The Effect of Alpha-Lipoic Acid on Myringosclerosis in a Rat Model

Arzu Tatar, Özgür Yörük, Deniz Ünal, Jale Selli, Muhammed Yayla, Zekai Halıcı

Department of Otorhinolaryngology-Head and Neck Surgery, Ataturk University Medical Faculty, Erzurum Turkey (AT, OY)
Department of Histology and Embryology, Ataturk University Medical Faculty, Erzurum, Turkey (DU, JS)
Department of Pharmacology, Ataturk University Medical Faculty, Erzurum, Turkey (MY, ZH)

OBJECTIVE: Alpha-lipoic acid (ALA) is an anti-inflammatory and antioxidant agent. In this study, the effects of ALA on the development of myringosclerosis in myringotomized rats were investigated.

MATERIALS and METHODS: The rats were studied in five groups: each group included 6 rats (for a total of 30 rats). After a myringotomy was performed, the groups were administered a 25-mg/kg/day and 50-mg/kg/day dose of ALA for 15 days. The eardrums of the rats were examined histopathologically. We made the histopathological assessment from 0 to 3 on a 4-point scale by scoring the degree of the histopathological changes. In addition, superoxide dismutase, malondialdehyde, and glutathione peroxidase levels were measured in blood samples taken from the rats.

RESULTS: In histopathological examinations, a high dose (50 mg/kg/day) of the ALA-applied group had histopathological results similar to those of the healthy group. In this group, we detected minimal collagen deposition, slight thickness in the lamina propria, and minimal epithelial disruption. In biochemical examinations, the maximum values of superoxide dismutase and glutathione peroxidase were observed in the group that received 50 mg/kg/day of ALA.

CONCLUSION: In this study, the effect of ALA on myringosclerosis development was observed when a dose of 50 mg/kg/day was administered, and a recovery that showed a nearly intact tympanic membrane occurred at the end of 15 days of use. If an antioxidant were to be added to the treatment protocol of tympanic membrane defects, ALA may be a good candidate.

KEY WORDS: Myringotomy, myringosclerosis, alpha lipoic acid, antioxidant

INTRODUCTION
Tympanosclerosis (TS) is an irreversible, nonspecific degenerative process characterized by an increase in collagen fibers and a deposit of extracellular calcium in the lamina propria of the middle ear mucosa and the tympanic membrane because of hyaline degeneration and progressive fibroblast infiltration[1]. Even though TS is usually asymptomatic, it can cause hearing loss when it has a wide area of involvement and when it limits ossicle movement[2]. The involvement of TS in the tympanic membrane and the emergence of a whitish chalky patch are conditions often seen clinically, and such conditions are called myringosclerosis (MS). MS or TS frequently follows a myringotomy, ventilation tube insertion, recurrent otitis media, physical trauma, and middle ear infections. In addition, a genetic predisposition, immunological sensitivity, and local metabolic changes are factors that predispose a person to the development of TS[3-5]. Although the etiopathogenesis of MS is not fully understood, recent studies have reported that reactive oxygen samples (ROS), mechanical injury, and inflammatory diseases are the main factors in the development of MS, and antioxidant agents, free radical connective substances, and anti-inflammatory agents are effective in preventing MS[4, 6]. Some studies reported that in the event of a defect in the tympanic membrane, an increase in the concentration of oxygen in the middle ear results in a rise in the amount of ROS, which leads to MS[3, 4]. In addition, the additive effect of ROS on myringosclerosis was detected in myringotomized rats[6]. Ferlito[7] reported that TS was strictly connected to inflammation and that it was the end product of inflammation, in spite of the severity of TS changes in the type of inflammation (serous, mucous, and purulent inflammation). Recently, Dundar et al.[8] presented a study about the effects of ascorbic acid and N-acetyl cysteine on the development of MS in myringotomized guinea pigs. By assessing sclerosis and inflammation scores, tympanic membrane thicknesses, and the expression of VEGF, TGF-β, iNOS, and IL1-β immunohistochemically, they showed that reduced inflammation scores led to a decrease in MS development. However, to our knowledge no research has shown the effectiveness of ALA on myringosclerosis, even though the potent antioxidant and anti-inflammatory effects of ALA were known.

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Corresponding Address:
Arzu Tatar, Department of Otorhinolaryngology-Head and Neck Surgery, Ataturk University Medical Faculty, Erzurum Turkey
Phone.: +90 506 248 1222; E-mail: berke327@mynet.com
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Alpha-lipoic acid is an anti-inflammatory, anti-oxidant substance that is now popular and was used for many years as a pharmacological agent without serious potential side effects\[^{[9]}\]. ALA, which is the natural cofactor of the mitochondrial dehydrogenase complex, is a physiological component of mitochondrial membranes and a natural antioxidant substance\[^{[9]}\]. After the determination of its antioxidant and anti-inflammatory effects, the beneficial effects of ALA were revealed in studies investigating its effectiveness against many diseases, such as osteoporosis, sepsis, ulcers, and inflammation\[^{[10-13]}\]. In addition, studies are available that show the effectiveness of ALA in healing wounds in different types of tissues\[^{[14,15]}\].

This study intends to investigate the effects of ALA in preventing and reducing the development of myringosclerosis by using histopathological data of an in vivo experimental model and biochemical measurement data of inflammatory mediators [superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH)] that are indicators of antioxidant and anti-inflammatory activity.

**MATERIALS and METHODS**

**Experimental Design**

Thirty 16-week-old male Wistar rats were used for this research, each weighing 250 g to 290 g, obtained from Ataturk University’s Experimental Animal Laboratory of Medicinal and Experimental Application and Research Center. Animal experiments and procedures were performed in accordance with national guidelines for the use and care of laboratory animals and were approved by Ataturk University’s local animal care committee. Rats were housed in standard plastic cages, which had sawdust bedding, in an air-conditioned room at 22°C under controlled light (14-h light/10-h dark cycle). Standard rat food and tap water were given ad libitum.

Alpha-lipoic acid (300-mg caps) (Sigma-Aldrich, Munich, Germany) and all chemicals for laboratory experimentation (Life Time Nutritional Specialties, CA, USA) were obtained commercially.

Thirty pathogen-free animals were anesthetized with ketamine hydrochloride (50 mg/kg; IM) and examined otomicroscopically. The exclusion criteria for the study were an external or middle ear infection before or after surgery and tympanic membrane perforations, adhesions, or retractions.

To demonstrate the development or prevention of myringotomy, a myringotomy animal model was studied on rats as described in the literature\[^{[16,17]}\]. The rats were divided randomly into 5 groups, each containing 6 rats: A, the healthy control group; B, the myringotomy-applied and non-treated group; C, the myringotomy- and physiological saline solution-treated group; D, the myringotomy- and ALA (low dose)-treated group; and E, the myringotomy- and ALA (high dose)-treated group. Myringotomies were performed with an ear speculum in both ears using a sterile pick under the otomicroscope (Opmi 1, Zeiss, Germany) at the upper posterior quadrant of the tympanic membrane near the handle of the malleus. The groups were kept separated in different cages.

Alpha-lipoic acid at 25 mg/kg/day was administered to group D and 50 mg/kg/day was administered to group E starting on the day of the myringotomy surgery. In group C, the rats received a 1.5 ml/day saline solution. In group B and group A, the rats received no treatment with any agent and were evaluated as the control groups. Nasogastric feeding tubes were used for the administration of agents via an oroal gastric approach for 15 days. The dose of the ALA supplement was determined according to the literature\[^{[18]}\]. All rats were anesthetized 15 days after the start of the study. The rats were killed by intraperitoneal injections of pentobarbital (80 mg/kg) and decapitated. Blood samples were collected from the abdominal aorta and centrifuged (2860 g for 5 min at 4°C). The serum was then frozen at -80°C until MDA, GSH, and SOD were measured to evaluate antioxidant activity.

**Histopathologic Evaluation**

On the 15th day, the rats were killed painlessly by high-dose pentobarbital (80 mg/kg, intraperitoneal injection). The tympanic membrane and surrounding bony annulus were removed together (Figure 1). Bulla tissues were fixed in 10% neutral-buffered formaldehyde for 72 h. After fixation, tissues were decalcified with 6% nitric acid for 7 days at room temperature. Sodium bicarbonate was applied for 3 h in a neutralization process to the decalcified specimens. Then, histological processing was applied. The specimens were dehydrated with a decreasing series of alcohol, cleared with xylene, and then embedded in paraffin. Five-micron-thick and five sections were obtained from each paraffin block, which was randomly cut, and stained with Mallory’s triple stain, modified by Crossman, for histopathologic examination. Stained specimens were visualized and evaluated under a light microscope (Table 1). The blinded pathologist evaluated the stained specimens with a light microscope (Figure 2).

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**Table 1. The biochemical results of superoxide dismutase, glutathione peroxidase, and malondialdehyde of all groups**

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD U/mL (mean±SD)</th>
<th>GSH mmol/mL (mean±SD)</th>
<th>MDA nmol/mL (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>55.49±15.72</td>
<td>4.83±0.51</td>
<td>2.81±0.70</td>
</tr>
<tr>
<td>Group B</td>
<td>50.18±10.95</td>
<td>3.33±1.24</td>
<td>2.02±0.25</td>
</tr>
<tr>
<td>Group C</td>
<td>48.41±9.09</td>
<td>4.08±0.99</td>
<td>2.94±0.69</td>
</tr>
<tr>
<td>Group D</td>
<td>55.12±5.71</td>
<td>3.80±1.44</td>
<td>2.39±0.15</td>
</tr>
<tr>
<td>Group E</td>
<td>57.79±7.42</td>
<td>5.33±1.01</td>
<td>2.15±0.35</td>
</tr>
</tbody>
</table>

**Figure 1.** The tympanic membrane of a subject of group B; the arrows show the myringotomic area.

**Figure 2.** Stained specimens with a light microscope (Mallory’s triple stain, modified by Crossman).
Statistical analysis was performed by Statistical Package for Social Sciences (SPSS) 15.0 software (SPSS Inc., Chicago, IL, USA). The means were undertaken separately for the five groups. Comparison of variables of the groups was done using analysis of variance (ANOVA) and Scheffe’s post hoc test. A \( p \)-value less than 0.05 was considered statistically significant.

RESULTS

Biochemical Results
All results of SOD, MDA, and GSH are shown in Table 1. The mean level of SOD was the lowest in group C and the highest in group E; the mean level of GSH was the lowest in group B and the highest in group E; and the mean level of MDA was the lowest in group B and the highest in group C (Table 1).

Histopathologic Results
Using a 4-point scale, we arrived at scores from 0 to 3 in scoring the degree of collagen deposition, increase in the thickness of the lamina propria, disruption of the epithelium, polymorphonuclear leucocyte infiltration, and angiogenesis. Scores meant the following: 0, absence of findings; 1, presence of findings at a minimal level; 2, presence of findings at a moderate level; and 3, presence of findings at an excessive level. Our histopathological findings were staged and are summarized in Figure 2 and Table 2.

Healthy group results: A normal tympanic membrane with its specific epithelium and a thin lamina propria were seen (Figure 2A).

Myringotomy-applied group results: In this group, Masson-dyed bul- la sections, an increase in the thickness of the lamina propria of the tympanic membrane, and collagen deposition were detected conspicuously (Figure 2B).

Myringotomy- and normal saline-applied group results: Similar to the myringotomy-applied group degeneration findings, increased thickness and collagen deposition in the lamina propria occurred. In addition, angiogenesis, as a finding of inflammation, and disruption of the tympanic membrane epithelium were conspicuously seen (Figure 2C).

Myringotomy- and ALA (low dose)-applied group results: The results showed that the perforated edges of the tympanic membrane had more of an increased thickness in the lamina propria and increased collagen deposition than the myringotomy-applied group and myringotomy+normal saline-applied group, and there was interstitial edema (Figure 2D).

Table 2. Histopathological findings for each group were assessed as five categories.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Collagen deposition</th>
<th>Increase of lamina propria thickness</th>
<th>Disruption of epithelium</th>
<th>PNL infiltration</th>
<th>Angiogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group B</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Group C</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Group D</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Group E</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

PNL: Polymorphonuclear lymphocyte
-: Absence of finding
+: Presence of finding at minimal level
++: Presence of finding at moderate level
+++: Presence of finding at excessive level

Figure 2. Summary of the histopathological findings; picture a (1-3) refers to group A; picture B (1-3) refers to group B; picture c (1-3) refers group C; picture d (1-3) refers to group D; and picture e (1-3) refers to group e
Myringotomy- and ALA (high dose)-applied group results: When compared with other groups, this group had an appearance similar to that of the healthy group. There was minimal collagen deposition and slight thickness in the lamina propria. There was minimal epithelial disruption (Figure 2E).

**DISCUSSION**

Although the histological and clinical signs of MS have been well defined, the prophylaxis and treatment are still waiting to be resolved because of the inability to determine the etiopathogenesis exactly. Many hypotheses have been proposed to explain the MS formation process. The most accepted view is that the increase in the production of oxygen free radicals and insufficient antioxidant defense mechanisms may lead to the development of MS when a hyperoxic environment occurs in the middle ear [3, 4, 8].

The oxygen concentration in the middle ear cavity is approximately 5% to 10%. The conditions that damage the integrity of the middle ear, such as myringotomy, cause the oxygen concentration to rise to 21% by causing the ambient air to pass into the middle ear cavity [18]. Previous studies have shown that an increased oxygen concentration causes the development of myringosclerosis by increasing the production of oxygen free radicals [19, 20].

A study by Mattson et al. [21] reported that sclerotic changes occurred within 9 hours of a myringotomy, and an intense inflammatory response was observed significantly within 12 to 24 hours. In the next phase, sclerotic changes in the eardrum were reported. In another study, myringotomized rats were exposed to different oxygen concentrations, and it was determined that myringosclerotic lesions formed more at higher oxygen concentrations [21]. Thus, it has been demonstrated that oxygen free radicals that increase in a hyperoxic environment have a role in the formation of myringosclerosis. Another study by the same researchers indicated that the use of free radical scavengers reduced or inhibited the development of myringosclerosis [30].

After the revelation of the role of oxygen free radicals in the pathogenesis of MS, thanks especially to the study of Mattson et al. [21], many studies showed that this damage, caused by oxygen free radicals, could be successfully reduced or prevented by using antioxidant, free radical connective substances and anti-inflammatory agents that have been reported in the literature. Of these, ascorbic acid, vitamin E, selenium, L-carnitine, N-acetyl cysteine, caffeic acid, ginkgo biloba, pomegranate, N-nitro L-arginine, steroids, and melatonin can be considered [6, 17, 21-29].

Organisms have enzymatic antioxidant mechanisms, such as superoxide dismutase, catalase, and glutathione peroxidase, and non-enzymatic antioxidant mechanisms, such as vitamin C and vitamin E, working as scavengers against the harmful effects of ROS. Oxidative stress is defined as an increase in oxidants and/or a reduction of antioxidant capacity. Antioxidant enzymes, such as SOD and CAT, are involved in the removal of superoxide anions and peroxides. The SOD and CAT system is the first defense mechanism of tissues against oxygen toxicity. An increase in SOD and CAT activities is seen with the use of exogenous antioxidants. Lipid peroxidation is one of the most important expressions of oxidative stress induced by ROS. MDA was used as a marker for lipid peroxidation caused by increased oxygen free radicals in various diseases and as an indicator for oxidative damage [27, 30].

Alpha-lipoic acid is a substance that is a physiological component of mitochondrial membranes, is a natural cofactor of mitochondrial dehydrogenase, and has both potent in vitro and in vivo antimicrobial properties. ALA performs its antioxidant effect with four different mechanisms: ROS-scavenging activity; the regenerative capacity of endogenous antioxidants, such as glutathione, vitamin C, and vitamin E; metal-chelating activity; and the ability to repair oxidized proteins [9, 10]. Several experimental studies have shown that ALA increases the antioxidant capacity of tissue. Therefore, ALA treatment can be used on patients with cirrhosis of the liver, mushroom poisoning, heavy metal poisoning, and diabetic polyneuropathy. Previous studies have reported that the exogenous use of ALA and other antioxidants increases SOD activity and GSH levels and decreases MDA levels [11-13]. In our study, it was seen that SOD and glutathione levels increased in the group treated with ALA as compared to what happened in the control and saline groups. Again, MDA levels that increased in the case of oxidative damage were observed to be lower as compared to those of the control and saline groups. This situation was consistent with the literature.

Oxidative stress leads to the release of acute phase proteins and affects the inflammatory cascade by causing the mucosal infiltration of polymorphonuclear cells, and the activation of TNF-alpha and IL-6. Proinflammatory cytokines are also associated with the release of oxygen free radicals. The relationship between oxygen free radicals and MS is well defined. ALA may be beneficial in preventing the development of MS by affecting the release of inflammatory cytokines in other words, showing an anti-inflammatory effect [11, 15, 31]. In many previous studies, the beneficial effects of anti-inflammatory agents on MS have been shown [6, 21, 26, 27, 32].

In most of the experimental studies on myringosclerosis, histological and otomicroscopic evaluations were preferred. However, a study evaluating the histological and otomicroscopic findings of myringosclerosis reported that otomicroscopic findings did not correlate to findings by light microscopy, and therefore, otomicroscopy was not a good method for the diagnosis of myringosclerosis [33]. In their study on MS, Song et al. [17] showed that there were differences between histological and otomicroscopic findings. In their study, Selcuk et al. [34] showed that calcium channel blockers, used locally, reduced the formation of MS and that tympanosclerosis could not be detected 1 week after administration otomicroscopically, but histological tympanosclerosis developed. In our opinion, otomicroscopic findings may give different results, depending on the difference in personal reviews. Therefore, we used a histopathological evaluation in our study, because it is an objective evaluation method. Although there are many experimental studies on MS, there is no objective, standard finding for a histopathological evaluation [33]. We also used a 4-point scale from 0 to 3 to evaluate the degree of collagen deposition, the increase in the thickness of the lamina propria, the disruption of the epithelium, polymorphonuclear leucocyte infiltration, and angiogenesis.

Many researchers evaluating the effectiveness of antioxidant and anti-inflammatory agents in MS have used tympanic membrane thickness as a parameter for histopathological evaluation and claimed that there was a positive correlation between TM thickness and the severity of myringosclerosis [25-29]. Kazikdas et al. [29] showed the preventive effect of melatonin on MS, which is an antioxidant that is saved from lipid peroxidation and reduces proinflammatory cytokine...
levels. Kinis et al. [32] demonstrated a decrease in tympanic membrane thickness and in the severity of inflammation in myringotomized rats in a group where caffeic acid-the anti-oxidant and anti-inflammatory effects of which are known-was administered intraperitoneally; Kinis et al. [32] also showed a correlation between the severity of the inflammation and the thickness of the tympanic membrane. The lamina propria is one of the three layers of the tympanic membrane (which consists of the lateral keratinized squamous epithelium layer, the middle lamina propria layer, and the medial mucosal layer) [32]. Increased fibroblast proliferation and inflammation intensity increase the thickness of the lamina propria [28]. In our study, the thickness of the lamina propria was found to decrease significantly in group E compared to what occurred in the control and saline groups, probably because of the anti-inflammatory and antioxidant effects of ALA.

Another parameter that is preferred in evaluating MS is inflammatory cell infiltration. Kahya et al. [33] evaluated the density of inflammatory cells in the middle ear in myringotomized rats to which pomegranate was applied; the study demonstrated that the density of inflammatory cells and MS were reduced compared to what happened with the control and saline groups. Similarly, Dogan et al. [28] showed that the severity of inflammation and MS lessened in the treatment group after the administration of L-NAME. In our study, we determined that inflammatory cell density was lower in group E as compared to the control and saline groups. Again, collagen deposition was used as the parameter in many studies and as a marker of fibroblast proliferation, which is characteristic of myringosclerosis; it was shown to be effectively reduced with the use of antioxidants and anti-inflammatory agents. Our study detected that collagen deposition decreased significantly in group E compared to what happened in the other groups.

Angiogenesis was used as a parameter in some studies of myringosclerosis; some authors associated angiogenesis with myringosclerosis, and still, other authors reported that they had found the relationship [25, 35]. In our study, the severity of angiogenesis did not differ significantly in any group. It suggested that there might not be a relationship between the severity of myringosclerosis and angiogenesis.

Histologically significant improvement was not detected in the group treated with low doses of ALA (25 mg/kg/day) among our experimental groups. The result obtained in our study by the low-dose treatment may be explained with treatment applied under effective dose. In fact, the beneficial effects of ALA, the dose range of which was 50-150 mg/kg, were reported to emerge in both human and animal studies at a 30-mg/kg dose [13].

Our study showed that 50 mg/kg/day of ALA administered orally to myringotomized rats remarkably prevented the development of myringosclerosis. In addition, the dose of ALA provides clinical advantages, with its oral administration and low incidence of side effects. As a result, we think that the use of 50 mg/kg/day of ALA for myringosclerosis prophylaxis in humans might be appropriate, especially after myringotomy and VT application.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Ataturk University (approval no: 2013.1.64)

Peer-review: Externally peer-reviewed.


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Conflict of Interest: No conflict of interest was declared by the authors.

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