Experimental Study / Deneysel Çalışma

The effect of melatonin on experimentally-induced myringosclerosis in rats

Sıçanlarda deneysel olarak oluşturulan miringosklerozda melatoninin etkisi

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Objectives: This study determined the preventive effect of melatonin on the occurrence of experimentally-induced myringosclerosis of the tympanic membrane (TM).

Materials and Methods: Twenty Wistar albino-type rats weighing approximately 300 g each were randomly separated into two groups and myringotomized on the left TMs: group 1 rats (n=6) received intraperitoneal melatonin injections 10 mg/kg/day whereas group 2 rats (n=12) were treated with physiological serum only. The remaining two rats were served as the control group for histological comparison and standardization. After 15 days of treatment, myringotomized membranes were examined by otomicroscopy and harvested for histopathological evaluation. The functional effect of myringosclerotic plaques in the TMs of the two groups were compared with tympanometric measurements.

Results: Tympanic membranes in group 2 revealed extensive myringosclerotic plaques, on the other hand, TMs in group 1 showed faint or no existence of myringosclerosis. The mean magnitude of the maximum admittance from group 2 measured by tympanometry reduced to about 40% of the values obtained from group 1 (Z=–2,067, p=0.041). The mean magnitude of the maximum admittance from melatonin group was very close to the mean tympanometric value of non-myringotomized Wistar albino rats, demonstrating a functional outcome.

Conclusion: The occurrence of myringosclerosis following experimental myringotomy can be hindered by systemic melatonin treatment.

Key Words: Experimental; melatonin; myringosclerosis; tympanogram.

Amaç: Bu çalışmada melatonin uygulamasının, timpanik membran (TM)'larda deneysel olarak meydana getirilen miringosklerozun oluşumunu engelleyici etkisi araştırıldı.

Gereç ve Yöntemler: Herbiri yaklaşık 300 g olan 20 adet Wistar albino cinsi sıçan randomize olarak iki gruba ayrıldı ve sol taraf TM'lerine miringotomi uygulandı: Grup 1 sıçanlara (n=6) 10 mg/kg/gün intraperitoneal melatonin injeksiyonu yapıldı, grup 2 sıçanlara (n=12) ise sadece serum fizyolojik uygulandı. Geriye kalan iki sıçan ise histolojik karşılaştırma ve standardizasyon amacıyla kontrol grubu olarak ayrıldı. On beş günlük tedavi süresinin sonunda miringotomi uygulanan membranlar otomikroskopik olarak incelendi ve histopatolojik inceleme için ayrıldı. Her iki gruptaki miringosklerotik plakların TM'ler üzerine fonksiyonel etkisi timpanometrik ölçümlerle karşılaştırıldı.

Bulgular: Grup 2'deki TM'lerde miringosklerotik plaklar yaygın bir şekilde izlenirken, diğer yandan grup 1'deki TM'lerde miringoskleroz ya hafif izlendi ya da hiç izlenmedi. Timpanometrik ölçümler ile grup 2'de elde edilen ortalama maksimum admitans değeri, grup 1'e göre yaklaşık %40 düşük idi (Z=–2,067, p=0.041). Melatonin grubunda ölçülen ortalama maksimum admitans değeri miringotomi uygulanmamış, normal Wistar albino sıçanların standart ortalama timpanometrik değerlerine çok yakındır ve fonksiyonel bir sonuç ifade etmektedir.

Sonuç: Deneysel miringotomi sonrasında izlenen miringoskleroz oluşumu sistemik melatonin tedavisi ile engellenebilir.

Anahtar Sözcükler: Deneysel; melatonin; miringoskleroz; timpanogram.

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Myringosclerosis (MS) is defined as the deposition of calcified plaques in the lamina propria of the tympanic membrane (TM) and clinically appears as white chalky patches. Histologically there is an increase in collagen fibers and fibroblasts as well as hyaline degeneration and extracellular calcium deposition within the lamina propia. Ventilation tube insertion, middle ear infection treated with myringotomy, genetic tendency and immunity are some of the hypothetical reasons blamed for this reaction.[1,2] Trauma to the TM resulting from myringotomy or tube insertion, accompanied with the increased production of free oxygen radicals are currently thought to be the most important factor that contribute to the formation of MS.[3]

The relationship between oxygen-derived free radicals and occurrence of MS has been proven in experimental models and previous reports showed that the formation of MS following experimental myringotomy could be reduced by application of various free radical scavengers.[3-6] Melatonin, the chief secretory product of the pineal gland, has anti-oxidant, free-radical scavenging and anti-inflammatory effects against various pathophysiologic conditions.[7] Melatonin has been reported to alter the activities of enzymes which improve the total antioxidative defense capacity of the organism, i.e., superoxide dismutase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, and nitric oxide synthase. Melatonin, because of its small size and high lipophilicity, crosses biologic membranes easily, thus reaching all compartments of the cell. Melatonin has a strong protective effect in many tissue damaging processes such as enhanced oxidative stress in rats. When compared under in vivo conditions of high oxidative stress, melatonin has proven superior to vitamins C and E in reducing oxidative damage.[8]

The aim of this study was to investigate the protective effect of intraperitoneal melatonin injection on MS in the TMs of myringotomized rats by using otomicroscopy, tympanometry and histopathology. Tympanometric measurement in myringosclerotic ears is a novel method for grading of severity of MS from a functional point of view.

MATERIALS AND METHODS

Animal care

Female Wistar albino rats reproduced in Dokuz Eylül University Multidiciplinary Laboratories, weighing approximately 300 g each, were examined otomicroscopically, and 20 animals with bilateral normal TMs were included in this study. Animals were housed in a controlled environment with a temperature of 20 ºC to 22 ºC and with 50 to 70% relative humidity, with a 12 hour light-dark cycle and were given pellets and water ad libitum. All animal care and procedures were performed humanely. The study protocol was approved (No. 04/06/19) by the Committee for Ethics in Animal Experiments in Dokuz Eylül Medical School and this study was carried out in compliance with the guidelines for animal experimentation at the Department of Laboratory Animal Science of Dokuz Eylül University (İzmir, Turkey).

Experimental design

All animals were anesthetized with ether and 18 animals were randomly selected and under otomicroscopic examination (Opmi 1, Carl Zeiss, Meditec Company. Oberkochen. Germany), a standard perforation, standardized in size, within the posterosuperior quadrant of the left TMs, was made with a sterile pick through an ear speculum. No intra-tympanic bleeding was observed during this procedure. The remaining two rats served as the control group for histological comparison, sacrificed and decapitated painlessly under lethal dose ether. The TMs and surrounding bony annulus were removed altogether by microdissection under otomicroscopy.

The animals were seperated into two groups, six rats (Group 1) were treated with intraperitoneal injections of melatonin (10 mg/kg/day) for two weeks. Melatonin (Sigma, St. Louis, Mo., USA) was dissolved in absolute ethanol and diluted with physiological saline. The concentration of ethanol in the final solution was 5%. Group 2 consisted of the remaining 12 rats, were injected with physiological saline only. Otomicroscopy was carried out on the 15th day and formation of MS was noted before sacrification in both group 1 and 2. The otomicroscopic occurence and extent of MS in the pars tensa of TM were evaluated semiquantatively as follows: (0) no visible MS, (+) white halo around umbo, (++) white halo around umbo and white line beside the handle of the malleus and along annulus, (+++) confluent whitish deposits, forming a horseshoe-shaped pattern.[9]

Tympanometry

To assess the tympanometric values in group 1 and 2 on day 15, the pressure and peak admittance

of the left middle ears were measured using a semiquantitative computed clinical admittance meter using a probe frequency of 226 Hz (Amplaid 750; Amplaid, Milan, Italy). A small neonatal rubber adapter was placed over the probe and the tympanogram was recorded by varying pressure between 400 and –200 mm H_2O. The provided information contained the peak pressure at maximum admittance and the magnitude of the admittance. Normal tympanometric values of Wistar albino rats were documented in a previous research using the same MS model.[10]

Histological preparation of the tympanic membrane

When otomicroscopic and tympanometric measurements were conducted in group 1 and 2 on 15th day of the experiment, all animals were sacrificed and decapitated painlessly after a high dose of pentobarbital (80 mg/kg by intraperitoneal injection). The TMs and surrounding bony annulus were fixed overnight in 10% formaldehyde solution and then decalcified with ethylenediaminetetraacetic acid (EDTA). After decalcification, specimens were embedded in paraffin, sectioned on 5 μm slices and stained with toluidine blue for studies under the light microscope. Toluidine blue staining was used to demonstrate the sclerotic changes in the connective tissue of lamina propia. Stained specimens were evaluated by a blinded histologist and photographs were taken by using a photomicroscope (Olympus BH-2, Japan).

Statistical analysis

Statistical analyses were performed under SPSS (SPSS Inc., Chicago, Illinois, USA) for Windows version 11.0 and $p<0.05$ was accepted as significant. Mann Whitney U-test was used for comparison of unpaired tympanometric from group 1 and 2 on day 15.

RESULTS

Otomicroscopy: On day 15, all TMs in group 1 were closed and the area of the healed perforation was opaque. The vascular reaction naturally observed during the healing process had vanished, although some capillaries could still be detected in the vicinity of the traumatized area. In otomicroscopic evaluation, TMs in group 2 revealed varying degress of myringosclerotic plaques, on the other hand, TMs in group 1 showed faint or no existence of MS. The myringosclerotic deposits were located predominantly in the anterior TM quadrant and the areas of healed perforation were observed in the posterosuperior quadrants. The otomicroscopic occurence and extent of MS in pars tensa of TM in group 2 were revealed semiquantatively as follows: (0) in one ear, (+) in one ears, (++) in two ears, (+++) in two ears and in group 1, (0) in three ears, (+) in two ears and (+++) in one ear.

Tympanometry: All TM perforations of group 1 and 2 had healed and closed prior to tympanometric evaluation. The peak pressure at maximum admittance and the magnitude of the admittance obtained from varying degrees of myringosclerotic TMs between group 1 and 2 on day 15 are listed in table 1. In all ears from group 2, the magnitude of the maximum admittance was reduced to about 40% of the values obtained from group 1 and this reduction was statistically significant ($Z=–2.067$, $p=0.041$). Nevertheless the mean magnitude of the maximum admittance from group 1 are very close to the standardization values of Wistar albino rats, which predicts a functional outcome. The extent of MS encountered in both groups did not correlate with the degree of reduction in tympanometric peak pressures due to limited sample size.

Histopathological examination: Normal TMs from the control group for histological comparison were thin and free of inflammatory cells under light microscopy and lamina propria under simple squamous epithelium was clearly perceptible in histological specimens (Figure 1a, b). Extensive sclerotic lesions were found in TMs of group 2 where these sclerotic deposits as well as fibroblasts were located in the lamina propria and increased thickness of the TM was noted (Figure 2a, b). The fibroblastic activity in the lamina propria was also present in histological specimens from group 1. In contrast to group 2, histological specimens of TMs from group A showed none or slight MS and TMs

Table 1. Comparison of maximum admittance values between group 1 and 2 on day 15

	n	Minimum	Maximum	Mean±SD
Group 1	6	0.25	0.60	0.3567±0.13064
Group 2	12	0.08	0.43	0.2158±0.11836

SD: Standard deviation; Z= –2,067; p=0.041; Group 1: Melatonin group; Group 2: Control group.

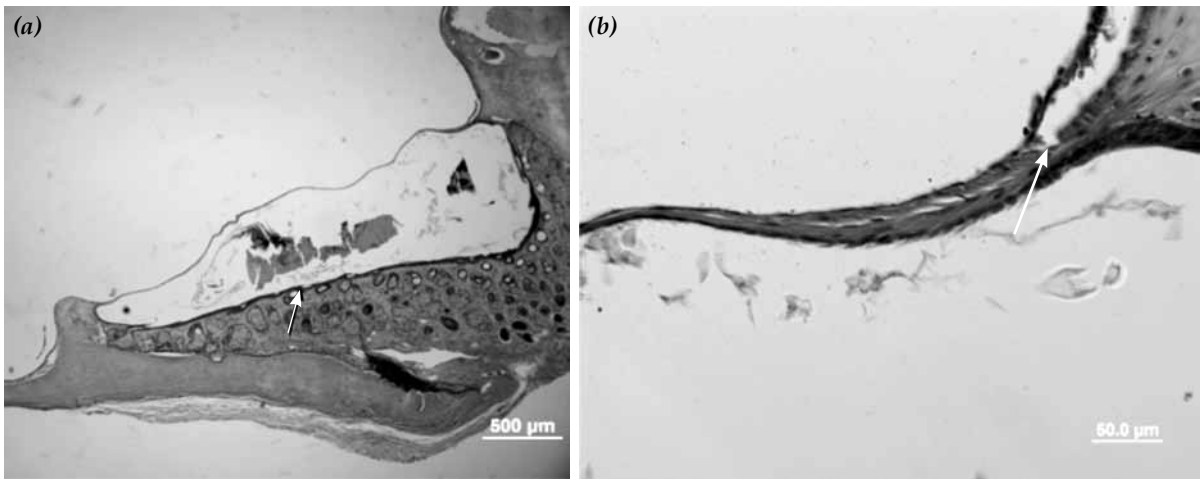

Figure 1. ***(a)*** *Light photomicroscopy of intact tympanic membrane with accompanying external auditory canal epithelium (white arrow) from non-myringotomized group (H-E x 132).* ***(b)*** *Detail of figure 1a. Arrow indicates the junction of fibrous and bony annulus accompanied with normal tympanic membrane without fibroblastic proliferation and myringosclerosis (H-E x 660).*

from the melatonin treated group were thinner than the saline treated group (Figure 3a, b).

DISCUSSION

A myringotomy admits ambient air into the middle ear cavity, resulting in a relatively hyperoxic condition. The oxygen concentration in the middle ear cavity is approximately 5 to 10%, which is much lower than that in ambient air. However a myringotomy permits passage of ambient air, containing 21% O2, into the middle ear cavity, resulting in a relatively hyperoxic condition. The result of a myringotomy therefore is an increased production of free oxygen radicals, initiating irreversible tissue damage involving fibrosis, hyalin degeneration and finally apoptosis as observed in MS.[11] The mechanical pressure of the ventilation tube, trauma to the myringotomy site, raised partial pressure of oxygen within the middle ear after tube insertion, and hemorrhage during the procedure are blamed for calcification of the TM.[6] Our study revealed that systemic administration of melatonin hindered the formation of MS in myringotomized rats. To our knowledge, this is the first study in the literature to evaluate the effectiveness of melatonin in the prevention of MS.

An increased oxygen concentration in ears with myringotomized TMs has been shown to increase the extent of myringosclerotic deposits.[12] Furthermore, this sequence was successfully arrested by systemic or topical administration of free radical scavengers in the injured tissue.[4-6,13,14]

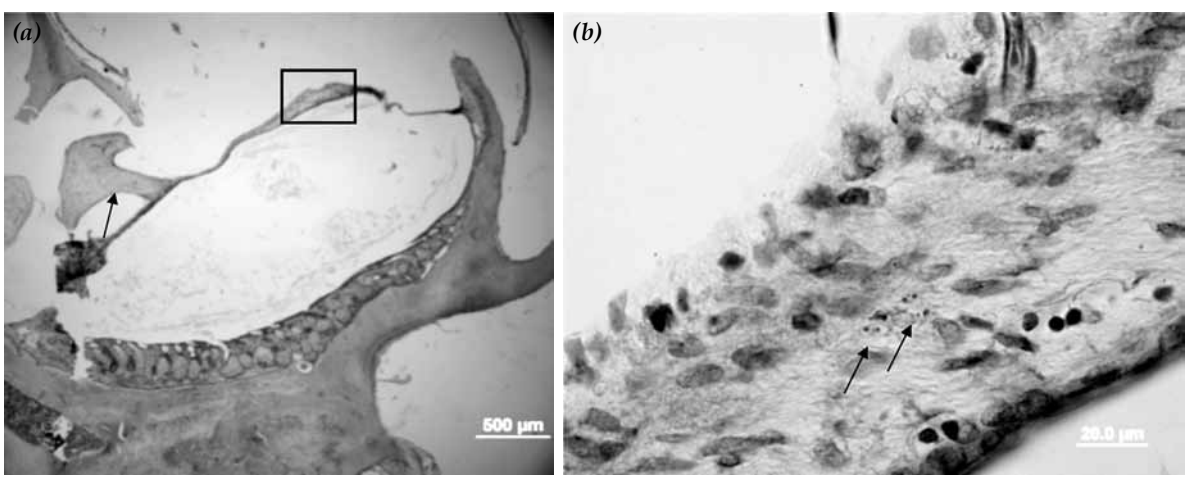

Figure 2. ***(a)*** *Light photomicroscopy of thickened pars tensa from group 2. Arrow indicates manibrium mallei (H-E x 132).* ***(b)*** *Detail of boxed area in figure 2a. Note a sample of extensive sclerotic lesions in lamina propria (arrows; H-E x 1320).*

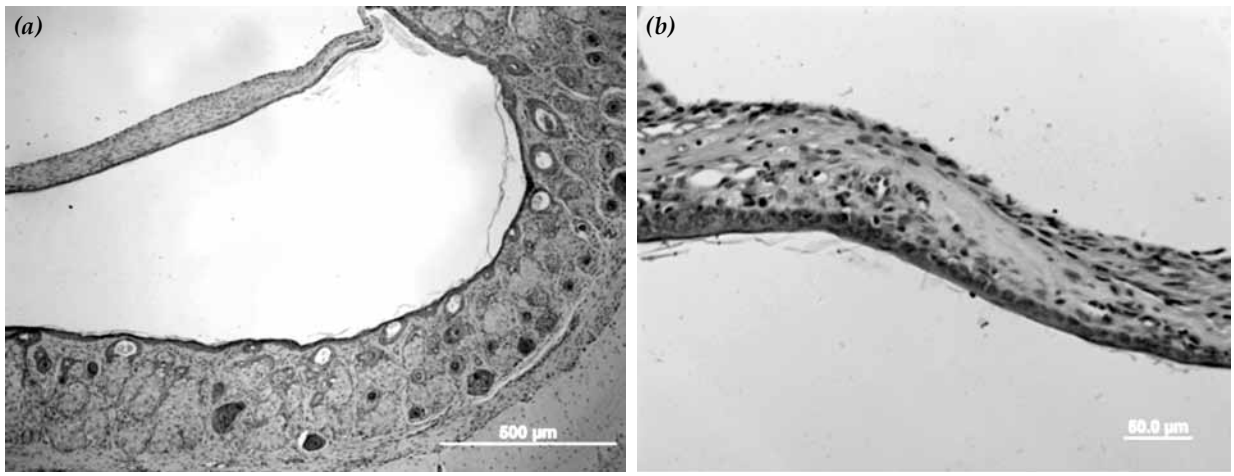

Figure 3. ***(a)*** *Light photomicroscopy of pars tensa from group 1 (H-E x 330).* ***(b)*** *Detail of figure 3a. Note normal epithelial components of slightly thinner tympanic membrane and no myringosclerosis (H-E x 660).*

However melatonin is distinct from classical antioxidants. The administration of melatonin, via any route, results in a rapid rise in blood melatonin concentrations. Since melatonin has both highly lipophilic and hydrophilic properties, in contrast to standard antioxidants, it passes rapidly through all biological membranes and enters the cells and their subcellular compartments.[15] The widespread subcellular distribution of melatonin may allow it to reduce oxidative damage in both the lipid and aqueous environments of the cell. This is an advantage for melatonin over some other antioxidants, which penetrate cells more slowly, since it has been shown that experimental MS in TM starts to develop microscopically within nine hours after myringotomy.[16]

Since lipids are in high concentrations in cellular membranes, damage to polyunsaturated fatty acids (PUFA) leads to variety of functional changes in the membranes such as lipid peroxidation, mediated by oxygen free radicals, which is believed to be an important cause of destruction and damage to cell membranes and has been suggested to be a contributing factor in the development of tissue damage. Numerous in vitro and in vivo studies have documented the ability of both physiological and pharmacological concentrations of melatonin to protect against lipid peroxidation. Its antioxidative profile extends to the activation of other antioxidative systems, such as glutathione peroxidase.[8,17] Therefore, melatonin has a high therapeutic potential.

It is clear that otomicroscopy allows only for visual identification of sclerosis seen in the TM, but histopathological evaluation demonstrates the calcification process in the lamina propria. Previous studies showed that melatonin reduced Ca^{2+}-dependent excitotoxicity in sclerotic tissue.[8,17] The antisclerotic properties of melatonin may be related, in part, to the protection of cells from calcium overload through the inhibition of calcium flux.[18]

Proinflammatory cytokines participate in middle ear inflammatory response.[19] Melatonin also reduces levels of proinflammatory cytokines such as: interleukin (IL)-6, IL-12, tumor necrosis factor-alpha (TNF-alpha), interferon-gamma (IFN-□).[20] This action of melatonin is mainly mediated through the modulation of cytokine production via binding to specific receptors expressed by different immune cells.[21] Melatonin was well tolerated over the entire observation period. No side effects have been reported or detected thus far.

Tympanometry is a well-established method for physiological assessment of the middle ear and has been used to study the middle ear pressure, the presence or absence of fluid in the middle ear and conditions of ossicular chain, but it can also give reliable information about the mechanoacoustic conditions of the whole TM, thus the character of the tympanogram reflects the mechanoacoustic properties of the total area.[22] Tympanometric measurements in MS models are underestimated in current studies and should be used to highlight the functional outcome of these experiments. In the present study, the mean magnitude of the maximum admittance from melatonin group 1 is very close to normal tympanometric values of

non-myringotomized Wistar albino rats. These findings suggest that systemic administration of melatonin reduces the formation of MS, even in functional manner measured by tympanometry.

In conclusion, intraperitoneal application of melatonin may be efficient in the treatment of MS, by acting as a free radical scavenger. However this is an experimentally-induced MS model in rats and this model might result with different findings in human beings. Thus further studies are needed to evaluate clinical applications of melatonin.

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