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Original Article

Does Resveratrol Have a Protective Effect on Tympanosclerosis Formation in Experimentally Induced Otitis Media?

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OBJECTIVE: To evaluate the effects of resveratrol on tympanosclerosis in an experimental rat model by otomicroscopic, histopathological, and immunohistochemical examinations, and measurement of malondialdehyde levels.

MATERIALS and METHODS: Twenty albino Wistar rats were used in the study. Four were randomly selected as negative controls, with no treatment or intervention. The ears of 16 rats were inoculated with *Streptococcus pneumonia* Type 3. They were divided into two Groups: resveratrol, at 20 mg/kg/day dissolved in 10% ethanol, was administered orally to the resveratrol Group for 6 weeks; 10% ethanol was administered to the control Group in the same manner. Blood samples were taken for malondialdehyde measurements after the first and sixth weeks. After otomicroscopic examinations, rats were sacrificed at the end of 6 weeks for histopathological and immunohistochemical (matrix metalloproteinases 2 and 9) examinations. Livers of rats were removed for malondialdehyde measurements.

RESULTS: Otomicroscopic and histopathological evaluations revealed no significant difference between the Groups. Plasma malondialdehyde level of the resveratrol Group in the first week was lower than that of the control Group ($p<0.05$). There was no difference in plasma and liver tissue malondialdehyde levels after 6 weeks. There was a significant difference in staining for matrix metalloproteinase 9 of tympanic membranes between the resveratrol and control Groups ($p<0.05$).

CONCLUSION: Resveratrol has potential antioxidant activity and significantly decreases malondialdehyde levels in the first week. It has an inhibitory effect on matrix metalloproteinase 9. However, it appears to be ineffective in the prevention of tympanosclerosis when used alone, according to otomicroscopic and histopathological evaluations.

KEY WORDS: Tympanosclerosis, resveratrol, metalloproteinase, Malondialdehyde (MDA)

INTRODUCTION

Tympanosclerosis (TS) is a potential complication of both acute and chronic otitis media, and also ventilation tube insertion. Tympanosclerosis affects the tympanic membrane (TM) and middle ear, and is characterised by hyaline degeneration in the lamina propria and formation of calcium plaques. Although the aetiology of TS has not been demonstrated exactly, it has been suggested that multiple factors, such as nitric oxide, oxygen free radicals, cytokines, inflammatory mediators, and growth factors contribute to the pathogenesis of TS [1-5].

After several studies pointed to a relationship between free oxygen radicals and the development of TS [3, 6], the preventive effects of different antioxidants were investigated. L-carnitine, N-acetylcysteine, caffeic acid, ginkgo biloba, and alpha-tocopherol are the most studied antioxidants in experimentally induced myringosclerosis after myringotomy [7-11]. Malondialdehyde (MDA) is one of the products of lipid peroxidation caused by reactive oxygen species and is extensively studied as a biomarker of oxidative stress in TS [5, 7, 10, 11].

Resveratrol is a polyphenolic compound present in over 70 plants and fruits, especially in fresh grapes, grape juice, and wine. Resveratrol has anti-inflammatory, antioxidant, anticarcinogenic, and anti-ageing properties [12, 13]. Although the protective effects of resveratrol against cisplatin-induced ototoxicity and noise-induced hearing loss were reported in recent studies [14-18], there have been no published studies on the possible role of resveratrol in the prevention of TS.

The matrix metalloproteinase (MMP) family is a large Group of proteins involved in the breakdown of extracellular matrix in both physiological and disease processes. MMP-2 and MMP-9 degrade Type 4 collagen, the major component of basement membranes [19, 20].

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Guo et al. [21] reported changes in expression of TGF-β1 and MMP-9 in the formation of TS. In addition, several studies have reported that resveratrol affects MMP activity in adipose and neuronal tissues [22, 23].

Against that background, the aim of this study was to evaluate the effects of resveratrol on TS in an experimental rat model through the use of otomicroscopic, histopathological, and immunohistochemical (MMP-2 and MMP-9) examinations, and also by the measurement of MDA levels in plasma samples and liver tissues.

MATERIALS and METHODS

Animals

Twenty healthy, female, albino Wistar rats, weighing 250-300 g and with intact tympanic membranes, were included in this study, which was approved by the Local Experimental Animal Studies Ethics Committee (HADYEK, 2013/03). Animals were kept under standard laboratory conditions and food and water were provided *ad libitum*.

Pharmaceuticals

A solution of resveratrol (Solgar Vitamin and Herb Company, UK) was prepared every day at the final concentration of 20 mg/kg in 10% ethanol and was given daily to the resveratrol Group via orogastric gavage. The same volume of 10% ethanol was given to the control Group. The content of resveratrol was analysed and confirmed by high-performance liquid chromatography.

Experimental Design

Before they were included in the study, all animals were anaesthetised with intramuscular ketamine (50 mg/kg) and xylazine (10 mg/kg), and an otomicroscopic examination was performed. Only rats with intact tympanic membranes and healthy external and middle ear were included in the study. Four rats were randomly selected to serve as negative controls, with no treatment and no intervention. Both ears of the other 16 rats were inoculated, using a 27 gauge needle, with an approximately 0.02 mL suspension of *Streptococcus pneumonia* Type 3 (49619 ATCC) at a concentration of 10^9 CFU/mL. These 16 rats were divided equally into two Groups. Resveratrol at 20 mg/kg dissolved in 10% ethanol (5 mg/day, 1 mL) was administered daily to the resveratrol Group via orogastric gavage, starting on the day of intervention and continuing for 6 weeks [24]. The same volume of 10% ethanol was administered to the control Group in the same manner. At the end of the first week, the presence of acute otitis media was determined in the experimental Groups by otomicroscopic examination. At that time, blood samples were taken from the dorsal tail veins of all rats for MDA measurements.

All rats were anaesthetised after 6 weeks and otomicroscopic examinations were performed again. Sclerotic lesions were scored on the scale previously described by Mattsson et al. [25]: 0 (no visible myringosclerosis, MS); 1 (occasional MS); 2 (moderate MS); or 3 (severe MS).

Blood samples were obtained by cardiac puncture for comparison of MDA, and the rats were then sacrificed by intraperitoneal pentobarbital (80 mg/kg) injection at the end of 6 weeks. Rats were decapitated and tympanic bullas were opened. Livers of rats were also removed for measurement of their MDA levels.

Histopathological Analysis

Tissue samples from the external auditory canal, middle ear, and inner ear were fixed in 10% neutral-buffered formalin and embedded in paraffin. The samples were then sectioned at a thickness of 4-6 μm and stained with haematoxylin and eosin for microscopic examinations. A pathologist blinded to the study Groups performed the histopathological evaluations. All microscopic slides were examined with the aid of a Nikon Eclipse E600 light microscope and scored according to the degree of tympanic membrane integrity, membrane thickening, oedema formation in the tympanic membrane, presence of exudate in the middle ear, and inflammatory reaction in the middle ear mucosa. Lesions were scored as absent (-), weak (+), moderate (++), or severe (+++).

Immunohistochemistry Procedures

All samples were sectioned at a thickness of 5 μm and placed on 3-aminopropyltriehoxysilane (Sigma, St. Louis, MO, USA)-coated slides and stained by the streptavidin-biotin-peroxidase complex technique (Histostain Plus Kit; Zymed, catalogue no: 85-8943, California, USA). Rabbit polyclonal anti-MMP-2 antibody (1/100; Abcam, cat no: ab37150, UK) and MMP-9 antibody (15 μm/mL; Novus Biologicals, catalogue no: NBP1-57940, USA) were used as primary antibodies. Aminoethylcarbazole was used as the chromogenin H_2O_2 for 10 minutes, which was checked by visual observation with a microscope. The sections were counterstained with Mayer's haematoxylin for 20 seconds and rinsed gently with tap water. Subsequently, the sections were mounted with an aqueous mounting medium. Immunohistochemical MMP-2 and MMP-9 antibody staining of the tympanic membrane and middle ear mucosa were evaluated semi-quantitatively, according to intensity differences between each experimental Group; namely, staining intensity was recorded as faint (-), mild (+), moderate (++), or strong (+++).

Malondialdehyde (MDA) Level Determination

The liver tissues were homogenised in 50 mM cold KH_2PO_4 solution (1:5 w/v) with a Dounce homogeniser and then centrifuged at 4,000 rpm for 20 minutes. All procedures were carried out at 4°C. Plasma and tissue MDA levels, as an index of lipid peroxidation, were determined by means of the thiobarbituric acid method [26]. Standards were prepared in the range of 0.125-10 μM, using 1,1,3,3-tetraethoxypropane in ethanol. Linear regression was R^2=0.999. Standards and samples of plasma and tissue supernatants were treated with 20% trichloroacetic acid and heat derivatised at 95°C for 30 minutes with 0.67% thiobarbituric acid. The plasma samples were rapidly cooled and extracted with 4 mL n-butanol. Twenty microlitres of supernatants were injected into the HPLC-FLD system (excitation wavelength=515, emission wavelength=553) and separated on an Inertil® ODS-3V, 4.6x250 mm, 5 μm (GL Sciences Inc., Tokyo, Japan). Determination of the protein content was based on the Bradford method [27], using the Bradford reagent (Sigma-Aldrich, St Louis, MO, USA).

Statistical Analysis

Statistical analysis was performed by using SPSS for Windows, version 21.0, using the Chi-square test, independent sample t-test, and Mann-Whitney U test. A p value of<0.05 was considered statistically significant.

RESULTS

Otomicroscopic Evaluation

After 6 weeks of daily treatment, otomicroscopic examination was performed blindly by the same ear-nose-throat specialist. Sclerotic lesions were scored on a four-point scale, as shown in Table 1 [26]. There was no significant difference between the Groups for the development of myringosclerosis (Chi-square test, p=0.149).

Histopathological Evaluation

There was no statistically significant difference between the experimental Groups for the histopathological parameters (Tables 2 and 3; Figures 1e, 1f, and 2d).

Immunohistochemical Evaluation

There was no statistically significant difference between the Groups for MMP-2 staining (p>0.05). There was moderate staining of tympanic membranes for MMP-2 in six ears of the resveratrol Group and four ears of the control Group (Figures 1a and 2c), and mild staining of middle ear mucosa in three ears, one in the resveratrol Group and two in the control Group (Figure 1c). On the other hand, there was a statistically significant difference between resveratrol and control Groups in MMP-9 staining of tympanic membranes (Table 4), with increased staining in the control Group (p=0.005; Figures 1b and 2a). There was also increased staining of MMP-9 (Figures 1d and 2b) in the middle ear of the control Group but the difference was not statistically significant (Table 5).

MDA Evaluation

Levels of MDA in plasma according to weeks, including median, minimum, and maximum values of the study Groups, are shown in Table 6. Median MDA levels of the negative control Group were 0.283 µM/mL (min 0.220, max 0.417) in plasma, and 0.0115 µM/mg protein (min 0.008, max 0.017) in liver tissue.

When the MDA levels were compared, there was a significant difference between the resveratrol and control Groups for the first week (p<0.05). High MDA levels were determined in the control Group. However, this difference disappeared at the end of the experiment (p>0.05).

Median MDA levels in liver tissue were 0.135 µM/mg protein (0.009-0.026) in the resveratrol Group and 0.13 µM/mg protein (0.01-0.034) in the control Group. There was no significant difference between MDA levels of the Groups in liver tissues (p>0.05).

DISCUSSION

Tymponosclerosis is a degenerative healing process of the tympanic membrane and middle ear. All kinds of otitis media and interventions to the tympanic membrane can trigger the pathophysiological

Table 1. Otomicroscopic evaluation of tympanic membranes

Groups	Number of 3 ears	0 (no MS)*	1 (Occasional MS)	2 (moderate MS)	3 (severe MS)
Resveratrol	16	8	2	2	4
Control	16	2	3	3	8

*MS: myringosclerosis; Chi-square test p=0.149

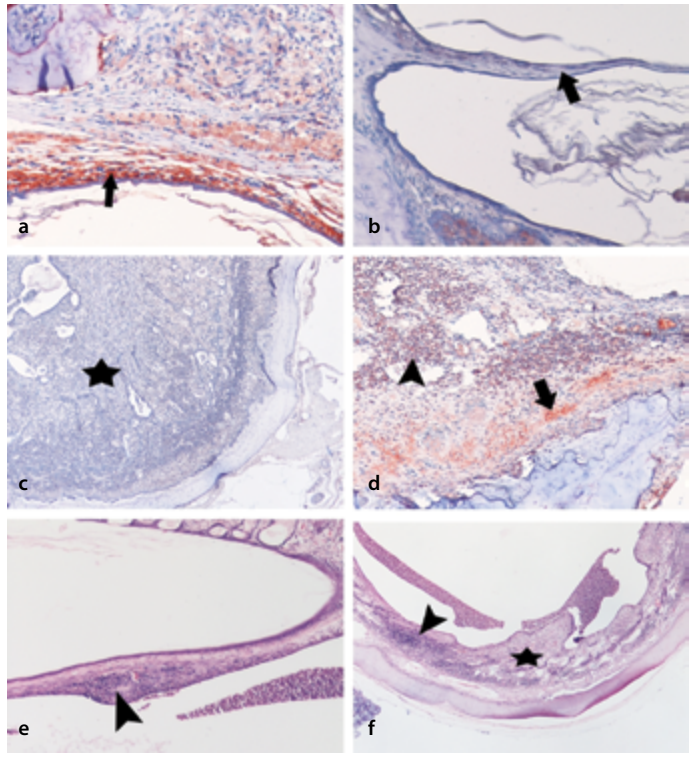

Figure 1. a-f. Histological and immunohistochemical evaluations of the tympanic membrane and middle ear in the resveratrol group. Moderate immunohistochemical staining of MMP-2 in the thickened tympanic membrane (arrow), x20 magnification (a); no immunopositive reaction of MMP-9 in the thickened tympanic membrane (arrow), x20 magnification (b); no immunopositive staining of MMP-2 in the thickened mucosa of the middle ear (star), x4 magnification (c); thickened mucosa of the middle ear (arrow) and infiltrated mononuclear cells (arrowhead) stained strongly with MMP-9 antibody, x10 magnification (d); thickening of the tympanic membrane due to mononuclear cell infiltration (arrowhead), x10 magnification, HE (e); thickened middle ear mucosa (star) and mononuclear cell infiltration (arrowhead), x4 magnification, HE (f)

MMP: matrix metalloproteinase

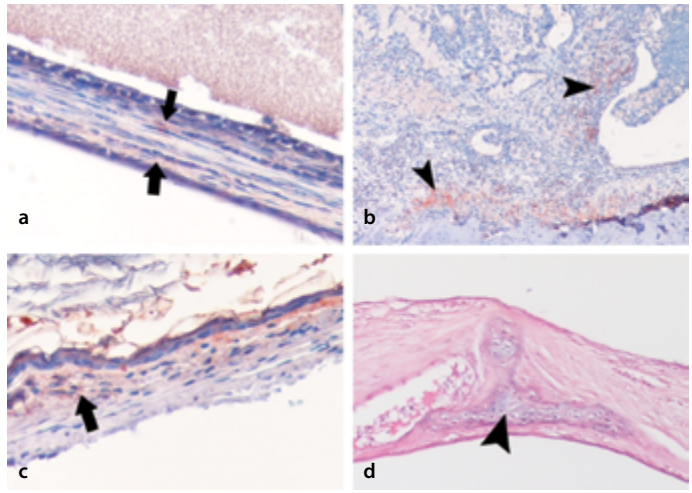

Figure 2. a-d. Histological and immunohistochemical evaluations of the tympanic membrane and middle ear in the control group. Weak immunohistochemical staining of MMP-9 in the thickened tympanic membrane (arrow), x40 magnification (a); moderate positive immunostaining of MMP-9 in the middle ear mucosa and inflammatory cells (arrowheads), x20 magnification (b); moderate immunopositive staining of MMP-2 in the tympanic membrane (arrow), x40 magnification (c); cartilaginous metaplasia of the tympanic membrane (arrowhead), x20 magnification, HE (d)

MMP: matrix metalloproteinase

Table 2. Histopathological evaluation of tympanic membrane thickness

Groups	Number of ears	Absent (-)	Weak (+)	Moderate (++)	Severe (+++)
Resveratrol	16	2	4	3	7
Control	16	3	4	5	4

Mann-Whitney U test p=0.232

Table 3. Histopathological evaluation of inflammation in middle ear mucosa

Groups	Number of ears	Absent (-)	Weak (+)	Moderate (++)	Severe (+++)
Resveratrol	16	-	3	2	11
Control	16	1	1	1	13

Mann-Whitney U test p=0.487

Table 4. Immunohistochemical evaluation of MMP-9 in tympanic membranes

Groups	Number of ears	Absent (-)	Weak (+)	Moderate (++)	Severe (+++)
Resveratrol	16	15	-	-	1
Control	16	7	7	-	2

Mann-Whitney U test p=0.005

MMP: matrix metalloproteinase

Table 5. Immunohistochemical evaluation of MMP-9 in middle ears

Groups	Number of ears	Absent (-)	Weak (+)	Moderate (++)	Severe (+++)
Resveratrol	16	3	6	2	5
Control	16	2	6	4	4

Mann-Whitney U test p=0.844

MMP: matrix metalloproteinase

Table 6. Plasma MDA levels at the end of the first and sixth weeks (median, minimum, and maximum values)

Groups	MDA* in 1st week	MDA in 6th week
Resveratrol	0.462 (0.357-2.136)	1.473 (1.102-2.168)
Control	1.93 (1.088-4.468)	1.303 (0.812-2.011)
P** value	p=0.004	p=0.325

*MDA in plasma μM/mL, **independent sample t-test

MDA: malondialdehyde

events that result in TS. TS affects the connective tissue layer and is characterised by an increase in collagenous fibres and hyaline degeneration. Many factors involved in wound healing may play a role in this pathological process [1-4].

Although the exact aetiology is unknown, many hypotheses have been put forward. One of them suggests that oxygen-derived free radicals may be an aetiological factor in the development of TS [3, 5]. However, Atmaca et al. [28] demonstrated that exogenous nitric oxide application did not alter the occurrence of TS. Consequently, the use of antioxidants in its prevention came to prominence and different antioxidants were studied [6-11]. In those studies, myringotomy was used to induce myringosclerosis and in most, the tympanic membrane was evaluated to assess its development. The antioxidants used in those studies were reported to be effective in the prevention of myringosclerosis [6-11].

In the current study, the inoculation of Type 3 *S. pneumonia* through the tympanic membrane of rats successfully induced otitis media. Earlier studies demonstrated that this procedure resulted in histological changes in the majority of animals within 4-6 weeks [21, 29]. Diffuse TS and inflammation were observed on the haematoxylin and eosin slides after 6 weeks.

Resveratrol is a natural polyphenol present in various plants, including grapes and peanuts that exhibits a wide range of biological activities. It modulates lipid and lipoprotein metabolism and is a potent chemopreventive agent. It also has antioxidant and anti-inflammatory properties [12, 13]. Seidman et al. [17, 18] demonstrated the effectiveness of resveratrol against noise-induced hearing loss in two studies. They showed that resveratrol significantly reduced the formation of reactive oxygen species (ROS) and inhibited cyclooxygenase-2 expression following noise exposure [18]. Resveratrol was also demonstrated to have a protective effect against cisplatin ototoxicity [14-16]. In those studies, the protective effects of resveratrol were thought to be due to its potent antioxidant properties. Yumusakhuylu et al. [14] reported decreased ROS level in the resveratrol-treated Group. In addition, Kavas et al. [30] studied the effects of resveratrol on the oxidant/antioxidant systems of rats and observed statistically significant increases in superoxide dismutase and catalase activity, and in plasma copper and red blood cell zinc concentrations, as well as statistically significant decreases in the concentrations of the lipid peroxidation product MDA, and plasma zinc and copper in red blood cells in the resveratrol-administered Group.

Reactive oxygen species attack polyunsaturated lipids in organisms and cause lipid peroxidation. This results in undesirable effects like decrease in membrane fluidity, increase in membrane permeability, and toxic end products. MDA is one of the products of lipid peroxidation and is frequently used as a marker in studies. It also has toxic effects such as inactivation of enzymes and reacts with DNA [31]. In the present study, MDA levels were found to be significantly lower in the resveratrol-treated Group than in the control Group in the first week, when acute otitis media was observed otomicroscopically. Even in the presence of acute infection, the reduced level of MDA in the resveratrol-treated Group may be evidence for its potential antioxidant activity. However, this difference disappeared at the end of 6 weeks when signs of acute infection disappeared. In earlier studies on myringosclerosis, there were also significant differences in MDA levels between antioxidant-administered Groups and control Groups [7, 10, 11]. In those studies, MDA levels were measured on days 10, 15, and 28 after myringotomy. Those results, together with results of the present study, suggest that oxidative stress may be active in the early stages of injury.

Trans-resveratrol-3-O-glucuronide and trans-resveratrol-3-sulphate are the most abundant metabolites of resveratrol; the metabolism of resveratrol is performed principally by the enterohepatic system [12]. In the present study, MDA levels were measured in the liver tissues of all rats. No significant differences between Groups were recorded.

This result is consistent with the literature in showing that oral administration of resveratrol is not harmful at the dosage and for the period administered [12, 13, 32].

In the present study, neither otomicroscopic examination nor histopathological evaluation revealed significant differences between Groups. These results contradict those of studies showing the effectiveness of antioxidants against myringosclerosis [6-11]. In these studies, myringotomy was used as the method to trigger the process of myringosclerosis and the duration of those studies ranged from 10 to 28 days. The current study differs from those studies in the following aspects: a rat model of acute otitis media induced by bacterial inoculation was used; and the study was performed over 6 weeks. Guo et al. [22] stated that only slight sclerotic changes in the tympanic membrane appeared in week 2, and that extensive myringosclerosis was observed in week 6. In the same study, vascularisation, junctional degeneration, and calcium crystals were also observed in the middle ear mucosa in week 6. Tympanosclerotic changes began to appear only after 2 weeks. Therefore, short-term studies might be inadequate to observe the development of TS.

The MMP family is a large Group of proteins involved in the breakdown of extracellular matrix in both physiological and disease processes. MMP-2 and MMP-9 degrade Type 4 collagen, the major component of basement membranes. Although MMP-9 is mainly stored in neutrophils, eosinophils, macrophages, and epithelial-derived cells, the major source of MMP-2 is mesenchymal cells, mainly fibroblasts [20, 21, 34]. MMP overexpression is involved in the degradation of the extracellular matrix and the formation of fibrosis [33]. Therefore, their high levels are consistent with poor wound healing [33, 34].

Several studies have shown that resveratrol may be involved in the regulation of MMP-2 and MMP-9 activity [22, 23]. Gao et al. [22] showed that resveratrol reduced the elevated level of MMP-9 induced by cerebral ischaemia-reperfusion in mice, and Kang et al. [23] demonstrated that the activity of MMP-2 and MMP-9 decreased significantly at different resveratrol concentrations, with MMP-9 being more significantly affected than MMP-2.

The possible role of MMP-9, together with TGF-β1, was investigated in a guinea pig model of TS [21]. Animals were inoculated with Type 3 *S. pneumonia* and sacrificed at six different times, namely 1, 2, 3, 4, 6, and 8 weeks post-inoculation, and with 10 subjects in each Group. The study reported increased expression of TGF-β1, with the development of TS, in 8 weeks, and also increased expression of MMP-9 from week 1 to week 4, which declined in week 6. These results indicate that TGF-β1 and MMP-9 are involved in the pathogenesis of TS [22].

In the present study, the role of resveratrol in the prevention of TS, together with its effect on MMP activity, were investigated by histopathological and immunohistochemical evaluations. The activity of MMP-2 and MMP-9 was evaluated in tissues by immunohistochemical staining 6 weeks after intervention. Although there was no significant difference in MMP-2 staining between the Groups, a statistically significant difference in MMP-9 staining of tympanic membranes was observed between the resveratrol-treated and control Groups. MMP-9 staining was stronger in the tympanic membranes of the control Group (Table 4). However, the intensity of MMP-9 staining in the middle ear mucosa was similar in both Groups (Table 5). High MMP-9 levels in the middle ear mucosa of both Groups can be explained by the high scores in inflammation of the middle ear that were confirmed by histopathological evaluation (Table 3). A study that showed increased expression of MMP-9 up to the sixth week in the development of TS supports this interpretation of the results [21].

In the present study, MMP-2 staining was faint in the middle ear mucosa of both Groups, and moderate in the tympanic membranes in only 10 of 32 ears. Although MMP-2 and MMP-9 have similar functions in the degradation of extracellular matrix and exhibit overlapping expression during tissue injury, studies have shown that the timing of their expression is very close but different [20, 34]. Koslowski et al. [34] reported that the activity of MMP-2 increased in the acute inflammatory phase, with the maximum at day 3, and that the activity decreased during chronic inflammation. However, it increased again at the beginning of the fibrotic phase. Goussev et al. [20] also demonstrated that the active form of MMP-9 was prominent 1 day after spinal cord injury and returned to normal by 14 days after spinal cord injury. The active form of MMP-2 appeared at 7 days and remained elevated for at least 21 days [20]. These studies also showed that MMP-2 levels continuously changed after the injury; they increased in the acute phase and decreased within a few weeks, and then increased again with the formation of fibrosis [19, 20, 34]. MMP-2 levels were evaluated at the end of 6 weeks in the present study, and this timing of the evaluation may have influenced the results. Although MMP-2 staining was faint in the middle ear still showing inflammation, it became more noticeable in the tympanic membrane showing marked sclerosis. These findings are consistent with the literature, which indicates increased MMP-2 levels during fibrosis formation [20, 34].

In conclusion the present study demonstrates that resveratrol has potential antioxidant activity and significantly decreases plasma MDA levels in rats in the first week. Resveratrol also has an inhibitory effect against MMP-9, as demonstrated by immunohistochemical evaluation of tympanic membranes. However, it appears to be ineffective in the prevention of TS when administered alone, according to otomicroscopic and histopathological evaluations. Additionally, MMP-9 plays an important role in TS formation.

Ethics Committe Approval: Ethics committee approval was received for this study from the ethics committee of Local Experimental Animal Studies (HADYEK, 2013/03).

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

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