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Original Article

The Impact of Systemic and Local Administration of Ascorbic Acid on Traumatic Perforation of Tympanic Membrane and Myringosclerosis

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OBJECTIVE: In the present study, tympanic membranes (TM) harvested from myringotomized rats were analyzed histopathologically to compare the systemic and local effects of ascorbic acid on the development of myringosclerosis.

MATERIALS and METHODS: Forty male Wistar-Albino rats weighing between 350-400 g were included in this study. Under otomicroscopic examination, a standard 2-mm myringotomy incision was made on the posteroinferior quadrant of the TM of both ears. Rats were randomized into five groups as control, topical ascorbic acid 50 mg/kg, systemic ascorbic acid 50, 100, and 200 mg/kg groups, each group containing eight rats. On the 15th day of the study, the rats were decapitated, and bullas of the rats were extracted. Sections were stained with hematoxylin-eosin and examined through light microscopy. Inflammation, distribution width of plaques, edema, and neovascularization were observed on the lamina propria. Thickness of the TM was evaluated under the microscope and scored semiquantitatively.

RESULTS: When intergroup comparisons of parameters related to total TM thickness were performed, differences between the control group and topical AA (ascorbic acid) or systemic treatment groups were found to be statistically significant ($p<0.005$). A statistically significant difference was detected among control, topical and systemic 200 mg/kg ascorbic acid groups for the edematous lamina propria ($p=0.003$ and $p<0.05$, respectively).

CONCLUSION: For the total TM thickness, systemic and topical ascorbic acid use was effective when compared with the control group. It has been concluded that systemic use of higher doses of (200 mg/kg) ascorbic acid is beneficial in the resolution of the edematous lamina propria.

KEYWORDS: Tympanic membrane, myringotomized rats, ascorbic acid, myringosclerosis

INTRODUCTION

Perforation of the tympanic membrane (TM) is a frequently seen clinical condition. Its estimated incidence is below 1%; however, its actual incidence is higher [1]. Among etiological factors of TM perforation, infection ranks on top, followed by trauma. Traumatic events generally include blows, stab wounds, barotraumas, exposure to loud noises, and surgical procedures (parasynthesis and application of ventilation tubes) [2].

Myringosclerosis is a degenerative pathology affecting TM. It is characterized by hyalinization and increase in collagen fibers in the lamina propria (LP) of the TM. Besides, accumulation of calcium and phosphorus on this structure leads to crystalization and sclerosis [3, 4]. Traumatic perforations or hyperoxidative condition and formation of free oxygen radicals are known to be effective on the development of sclerosis [5]. In many studies performed, various antioxidants have been tried to prevent the development of sclerosis after traumas or parasynthesis which injured TM and quantitative analysis of free oxygen radicals was perform to demonstrate a decrease in the development of sclerosis [5, 6].

Ascorbic acid (AA) is a potent reducing agent and an antioxidant [7]. The effect of topical application of ascorbic acid on the development of sclerosis in TM of rats was analyzed and alleviating effect of ascorbic acid on the development of myringosclerosis was demonstrated [8].

This study was presented at the 11th International Congress of Otorhinolaryngology and Head and Neck Surgery on 17-19 April, 2014, Ankara, Turkey.

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The relevant literature was reviewed and any study comparing effects of systemic and local applications of ascorbic acid on the traumatic eardrum and myringosclerosis was not encountered. However, in our study, TMs harvested from myringotomized rats were analyzed histopathologically with an intention to compare the systemic and local effects of ascorbic acid on the development of myringosclerosis.

MATERIALS and METHODS

The study was approved by the Ethics Committee of Ankara University Experimental Animal Research Laboratory (2013-8, File No. 2013-54). Forty male Wistar-Albino rats were included in the study. After induction of anesthesia with intraperitoneal injections of 50 mg/kg ketamine hydrochloride (Ketalar; Pfizer Warner Lambert, ABD) and 5 mg/kg xylazine hydrochloride (Rompun %2; Bayer HealthCare, ABD), 40 rats with normal bilateral TM as detected on otomicroscopic examination were included in the study. Under otomicroscopic examination, using ear speculum and a sterile pick, a standard 2-mm myringotomy incision was made on the posteroinferior quadrant of the TM of both ears. Rats were randomized into 5 groups as control, topical AA, 50 mg/kg and systemic AA 50, 100 and 200 mg/kg groups, each group containing eight rats (hence, 16 ears).

The first group which was determined as the control group did not receive any treatment. Ten mg ascorbic acid impregnated patches were placed on the perforation site on ears of 8 rats (totally 16 ears), immediately after myringotomy. Once daily doses of 10 mg ascorbic acid (Redoxon amp 500 mg/5 mL; Bayer Chemical Industry, Istanbul, Turkey) were administered for 14 days on the external ear canal of the rats. For 14 days, 200 mg/kg ascorbic acid was intraperitoneally (IP) administered.

On the 15th day of the study the rats were decapitated following high doses (80 mg/kg) of IP pentothal (Pental Sodium; I. E. Ulagay Pharmaceutical Industry, Istanbul, Turkey). Histopathological examination was performed on the 15th postoperative day, in accordance with previously performed and published studies [8-11].

After decapitation, bullas of the rats were extracted. Histopathological preparation and evaluation procedures of the study were performed. Sections were stained with hematoxylin-eosin (H&E) and examined under light microscope. Inflammation observed on LP of the TM were rated between 0 and 3 points and scored semiquantitatively (Table 1a). Because the evaluation of inflammation was based on semiquantitative scores, for statistical intergroup comparisons results were evaluated in 2 groups (Table 1b).

Histopathological assessments were performed by the same histopathologist. This histopathologist had previously participated in many studies of similar design [12].

For the measurement of total thickness of TM, microscopic images of H&E stained sections were transferred by means of a camera into a computerized data base After calibration, TM thickness was measured in microns at different fields of vision using ocular and carriage micrometers, and the average of these measurements was accepted as the thickness of the TM. For the measurement of total tympanic membrane thickness, microscopic images of H&E-stained cut sections were evaluated for tympanic membrane thickness in epithelial, and connective tissue of perforated, and non-perforated areas. On randomly selected 10 fields of vision, sections were measured in microns, and average of all these measurements was accepted as the tympanic membrane thickness. Thickness of the TM was evaluated under ×200 magnification using an oculometer mounted on the ocular of the microscope. Using images obtained from H&E stained sections, sclerotic lesions on the LP of the TM were evaluated. Findings were semiquantitatively scored between 0 and 2 points (Table 2a). Degrees of edema and neovascularization of LP were evaluated in three categories (Table 2b).

Table 1. Inflammation observed on lamina propria of the tympanic membrane (a) and grading (b)

Table 1a	
No inflammation	0
Mild inflammation	1
Moderate inflammation	2
Severe inflammation	3
Table 1b	
Absent–Mild	Grade 0 and 1
Moderate–Severe	Grade 2 and 3

Table 2. Sclerotic lesions observed on tympanic membrane (a). Degrees of edema and neovascularization of lamina propria (b)

Table 2a	
No myringosclerosis	0
Rarely located sclerotic lesions on lamina propria	1
Dense and wide sclerotic lesions	2
Table 2b	
Absent	0
Mild	1
Moderate	2
Severe	3

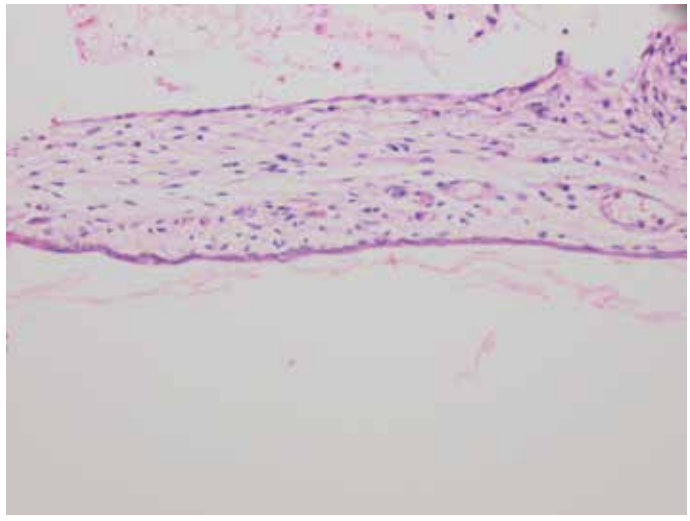

Figure 1. Histopathological appearance (×40 magnification) of a specimen in a topical ascorbic acid application group

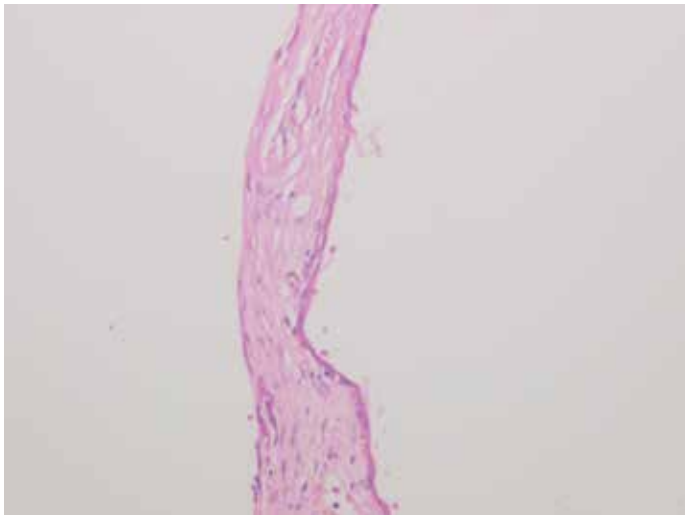

Figure 2. Histopathological appearance (×40 magnification) of a specimen in a systemic ascorbic acid application (50 mg/kg) group

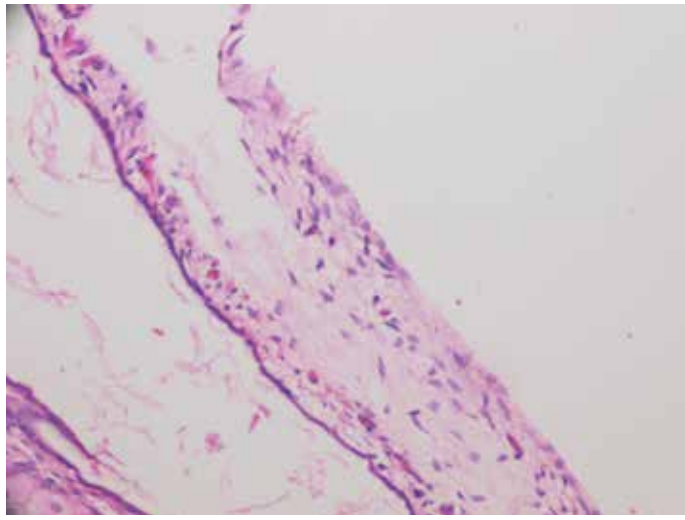

Figure 3. Histopathological appearance (×40 magnification) of a specimen in an untreated control group. Deficiency chronic inflammatory cells and edema

Before the study, the tympanic membranes of all rats were examined under general anesthesia. All rats with detected pathological findings were excluded from the study. Besides, rats with histopathologically detected dense polymorphonuclear leukocytic infiltration and fibrin accumulation in their prepared specimens were considered to be infected; hence, they were excluded from the study. Thus, other factors that might cause myringosclerosis were ruled out.

Statistical Analyses

Statistical analysis was performed using SPSS for Windows, version 20.0. (Statistical Package for Social Sciences; SPSS Inc., Chicago, IL, USA). Parametric tests were applied to data of normal distribution and non-parametric tests were applied to data of questionably normal distribution. The Mann-Whitney U-test was used to compare independent groups. The distribution of categorical variables in both groups was compared using the Pearson chi-square test. Data are expressed as mean±standard deviation (SD). All differences associated with a chance probability of 0.05 or less were considered statistically significant.

RESULTS

Distribution of the severity of inflammation did not differ among topical, systemic 50-100-200 mg/kg AA and the control groups ($p>0.05$). When intergroup comparisons of parameters related to total TM thickness were performed, differences between control group and topical AA or systemic treatment groups were found to be statistically significant ($p<0.005$). Besides, any statistically significant difference was not detected between topical and systemic treatment groups ($p>0.005$). Comparisons among systemic treatment groups revealed a statistically significant difference between 50 mg/kg AA and 100 mg/kg or 200 mg/kg AA groups ($p<0.005$). However, a significant difference was not detected between 100 mg/kg AA and 200 mg/kg AA groups ($p>0.005$). A statistically significant difference was not found among the five groups as for distribution of myringosclerotic plaque ($p>0.05$). A statistically significant difference was not found regarding LP edema among control, topical, and systemic 200 mg/kg AA; in addition, among control, topical, and systemic 100 mg/kg AA groups ($p=0.215$, $p>0.05$, $p=0.066$ and $p>0.05$, respectively). However a statistically significant difference was detected among control, topical, and systemic 200 mg/kg ascorbic acid groups ($p=0.003$ and $p<0.05$, respectively). As for neovascularization in the LP of the TM, a statistically significant difference was not observed among the five groups ($p>0.05$). Intragroup evaluations and total TM thickness are presented in Table 3a, 3b, and Table 4.

DISCUSSION

Myringosclerosis is characterized by calcification and hyalinization of the collagenic structure of the LP layer of the TM. Generally, it manifests following traumatic TM perforations or parasynthesis applied for the treatment of experimental otitis media (EOM) or the use of ventilation tube to balance areation of the middle ear [13, 14]. As a striking finding, as for the structure and location of plaques, microscopic examination of sclerotic lesions reveals no difference between rats and human beings [15, 16]. Another issue to be underlined is that irreversible tissue damage induced by free oxygen radicals is the most important factor in the development of myringosclerosis [5, 8, 10, 17].

Free oxygen radicals can be rendered ineffective by antioxidant subagents. Previous studies in literature have demonstrated that the use of vitamin E, ascorbic acid, selenium, N-acetylcysteine, L-carnitine can prevent the development of myringosclerosis [5, 8, 10, 17].

For the ameliorating effects of ascorbic acid on the healing process, many underlying mechanisms have been asserted. It has been reported that ascorbic acid applied on traumatic ear drum contributes to wound healing due to its antioxidant effect [8]. In addtion, increased amounts of Fe++ ions in the wound tissue adversely affect healing [18]. Application of ascorbic acid decreases Fe++ ions in the tissue and in turn effects wound healing favorably [19]. In our study, we preferred to prevent the formation of peroxynitrite with ascorbic acid. We aimed to suppress the production of superoxide and inhibit formation of peroxynitrite, using AA. Thus, we aimed to suppress the development of myringosclerosis by preventing tissue damage that is induced by peroxynitrite.

Ascorbic acid and other vitamins with antioxidant properties increase the levels of endothelial nitric oxide (NO) to mediate the functions

Table 3a. Inflammation observed on lamina propria and Sclerotic lesions observed on tympanic membrane among study groups

	Inflammation			Distribution of myringosclerosis			
	Absent+Mild	Moderate+Severe	Total	Absent	Rare	Dense	Total
Control	14 (100%)	0 (0.0%)	14 (100%)	5 (35.7%)	5 (35.7%)	4 (28.6%)	14 (100%)
Systemic 50 mg/kg AA	8 (72.7%)	3 (27.3%)	11 (100%)	1 (9.1%)	5 (45.5%)	5 (45.5%)	11 (100%)
Systemic 100 mg/kg AA	7	4	11	7	3	1	11
Systemic 200 mg/kg AA	9	6	15	1	13	1	15
Topical AA	9 (75.0%)	3 (25.0%)	12 (100%)	4 (33.3%)	6 (50.0%)	2 (16.7%)	12 (100%)
Total	47	16	63	18	26	13	63

AA: ascorbic acid

Table 3b. Degrees of edema and neovascularisation of lamina propria among study groups

	Edema					Neovascularization				
	Absent	Mild	Moderate	Severe	Total	Absent	Mild	Moderate	Severe	Total
Control	11 (78.6%)	3 (21.4%)	0 (0%)	0 (0%)	14 (100%)	5 (35.7%)	9 (64.3%)	0 (0.0%)	0 (0.0%)	14 (100%)
Systemic 50 mg/kg AA	5 (45.5%)	6 (54.5%)	0 (0%)	0 (0%)	11 (100%)	2 (18.2%)	9 (81.8%)	0 (0.0%)	0 (0.0%)	11 (100%)
Systemic 100 mg/kg AA	5	3	3	0	11	3	6	2	0	11
Systemic 200 mg/kg AA	2	12	1	0	15	4	5	5	1	15
Topical AA	6 (50%)	6 (50%)	0 (0%)	0 (0%)	12 (100%)	3 (25.0%)	8 (66.7%)	1(8.3%)	0 (0.%)	12 (100%)
Total	29	30	4	0	63	17	37	8	1	63

AA: ascorbic acid

Table 4. Total tympanic membrane thickness among study groups

Topical AA mean±SD (min-max) (micron)	2.21±1.42 (1-4)
Systemic 50 mg/kg AA mean±SD (min-max) (micron)	3.27±1.19 (2-6)
Systemic 100 mg/kg AA mean±SD (min-max) (micron)	1.31±0.81 (0,5-3)
Systemic 200 mg/kg AA mean±SD (min-max) (micron)	1.53±1.15 (0,5-4)
Control mean±SD (min-max) (micron)	4.42±2.61 (1-10)

SD: standard deviation; Min: minimum; Max: maximum; AA: ascorbic acid

of vascular endothelium [20]. During the inflammation phase of the wound healing process, abundant amounts of NO are released, and following the inflammatory process, the activity of NO tapers and disappears [20]. In our study, our motive behind using ascorbic acid was to decrease the impact of inflammatory response on LP of the TM through blockade of excessive synthesis of NO by inhibiting induced NO secretion. Based on study results, we were unable to observe a significant impact of topical or systemic use of ascorbic acid on inflammation when compared with the control group.

In a study that investigated the ameliorating effect of ascorbic acid on healing process of the ear drum, topical ascorbic acid was applied on experimentally perforated ear drums of rats, and a lesser degree of myringosclerotic development was observed, relative to the control group. The authors asserted that topical use of ascorbic acid on ear drum contributed favorably on wound healing due to its antioxidant activity [21].

In our study, we have concluded that topical application of AA does not prevent the development of a myringosclerotic plaque, while it has a significant effect on total membrane thickness when compared with the control group.

In literature, studies concerning the effects of the topical use of ascorbic acid on traumatic ear drum are available [8, 22]. However, we did not encounter any study which investigated the impact of systemic AA use on myringosclerosis.

In histopathological evaluations of the sections obtained from the control group, a decrease in the number of chronic inflammatory cells, and edematous cells in the lamina propria were observed. However an increase in fibroblastic activity, hyalinization, and collagenization were seen. These alterations were thought to be potential factors increasing total tympanic membrane thickness.

In many studies that investigated the antioxidant efficacy and wound healing properties of ascorbic acid different doses have been used [23-26]. In our study, we used spongostan impregnated with 10 mg topical ascorbic acid and intraperitoneal ascorbic acid at doses of 50-, 100-, and 200 mg/kg. In literature, we have not encountered any study that demonstrated the contribution of use of different doses, and time periods on treatment. Treatment period of 15 days, and drug dosages used are limitations of our study. We have indicated the limitations of our study.

In our study, we aimed to compare the effects of systemic and topical applications of ascorbic acid on the healing of traumatic ear drum and myringosclerosis. We concluded that the use of systemic AA decreased total membrane thickness when compared with the control group. However, a statistically significant difference was not observed in association with topical treatment group.

Besides, ascorbic acid affects wound tissue just like NO by increasing vasodilation and vascularization [27]. Spratley et al. [8] demonstrated increased vascularization of the perforated ear drums treated with AA. In the healing process of traumatic ear drum, neovascularization is required for the development of epithelial and fibrotic layers [28]. We have concluded that higher doses (ie. 200 mg/kg) of systemic ascorbic acid have a significant effect on edematous LP when compared with the other groups (p=0.003 and p<0.05, respectively).

The limitation of our study is that we performed only histopathological evaluation in other groups.

It is possible to perform audiological and electrophysiological tests along with histopathological tests to investigate the effectiveness of the drug, administered at various doses and for different periods of time.

In conclusion, systemic and topical ascorbic acid use was not effective regarding the extent of inflammation, distribution width of plaques, and neovascularization. Regarding TM thickness, systemic and topical ascorbic acid use was effective when compared with the control group. It has been concluded that systemic use of higher doses (200 mg/kg) of ascorbic acid is beneficial in the resolution of edematous LP.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Ankara University Experimental Animal Research Laboratory (2013-8, File No. 2013-54)

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

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