the most potent, natural antioxidants (2). Reduction of oxidative stress by ALA supplementation has been demonstrated in patients with diabetic neuropathy (2).

It has been shown that apoptosis of endothelial cells is an important part of the pathogenesis of vasospasm after SAH and protection of the endothelial cells from the process of apoptosis may attenuate vasospasm (13).

ALA is a thiol antioxidant (similar to glutathione) that has some well defined neuroprotective (2,9) and antiapoptotic (15) properties. It crosses the blood-brain barrier easily (2).

The beneficial effects of ALA on transient cerebral ischemia (9), autoimmune encephalomyelitis (15), Alzheimer’s disease (14), and traumatic brain injury (5) has been shown previously.

The major aim of this study is to determine antivasospastic, antioxidant and antiapoptotic properties of ALA in a rabbit model of SAH-induced vasospasm.

MATERIAL and METHODS

All procedures were approved by and conducted in strict accordance with the policies of the Selcuk University Experimental Medicine Research and Application Center Experimental Animal Ethics Committee (Project no:2008/13).

The material method used in this study was also used in our previous experimental study (4). Twenty-one male New Zealand rabbits weighing 2.0-3.0 kg were anesthetized using an intramuscular injection of ketamine HCL (35 mg/kg; Ketalar; Pfizer, Istanbul, Turkey) and xylazine (5 mg/kg; Rompun; Bayer, Turkey). All animals breathed spontaneously throughout the procedures. Arterial blood samples (PO2 and PCO2) were taken from each animal from the catheterized ear arteries for blood gas analysis during the procedures, and only those animals with PO2 greater than 70 mm Hg and PCO2 less than 40 mm Hg were included in the study.

Heart rate and systemic blood pressure were measured with the use of an ear artery catheter, and mean blood pressures before and after SAH were recorded. Core body temperature was monitored rectally and maintained at 37°C± 0.5°C with a heater. Mean physiological parameter values were not statistically significant between the groups (P>0.05).

Cerebral vasospasm model

The head of the rabbit was extended in the prone position. With the use of aseptic techniques, a midline nuchal incision was made, and dermal and subdermal tissues, fascia and paravertebral muscles were dissected to expose the atlantooccipital membrane. The atlanto-occipital membrane was dissected and a 25 gauge needle was inserted through the dura mater and the arachnoid membrane into the cisterna magna. 1.0 mL/kg of cerebrospinal fluid (CSF) was withdrawn and equal volume of fresh nonheparinized autologous arterial blood that was obtained from the ear artery injected into the cisterna magna within two minutes. Nuchal muscles and skin were closed with sutures after the needle was withdrawn. The animals were then placed in a head-down position for 15 minutes to facilitate blood settling around the basilar artery. After the recovery from anesthesia, and confirmation of vital signs, rabbits were left to their cages for the establishment of cerebral vasospasm.

Drug treatments and groups

The rabbits were randomly divided into three groups of 7 rabbits each: Group I (control), Group II (SAH only), Group III (SAH+ ALA treatment).

Group I (control group, n = 7) was a sham surgery group in which SAH was not induced. In this group, after induction of anesthesia, atlantooccipital membrane was exposed as described above and the cisterna magna was aseptically punctured by a 25-gauge needle, and 1 mL/kg of physiological saline (0.9% NaCl) was slowly injected into the cisterna magna after removal of the same amount of CSF.

In group II (SAH only group, n = 7), the SAH protocol was used to induce cerebral vasospasm as described above.

In group III (SAH + ALA group, n = 7), cerebral vasospasm was induced by SAH, as described above and the rabbits received ALA (Thioctacid-T ®, Baxter Oncology GmbH, Halle/ Westph., Germany).

ALA 100 mg/kg was given intraperitoneally. The treatment was started just before intracisternal blood injection and continued for 72 hours once a day.

This dosage and treatment regimen was decided according to the literature (2,4,13). The animals tolerated this dosage well without any important side effect.

Tissue preparation

The animals were sacrificed under general anesthesia 72 hours after the induction of SAH. Each rabbit was transcardiac
perfused as described below. The thorax was opened and a cannula was placed in the aorta, via the left ventricle. The right atrial appendage was opened and the descending thoracic aorta clamped. The vascular system was perfused with 300 ml of physiological saline under a pressure of 120 cm H2O.

After perfusion, the brain and brainstem were removed and each brainstem cut coronally into two pieces: the anterior part that contains basilar artery and the dorsal part that contains brainstem tissue.

**Biochemical procedures**

Brainstem tissues (dorsal part) of the rabbits were extracted after decapitation and rinsed with ice-cold PBS (phosphate buffered saline) containing heparin and blood and clot remnants were removed. Subsequently tissues were blotted on filter paper and stored in eppendorf tubes at -80ºC until biochemical analysis. Brain tissue samples were weighed and homogenized in 1.15% KCl (10% w/v, g/ml) on ice. These homogenates were centrifuged at +4 ºC and 20.000 g for 20 minutes. Supernatant obtained was used for SOD and GSH-Px analysis. Protein contents of tissue samples were determined with Bradford method (1). SOD and GSH-Px enzyme activities were given as U/mg protein.

**Measurement of malondialdehyde (MDA) level**

MDA levels were determined with the method of Okhawa (11). The principle of the assay depends on the reaction of lipid peroxidation products with thiobarbituric acid and formation of products named as thiobarbituric acid reacting substances that give maximum absorbance at 532nm wavelength. Serial dilutions of 1,1,3,3 tetraethoxypropane were used to obtain a standard absorbance versus concentration curve and MDA concentrations of the tissue samples were determined from this curve. MDA concentrations were given as nmol/g wet tissue.

**Measurement of tissue superoxide dismutase (SOD) activity**

Superoxide dismutase enzyme activities were determined with RANSOD (Randox, UK) superoxide dismutase assay kit. The method of the assay employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of the reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the conditions of the assay. SOD activities of the samples were given as U/mg protein.

**Measurement of tissue glutathione peroxidase (GSH-Px) activity**

Glutathione Peroxidase Assay (Northwest Life Science Specialties, USA) kit was used for the determination of tissue glutathione peroxidase activity and this assay is an adaptation of the method of Paglia and Valentine (12). The principle of the assay is as follows: Glutathione peroxidase (GSH-Px) catalyzes the reduction of hydrogen peroxide (H2O2), oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG is then reduced by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming NADP+ (resulting in decreased absorbance at 340 nm) and recycling the GSH. Because GSH-Px is limiting, the decrease in absorbance at 340 nm is directly proportional to the GSH-Px concentration. GPx activity is reported as units based on the definition: 1 unit of GSH-Px is the amount of enzyme necessary to catalyze the oxidation (by H2O2) of 1.0 μmole GSH to GSSG, per minute at 25 ºC, pH 7.0. GSH-Px activities of the tissue samples were given as U/mg protein.

**Histological morphometric analysis of the basilar artery**

In total, five artery sections were analysed per animal (Figure 1A-E). The morphometric and immunohistological analyses were done in a blind fashion by one pathologist. Morphometric measurements on all five segments of the basilar artery were performed by using the Image Analysis System (BAB Bs200ProP Image Processing and Analysis System, Ankara, Turkey). The luminal area was calculated from the perimeter of the luminal border and the area contained within the boundaries of the internal elastic lamina was neglected. The luminal area for each basilar artery was obtained by averaging these measurements. The mean±SEM value obtained from each artery was used as the final value for a particular vessel.
The wall thickness between lumen and external border of muscle layer was measured at four quadrants of each section of basilar artery. If an undulating luminal border was encountered, an extra measurement was done from the internal elastic membrane to the external border of the muscle layer. Results were given as mean ±SEM value for a particular vessel, after averaging five consecutive basilar artery segment values for cross-sectional area and wall thickness (Figure 2A-C).

**Staining for apoptosis: immunohistology**

The ApopTag ® peroxidase in situ apoptosis detection kit (Chemicon International, Apoptosis Detection Kit, S7101, USA) was used to demonstrate apoptosis in a cross-section of basilar arteries. Paraffin-embedded samples were deparaffinized and rehydrated in decreasing concentrations of alcohol. Samples were first treated with proteinase K (20 mg/ml) for 15 minutes at room temperature to increase permeability of the samples. After endogenous peroxidase activity was blocked with 3% H2O2 for 5 minutes, 75 ml equilibration buffer was applied on the sections. The samples were then treated with terminal deoxynucleotidyl transferase (TdT enzyme), which catalyzes polymerization of nucleotides to free 39-OH DNA ends in a template independent manner, and used to label DNA strand breaks, in a humidified chamber for 1 hour at room temperature. Sections were then incubated with antidigoxigenin peroxidase conjugate for 30 minutes. Then to develop color, peroxidase substrate was applied for 3–6 minutes. Next, they were counterstained with hematoxylin and mounted. Between steps, the slides were washed in phosphate-buffered saline (PBS). Sections of the arteries showed staining in the nuclei of the endothelial cells (Figure 3A-C). The apoptotic index was calculated as the number of the immunoreactive nuclei per total number of endothelial cells, expressed as a percentage.

**Statistical analysis**

The differences among three groups were tested by one-way analysis of variance (ANOVA) and the Bonferroni test was used for Post Hoc tests. Statistical analysis was performed by SPSS 11.0 for Windows. Differences of the parametric data were tested by one-way ANOVA.

Differences of apoptotic cells between groups were examined by the Bonferroni test. P<0.05 was considered statistically significant.

**RESULTS**

All 21 animals survived to complete the study. The animals in the SAH only group was hypoactive and lethargic. Rabbits in the control group and ALA treated groups were as active as they were before.

**Biochemical Results**

The average MDA levels was measured as 61.40± 16.60 nmol/ gr in the control group, 94.83± 3.69 nmol/gr in the SAH-only group and 68.07± 16.35 nmol/gr in the SAH+ALA group.

The average SOD values were measured as 1.64± 0.2 U/mg in the control group, 0.9± 0.1 U/mg in the SAH only group, 1.52± 0.32 U/mg in the SAH+ALA group.

The average GSH-Px levels were measured as 1.77± 0.33 U/mg in the control group, 0.71± 0.28 U/mg in the SAH only group, 3.06± 0.43 U/mg in the SAH+ALA group.

**Figure 2A-C**: Cross sections of basilar artery for arterial wall thickness and luminal area measurements. A: Control. B: SAH Only. C: SAH+ALA (Hematoxylin and eosin, bar=100µm).
SAH significantly increased the tissue MDA levels (P < 0.05) and significantly decreased the tissue SOD and GSH-Px activities (P < 0.05) when compared with controls.

ALA (100 mg/kg/day i.p.) treatment has shown protective effect via decreasing significantly (P < 0.05) the elevated MDA levels and also significantly (P < 0.05) increasing the reduced antioxidant enzyme activities (SOD and GSH-Px). The average MDA levels and SOD, GSH-Px activities of the groups are also shown at Figure 4,5,6 respectively.

**Morphometric and Immunhistological Results**

The average wall thickness and the average cross-sectional areas of the basilar artery, the mean percentages of apoptotic cells of the groups were shown in Table I.

![Figure 3A-C: Cross sections of basilar artery for immunohistological investigation. A: Control. B: SAH Only. C: SAH+ALA. The black arrows indicate immunoreactive apoptotic endothelial cells. (TUNEL, original magnification ×200).](image)

![Figure 4: The tissue MDA concentrations of the groups. In the graphic, tissue MDA concentrations were given per gram of wet tissue (nmol/g). Values were expressed as mean±SEM.](image)

![Figure 5: The tissue SOD enzymatic activities of the groups. In the graphic, tissue SOD enzymatic activities were given per mg protein (U/mg). Values were expressed as mean±SEM.](image)
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The agent itself and autooxidation of OxyHb to methemoglobin in the subarachnoid space leads to excessive production of ROS (7). One of the major consequences of ROS injury is the depletion of the cellular antioxidant glutathione, leading to oxidation of protein thiols to disulfides and the loss of enzymes having thiol groups (2).

Several antioxidant agents were used in experimental studies and beneficial effects on vasospasm have been demonstrated (7). Tirilazad, a non-glucocorticoid aminosteroid, was used in human trials and gave promising results (6,7).

The mechanism of action of ALA appears to be due to its ability to substitute for glutathione and four major antioxidant properties of ALA have been demonstrated: it has metal chelating capacity; it can scavenge reactive oxygen species; it can regenerate endogenous antioxidants, including vitamins E and C; and it can repair oxidative damage (2).

Cellular proliferation in the arterial wall and apoptosis of endothelial cells have an important role in cerebral vasospasm pathogenesis (7). Endothelial apoptosis has been observed after cerebral vasospasm and recently p53, a transcription factor that acts as a tumor suppressor, has been implicated in both apoptosis and cellular proliferation in relation to vasospasm (7).

Impairment of endothelium dependent vasorelaxation and directly exposure of vascular smooth muscle cells to spasmogens due to endothelial apoptosis can contribute vasospasm (7). Excessive ROS production can promote endothelial apoptosis by some direct and indirect mechanisms (10). It has been shown that prevention of apoptosis ameliorates cerebral vasospasm (4,7,13).

In this study ALA exert significant neuroprotective effects by inhibiting lipid peroxidation, enhancing the activity of SOD and GSH-Px and reducing morphological changes. Endothelial apoptosis was also prevented by ALA. This study shows treatment with ALA reduced vasospasm in a rabbit SAH model. Additional studies investigating especially the efficacy of delayed treatment are needed.

**DISCUSSION**

In this study systemic administration of ALA just before SAH establishment resulted in a reduction in both biochemical and morphological alterations of vasospasm. ALA treatment reduced the endothelial apoptosis and prevented the endothelial cell integrity.

ROS plays significant roles in the vasospasm pathogenesis after SAH (2). Macdonald and Weir suggested that oxyhemoglobin, which is a ferrous hemoglobin (OxyHb), is the central agent in the development of vasospasm (8). OxyHb is a spasmogenic agent itself and autooxidation of OxyHb to methemoglobin in the subarachnoid space leads to excessive production of ROS (7). One of the major consequences of ROS injury is the depletion of the cellular antioxidant glutathione, leading to oxidation of protein thiols to disulfides and the loss of enzymes having thiol groups (2).

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**CONCLUSION**

ALA therapy may present an alternative modality for the treatment of vasospasm due to SAH. Further studies are needed to implement this treatment option into the clinical practice.

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**Table 1:** Morphometric and Immunohistological Results

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average wall thickness (μ)</th>
<th>Average cross-sectional area (μm²)</th>
<th>Mean percentage of apoptotic cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.020± 2</td>
<td>254266.3850± 212</td>
<td>23.56 ±3.68</td>
</tr>
<tr>
<td>SAH Only</td>
<td>45.6237± 1</td>
<td>132265.9689± 692</td>
<td>77.29± 6.45</td>
</tr>
<tr>
<td>SAH+ALA</td>
<td>25.0503± 1</td>
<td>229949.37 ±178</td>
<td>41.50 ±2.78</td>
</tr>
</tbody>
</table>

The differences between the SAH group and the ALA treated group were statistically significant (P<0.05).

In the control group, the average cross-sectional area of the basilar artery was reduced by 47% after SAH as compared with the control (P<0.05). The mean cross-sectional areas of basilar artery were significantly different between the SAH group and the ALA treated group (P<0.05).

ALA leads to a statistically significant reduction in the mean percentage of apoptotic endothelial cells when compared with other groups (P<0.05).

**DISCUSSION**

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REFERENCES


