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OTOLOGY

Is there any effect of montelukast on prevention of myringosclerosis after myringotomy in a rat model?

Selda Kargin Kaytez[1] · Ali Kavuzlu[1] · Nihat Yumusak[2] · Ramazan Oçal[1] · Ozlem Akkoca[1]

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Abstract

Objectives In this study, our aim was to identify the possible effects of montelukast sodium (ML) on the prevention of experimentally induced myringosclerosis.

Materials and methods Twenty-eight female Wistar albino rats were used and they were divided into four groups randomly. Tympanic membranes (TM) of all animals were perforated and then group 1 received no treatment (control group), group 2 was treated with a topical saline solution, group 3 received topically ML and group 4 received orally ML. On the 15th day, all animals were euthanized. Tympanic membranes were evaluated otomicroscopically and histopathologically.

Results The histopathological findings, compared against a control and saline groups, showed the topically and orally ML groups had statistically significant differences of degree of myringosclerosis ($p < 0.002$) and median thickness of the TMs ($p < 0.001$). Suppression of inflammation was statistically significant only in the oral ML treatment group ($p < 0.002$).

Conclusion Oral and topically administration of ML reduced myringosclerosis formation in myringotomies rats.

Keywords Antioxidants · Myringosclerosis · Montelukast · Tympanic membrane

Introduction

Myringosclerosis (MS) is an irreversible degenerative improvement mechanism of the tympanic membrane (TM) which can result in dystrophic calcification and hyalinization in the fibrous layer [1]. It appears as a semicircular crescent or horseshoe-shaped white chalky patches generally at the anterior and posterior inferior quadrants of the TM [2]. Histopathologically characterized by an increase in collagen fibers due to progressive fibroblast infiltration as well as hyaline degeneration and extracellular calcium deposition within lamina propria [2–5]. Usually, MS is a caused by recurrent otitis media, chronic middle ear effusion, myringotomy, ventilation tube insertion, trauma and autoimmunity [6–8].

In the pediatric population, otitis media with effusion is a common disease and often necessitates a surgical intervention like myringotomy or ventilation tube insertion. The risk of MS especially increases in children who had ventilation tubes inserted [9, 10]. The clinical studies showed that the rate of MS is higher in TM with tympanostomy tube insertion than in TM with no tympanostomy tube insertion [10–12]. The incidence of myringosclerosis in children for whom ventilation tubes inserted is between 28 and 61% [13]. Several hypotheses have been proposed for the etiology of MS: traumatic perforations, the formation of oxygen-derived free radicals, immunologic sensitivity, metabolic disturbance and inflammatory reaction [2, 14, 15]. Recent studies have shown that formation of oxygen-derived free radicals because of hyperoxidative conditions may be the primary reason for the formation of MS [2, 14, 16, 17]. Therefore, MS may be reduced or prevented by administration of anti-inflammatory or antioxidant agents which can inhibit the harmful effects of oxygen-derived free radicals [4, 5, 18, 19].

Montelukast (ML) is a selective, antagonist of type 1 cysteinyl leukotriene (CysLT1) receptor. It is a licensed drug for asthma and allergic rhinitis. It has also been reported that antioxidant and anti-inflammatory effects are among the benefits of ML in a various experimental models [20–24].

✉ Selda Kargin Kaytez

[1] Department of Otorhinolaryngology-Head and Neck Surgery, Ministry of Health, Ankara Training and Research Hospital, Ankara, Turkey

[2] Department of Pathology, Harran University, Faculty of Veterinary Medicine, Şanliurfa, Turkey

ML was used orally in the literature by diluting with saline or distilled water [25, 26].

In our study, by creating tissue trauma in rats, we evaluated the effectiveness of ML in the prevention of myringosclerosis for the first time in the literature.

Materials and methods

Experimental material and animal maintenance

The experimental study complied with experimental ethical principles and animal protection laws according to the rules and regulations, approved by the Local Ethics Committee (Prot. no: 01/03/2018-0045). This study was conducted in compliance with the guidelines for animal experimentation at the Department of Laboratory Animal Science of Medical School. All animal care and procedures were performed humanely. The animals were kept in ordinary cages with free access to food and water and at a temperature-controlled (20 ± 2 °C) and humidity-controlled ($60 \pm 5\%$) room in which 12-h light/12-h dark photoperiods. They were provided standard laboratory food and tap water ad libitum and used after 1 week of quarantine and acclimatization.

Experimental design and surgical procedure

Twenty-eight female Wistar albino rats, weighing 250 g, at age 10–12 weeks, were used in this study. They were anesthetized with ketamine hydrochloride (50 mg/kg, intramuscular). The animals with bilateral normal tympanic membranes were included in the study under an otomicroscopic (Opmi 1, Zeiss, Germany) examination after anesthesia. Myringotomies were performed bilaterally in the posterior superior quadrant of TMs through an ear speculum with sterile pic under the otomicroscope by same authors. Intratympanic bleeding was not observed during this procedure. In the working process, if any signs of ear infection in the animals were detected, those were excluded from the study.

The rats were randomly divided into four groups (groups 1, 2, 3, 4). Each group included 7 rats. Group 1 served as the control group and was not administered any treatment. Group 2 received 0.1 ml saline solution topically in both ears. Group 3 received topically montelukast (ML) 10 mg/kg daily for 15 days in both ears (Notta tb 5 mg (5.2 mg montelukast sodium), Sanovel, Turkey, diluted with 0.2 ml saline). Saline solution and ML diluted with saline were 0.1 ml dropped into external ear canal using a syringe. In group 4, the animals received oral ML 10 mg/kg daily for 15 days Notta tb 5 mg (5.2 mg montelukast sodium), Sanovel, Turkey, with a feeding needle. The medicine was administered daily for 15 days. The doses of ML was selected on the basis of previous experimental studies [21, 22]. Two TMs of another rat were used for histopathologic comparison between the TMs of study groups and the healthy intact TM (Fig. 1).

Otomicroscopic examination

On the 15th day of the study, the tympanic membranes of 28 rats (56 ears) were examined through otomicroscopy under anesthesia with ketamine. The status of TMs was evaluated and myringosclerotic lesions were documented semiquantitatively by using 4-point scale: (0), no visible myringosclerotic plaques; (1), occasional MS with white halo around umbo; (2), moderate MS with white halo around umbo and white line beside the handle of the malleus and along the annulus; and (3), severe MS with confluent whitish deposits, forming a horseshoe-shaped pattern [2].

Histopathological examination

After the otomicroscopic evaluation, the rats were killed painlessly by high-dose pentobarbital (80 mg/kg, intraperitoneal injection). TM and surrounding bony annulus were removed. All histopathological evaluations were made by one pathologist who did not know what treatment was applied in the light microscope. They were fixed with 10% buffered neutral formalin overnight and decalcified with 10% nitric oxide solution. After decalcification specimens were dehydrated with graded alcohol (50%, 75%, 96%, 100%) baths and sampled including the myringotomy region. The specimens were embedded in paraffin. From these blocks, 4-µm-thick sections were taken with the Leica RM2125 RT and the first three sections and each tenth section were taken into the slides. Sections were prepared at the TM where myringotomies were performed. Prepared preparations

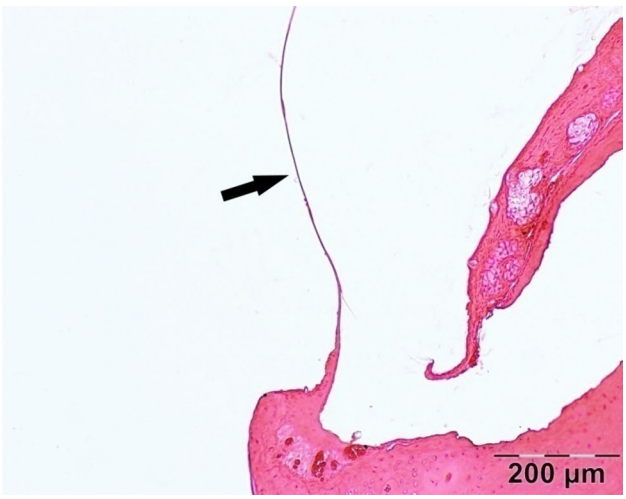

Fig. 1 Appearance of normal tympanic membrane (0 point scale) in (hematoxylin and eosin, original magnification × 200)

Table 1 Histopathologic examination of myringosclerosis

Group	Ear number	Myringosclerosis (MS)			
		No MS (%)	Occasional MS (%)	Moderate MS (%)	Severe MS (%)
1 (non-treated)	14	0 (0.0)	2 (14.3)	8 (57.1)	4 (28.6)
2 (topical saline)	14	0 (0.0)	8 (57.1)	2 (14.3)	4 (28.6)
3 (topical ML)	14	8 (57.1)	6 (42.9)	0 (0.0)	0 (0.0)
4 (oral ML)	14	10 (71.4)	4 (28.6)	0 (0.0)	0 (0.0)

were stained with hematoxylin–eosin (HE) and Masson's trichrome staining by passing through graded alcohol and xylol series. Masson's trichrome staining was used to evaluate the sclerotic changes in the connective tissue of lamina propria. All specimens were examined in a high-resolution light microscope (Olympus DP-73 camera, Olympus BX53-DIC microscope; Tokyo, Japan) 40× to 100× magnification.

The degree of sclerotic lesions and intensity of fibroblastic proliferation in the lamina propria of tympanic membranes were graded semiquantitatively as 0, no visible myringosclerosis; 1, occasional myringosclerosis; 2, moderate myringosclerosis; 3, serious myringosclerosis.

Total thickness measurement of tympanic membrane was obtained from H&E stained sections. Microscopic images were computerized with a camera (Olympus BX50, Olympus Optical Co., Tokyo, Japan). By calibrating according to the magnification used, tympanic membrane thicknesses were measured in 10 areas in terms of micrometers, the mean of the measurements was taken as tympanic membrane thickening.

Statistical analysis

Statistical analyses were performed using SPSS v.21 (SPSS, Inc. Chicago, IL, USA) and a value of $p < 0.05$ was accepted as significant. Histopathological MS scores in the lamina propria in each group were compared with Fisher's exact test. The median thickness of the TMs differences was analyzed by Kruskal–Wallis test. The Mann–Whitney *U* test was used to determine which groups median thickness of the TMs were different from the others. To find difference mean thickness of the TMs between the groups Bonferroni correction is applied.

Results

Otomicroscopic examination all TM perforations in four groups on the 15th day were healed and closed. In the otomicroscopic examination, we observed the calcific deposits in 42 TM. In 38 of them, we have observed calcific deposits and thickness after the histopathologic examination. Table 1 summarizes the results of histopathologic examination of among groups. More myringosclerotic plaques and thickness were seen in group 1 (non-treated) (Fig. 2) and group 2 (treated with topical saline solution) (Fig. 3), but the TMs of the rats of group 3 (treated with topical ML) (Fig. 4) and group 4 (treated with oral ML) (Fig. 5) showed significantly less or no sclerotic plaques and thickness. Fisher's exact test showed $p < 0.002$ in MS between the groups.

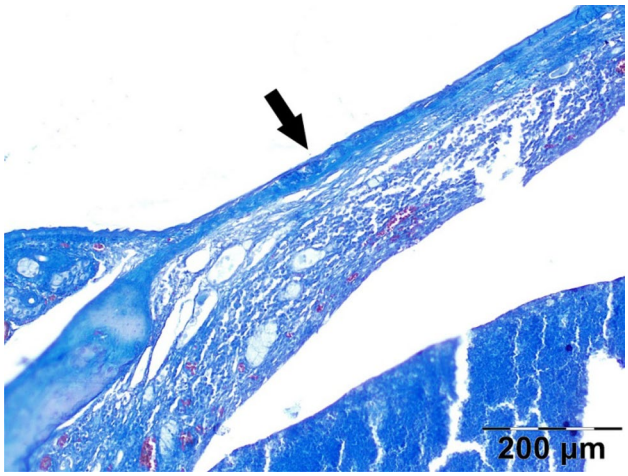

Fig. 2 Sclerotic areas and thickness in the tympanic membrane from group 1 (Masson's trichrome staining, original magnification × 200)

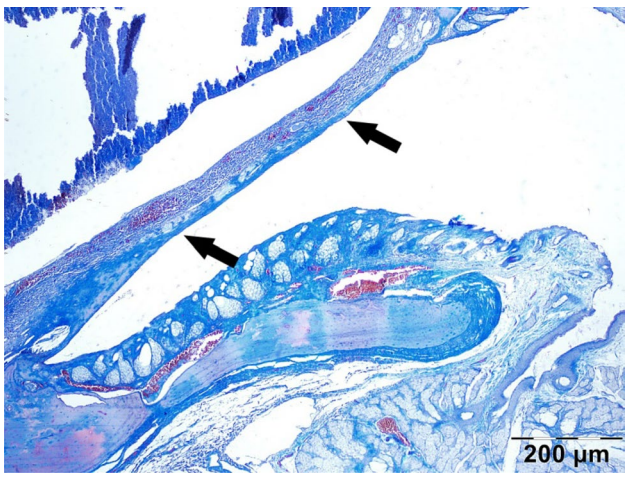

Fig. 3 Sclerotic areas and thickness in the tympanic membrane from group 2 (Masson's trichrome staining, original magnification × 200)

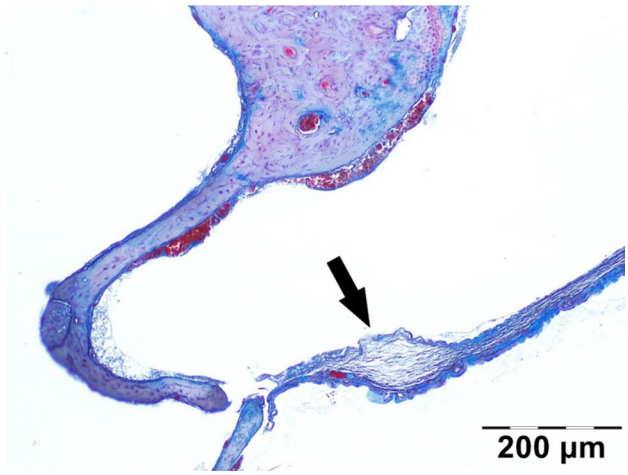

Fig. 4 Sclerotic areas and thickness in the tympanic membrane from group 3 (Masson's trichrome staining, original magnification × 200)

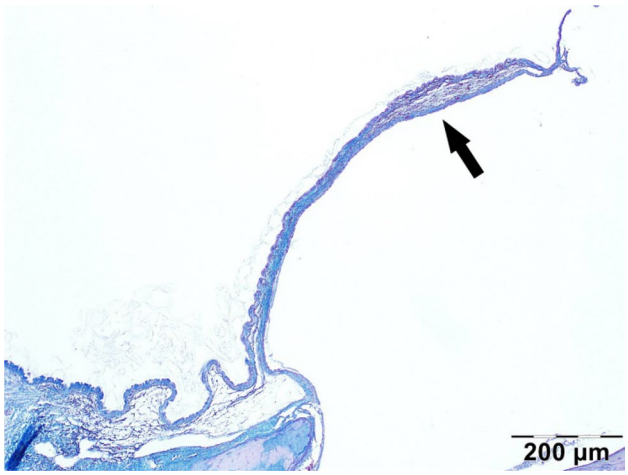

Fig. 5 Sclerotic areas and thickness in the tympanic membrane from group 4 (Masson's trichrome staining, original magnification × 200)

There were statistically significant differences between in group 1–3 ($p < 0.014$), in group 1–4 ($p < 0.002$) and in group 2–4 ($p < 0.024$). There was no significant difference between group 1 and group 2 ($p < 0.184$), group 2 and group 3 ($p < 0.062$), group 3 and group 4 ($p < 0.576$).

Median thickness has been determined in all TM. Table 2 summarizes the results of tympanic membrane thicknesses of among groups. The median thickness of the TMs of group 1 were 136.6 ± 37.3 µm; in group 2 were 212.7 ± 37.45 µm; in group 3 were 42.6 ± 8.42 µm and in group 4 were 31.1 ± 7.98 µm. The median thickness of the TMs differences was analyzed by Kruskal–Wallis test. Kruskal–Wallis test showed p 0.001 in terms of TM thickness between the groups. The Mann–Whitney U test was used to determine which groups median thickness of the TMs were different

Table 2 Thickness of tympanic membrane

Group	Number	Thickness of tympanic membrane (micrometer)			
		Min.	Max.	Mean	Std. deviation
1 (non-treated)	14	93	192	136.6	37.34
2 (topical saline)	14	83	180	121.7	37.45
3 (topical ML)	14	28	56	42.6	8.42
4 (oral ML)	14	17	42	31.1	7.98

from the others. According to the Mann–Whitney U test, significant differences were observed between group 1–3 ($p < 0.001$), group 1–4 ($p < 0.001$), group 2–3 ($p < 0.001$) and group 2–4 ($p < 0.001$). There was no significant difference between group 1–2 ($p < 0.383$) and group 3–4 ($p < 0.025$) according to the mean thickness of TM (Bonferroni correction p 0.0083).

Discussion

Myringosclerosis (MS) is irreversible, non-specific, the end result of chronic inflammation or trauma or infection of the TM that is characterized by the hyaline degeneration of the elastic fibers in the ear membrane and with the calcium deposition [2, 7]. The real cause and pathogenesis of it are not well-known but recent studies have emphasized that mechanical injury, oxygen-derived free radicals and inflammatory reactions may be the main factors in the formation of MS. Mattsson et al. [17] reported that under the light microscopic examination the sclerotic changes in tympanic membrane developed within 9 h and inflammatory response after 12–24 h after myringotomy. In the literature, the studies were reported that MS formation might be reduced or prevented by administration of antioxidants, free radical scavengers and anti-inflammatory agents [4, 5, 18, 19].

There have been many experimental studies attempting to prevent the development of MS by applying free radical scavengers topically such as ascorbic acid [19], *N*-acetylcysteine [18], copper zinc, superoxide dismutase plus catalase and deferoxamine [14]. Systemically applied drugs such as L-carnitine [27], selenium [5], caffeic acid phenethyl ester [3], vitamin E (alpha-tocopherol) [4], *Ginkgo biloba* [28] were effective to prevent MS formation. In the literature; topically applied antioxidants such as copper, zinc, superoxide dismutase, catalase, ascorbic acid and deferoxamine prevent or reduce the development of sclerotic lesions [14, 19]. Consequently, the findings have supported the hypothesis that the formation of reactive oxygen species contributed significantly to the development of MS. The development of MS after myringotomy can be

prevented by anti-inflammatory drugs such as the dexamethasone or topical fenspiride [17].

Cysteinyl leukotrienes (CysLTs) are synthesized from arachidonic acid by the metabolic pathways initiated by the 5-lipoxygenase enzyme mainly by mast cells, basophils, eosinophils, macrophages; however, they are also synthesized by neutrophils, thrombocytes, lymphocytes, endothelial cells, erythrocytes and act via CysLT1 and CysLT2 receptors [29, 30]. Montelukast (ML) is a selective, antagonist of the CysLT1 receptor [20]. It is a licensed drug for asthma and allergic rhinitis. Some studies reported that ML may have a beneficial effect on the improvement of otitis media with effusion [31, 32]. Increasing evidence has shown the anti-inflammatory, anti-fibrotic capacity and antioxidant protective effects of montelukast in various tissues [33–36]. Sener et al. have reported that ML's ability to inhibit neutrophil infiltration and apoptosis and also balances the oxidant–antioxidant status and regulates the generation of proinflammatory mediators [37].

In our study, we selected the myringotomy model to generate myringosclerosis in light of the above-mentioned literature on myringosclerosis, and we sacrificed the rats on day 15 to assess the clinical/histological findings.

Santos et al. reported that the sensitivity of the otomicroscopy has been found as 80%, and specificity has been found as 75%. They have reported that the histological findings are a better method in the diagnosis of myringosclerosis than otomicroscopy [38]. Thickness and inflammation also were observed in all TMs where histopathological MS has been found. We also believe that histological findings are a better method for diagnosing myringosclerosis than otomicroscopy.

When the histopathological findings of the study were examined, it was determined that myringosclerosis development and thickness of TMs after experimental myringotomy in rats were significantly decreased in topical and oral ML treatment groups. In this study, it was shown that the topical and oral application of montelukast (ML) reduced fibrosis and prevented the MS development. Previously, several studies revealing the anti-inflammatory, anti-fibrotic and antioxidant effects of ML were reported [33–36] but to our knowledge, this is the first study to evaluate the use of ML for prevention the development of MS.

Prevention and treatment effects of ML may be useful for humans who are at risk of developing myringosclerosis. Therefore, clinical research should be designed and undertaken to confirm this expectation.

Conclusion

To our knowledge, there have been no studies on the preventive effect of ML on myringosclerosis development; hence, this study represents the only study to interpret the association between ML and MS development. In light of our study's otomicroscopic and histological findings, it can be suggested that topical and oral ML administration may have a positive effect in preventing myringosclerosis formation. This effect may be related to the anti-inflammatory, anti-fibrotic and antioxidant effects of ML. ML is a licensed drug used in the treatment of asthma and allergic rhinitis and can also be useful in otitis media treatment. Therefore, it can be widely used in the prevention and treatment of myringosclerosis in people at risk of developing myringosclerosis.

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Funding There is no financing in this study.

Compliance with ethical standards

Conflict of interest There is no conflict of interest in this study.

Ethical approval The experimental study complied with experimental ethical principles and animal protection laws according to the rules and regulations in Turkey, approved by the Local Ethics Committee in Ankara, Turkey (Prot. no: 01/03/2018-0045). This study was conducted in compliance with the guidelines for animal experimentation at the Department of Laboratory Animal Science of Medical School.

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