The Journal of Laryngology & Otology (2011), ***125***, *370–375*.

doi:10.1017/S002221511000263X

MAIN ARTICLE

Antioxidant effect of pomegranate extract in reducing acute inflammation due to myringotomy

V KAHYA[1], A MERIC[1], M YAZICI[2], M YUKSEL[3], A MIDI[4], O GEDIKLI[5]

[1]Otorhinolaryngology Department, Bezmialem Vakif University Medical Faculty, [2]Otorhinolaryngology Department, Bakirkoy Training and Research Hospital, [3]Biochemistry Department, Marmara University, Vocational School of Health Related Professions, [4]Pathology Department, Maltepe University Medical Faculty, Istanbul, Turkey, and [5]School of Health Related Professions, Kirklareli University, Kirklareli, Turkey

Abstract

Objective: To assess the effect of pomegranate extract on acute inflammation due to myringotomy.

Design: Prospective, randomised study.

Subjects: Thirty Sprague–Dawley rats were divided into three groups. Group one constituted controls. Group two underwent myringotomy. Group three underwent myringotomy and also received 100 µl/day pomegranate extract, via gavage, one day before and two days after surgery. Following sacrifice 48 hours after myringotomy, the animals' right ears were used to determine the concentration of reactive oxygen species, using the chemiluminescence method; left ears were used for histological study.

Results: Reactive oxygen species levels were significantly decreased in group three compared with group two ($p < 0.01$). The density of inflammatory cells in group three was significantly less than that in group two ($p < 0.01$). Lamina propria thickness and vessel density were also significantly decreased in group three compared with group two ($p < 0.01$).

Conclusion: Our results indicate that oral pomegranate extract decreases reactive oxygen species concentration and acute inflammation in the tympanic membrane after myringotomy.

Key words: Myringotomy; Inflammation; Pathology; Antioxidants; Punicaceae

Introduction

Myringotomy is most often used to treat recurrent acute otitis media and chronic otitis media with effusion.[1] The most common sequela of myringotomy is myringosclerosis.[2] It has recently been shown that the development of myringosclerosis after myringotomy occurs concomitantly with an increased concentration of reactive oxygen species in the middle-ear cavity and an inflammatory reaction in the tympanic membrane.[3,4]

The protective mechanisms against reactive oxygen species and inflammation comprise enzymatic and nonenzymatic free radical scavengers.[5] Previous reports have shown that, following experimental myringotomy, the development of acute inflammation and myringosclerosis can be reduced by the topical application of various free radical scavengers such as vitamin E, ascorbic acid, L-carnitine and N-acetylcysteine.[6–9]

Pomegranate has been used for centuries to confer health benefits in a number of inflammatory diseases. It has been reported that consumption of pomegranate extract may lower cholesterol and mitigate the effects of atherogenesis and urolithiasis.[10–12] Pomegranate extract is a rich source of potent polyphenolic and flavonoid antioxidants.[13] The soluble polyphenol content of pomegranate extract varies between 0.2 and 1.0 per cent, depending on the horticultural variety, and includes mainly anthocyanins, catechins, tannins, and gallic and ellagic acids.[14]

The first aim of the current study was to assess the level of reactive oxygen species in the tympanic membrane of rats following myringotomy, using the chemiluminescence method. The second aim was to assess the protective effect of pomegranate extract on rat tympanic membrane following myringotomy, including the assessment of any tympanic membrane histopathological changes.

Materials and methods

Experimental design

The study was approved by the animal ethics committee of the Istanbul University Medical Faculty.

Thirty healthy Sprague–Dawley rats (weight, 250–300 g) were used. All animals had been kept in

Accepted for publication 20 July 2010 First published online 25 February 2011

a 14-hour light/10-hour dark cycle with free access to food and water.

The animals were anaesthetised with 50 mg/kg ketamine hydrochloride intraperitoneally. They were then examined and assessed otoscopically for evidence of ear disease. Any animal showing signs of ear disease was excluded from the study.

The animals were randomly assigned to three groups of 10 animals each.

In groups two and three, myringotomy was performed in the upper posterior quadrant of the tympanic membrane in both ears, with a sterile myringotomy lancet and aural speculum, under otomicroscopy (S1, 300 mm lens; Carl Zeiss, Oberkochen, Germany) and using a sterile technique.

The group two rats received no pre- or post-myringotomy treatment. However, the group three rats received 100 µl/day pomegranate extract via gavage for one day pre-myringotomy and two days post-myringotomy.

The group one rats constituted the control group, and did not receive myringotomy or any other treatment.

Forty-eight hours after myringotomy, all animals were sacrificed via injection with a lethal dose of ketamine hydrochloride intraperitoneally. The temporal bones were harvested and the tympanic bullae cracked with scissors. Under a dissecting microscope, the middle-ear mucosa and tympanic membrane were peeled off the underlying bone. The right tympanic membrane of each animal was used for luminol-enhanced chemiluminescence measurements, while the left tympanic membrane was used for histological study.

Pomegranate extract processing

Fresh pomegranates were washed, crushed and squeezed, and then treated enzymatically with pectinase to yield pomegranate extract and by-products, which included the inner and outer peels and seeds. (Pectinase hydrolyses α-1,4-galacturonide bonds in pectin, and thus improves extraction and filtration and prevents the formation of pectin gels.) The pomegranate extract was filtered, pasteurised, concentrated and stored at −18°C as described previously.[11,14] Concentrated pomegranate extract was diluted in water (20 ml of concentrated extract in 500 ml of distilled water). Therefore, a standardised average of 2.5 ml of the resulting solution contained 100 µl pomegranate extract, equivalent to 2.8 µmol total polyphenol.

Chemiluminescence

The tympanic membranes were washed with ice-cold saline solution and analysed immediately. After 10 minutes, the specimens were assessed for reactive oxygen species, using luminol chemiluminescence as described previously.[6] Chemiluminescence was measured at room temperature using a Mini Lumat LB 9506 luminometer (EG & G Berthold, Germany) in the presence of 0.2 mmol/l luminol containing phosphate-buffered saline 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid buffer (0.5 mol/l phosphate-buffered saline containing 20 mmol/l 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid). Counts were obtained at 5-second intervals over a period of 5 minutes. Results were recorded using relative light area per mg of tissue (rlu/mg) as the unit of measurement, expressed as the area under the curve for the 5-minute counting period.

Tissue specimens were drained and weighed at the end of each assessment. The average specimen weight was approximately 1–2 mg.

Histology

For histological study, temporal bones were fixed in 10 per cent formalin for four days. Specimens were then decalcified in 10 per cent formaldehyde for five days. Specimens were subsequently washed for 3 hours to remove acidity.

A tracking process was then performed for 13 hours using an automatic tissue tracking machine. After this processing, the specimens were embedded in paraffin, sectioned to a thickness of 3 µm with a microtome, and stained with haematoxylin and eosin. The sections were evaluated by a blinded pathologist using a light microscope (Olympus Bx-50, Olympus Optical, Hamburg, Germany).

On light microscopic examination, the inflammatory cell density, lamina propria thickness and tympanic membrane vessel density were evaluated semiquantitatively using the following grading system: 0 = absent, 1 = slightly increased, 2 = moderately increased and 3 = severely increased.

Statistical analysis

The NCSS 2007 and PASS 2008 statistical software programs (Kaysville, Utah, USA) were used for statistical analysis. Relevance of data to the standard distribution was determined by the Kolmogorov–Smirnov test. The significance of differences between experimental groups was analysed using the one-way analysis of variance test and the Tukey HSD (Honestly Significant Differences) test. The chi-square test was used to analyse qualitative data. Differences were considered significant when the probability was $p < 0.05$.

Results

Chemiluminescence

The mean luminol chemiluminescence level ± standard deviation (SD) of the tympanic membrane tissue specimens was 31.37 ± 3.63 rlu/mg in group one and 56.35 ± 7.08 rlu/mg in group two ($p < 0.01$) (Table I). In group three, the mean luminol chemiluminescence level ± SD was 44.69 ± 7.07 rlu/mg tissue; this was significantly lower than that of group two ($p < 0.01$). There was also a statistically significant difference between the mean chemiluminescence levels of groups three and one ($p < 0.01$) (Figure 1).

https://doi.org/10.1017/S002221511000263X Published online by Cambridge University Press

TABLE I
LUMINOL-AMPLIFIED CHEMILUMINESCENCE VALUES

Rat no	Group 1	Group 2	Group 3
1	32.7	48.9	37.5
2	35.8	49.9	31.8
3	30.1	62.9	42.1
4	35.4	53.9	45.9
5	34.7	51.6	41.7
6	30.0	49.4	43.1
7	31.1	69.3	63.7
8	27.7	53.3	50.9
9	32.1	54.2	56.0
10	24.1	60.1	51.2
Mean ± SD	31.37 ± 3.63	56.35 ± 7.08	44.69 ± 7.07

Data represent relative light units per mg tissue. No = number; SD = standard deviation

Histology

In the tympanic membrane specimens from group one animals, the observed structure of the tympanic membrane was normal, with an inner mucosal layer and a thin lamina propria, without inflammatory cells or angioneogenesis (Figure 2). In the group two tympanic membrane specimens, the lamina propria was oedematous, the number of tympanic membrane vessels had increased (Figure 3), and the tympanic membrane perforation edges showed a dense infiltration of inflammatory cells, especially neutrophils (Figure 4). However, this inflammatory infiltrate was greatly reduced in the group three specimens, compared with group two specimens (Figure 5).

In group three, the ratio of absent or slightly increased inflammatory cell density was found to be high. Conversely, in group two the ratio of severly increased inflammatory cell density was significantly high ($p < 0.01$)

In group three, absentor slightly increased inflammatory cell density was found. Conversely, severely increased inflammatory cell density was significantly high in group two ($p < 0.01$).

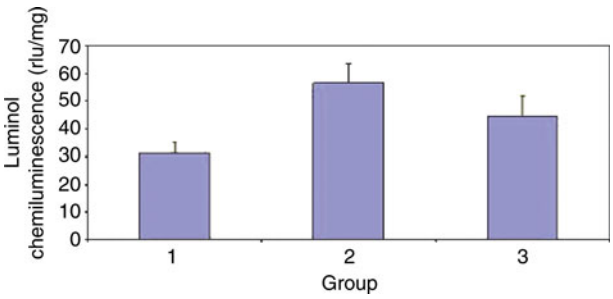

FIG. 1
Comparison of luminol chemiluminescence (representing reactive oxygen species concentration) for the three groups (mean ± std).

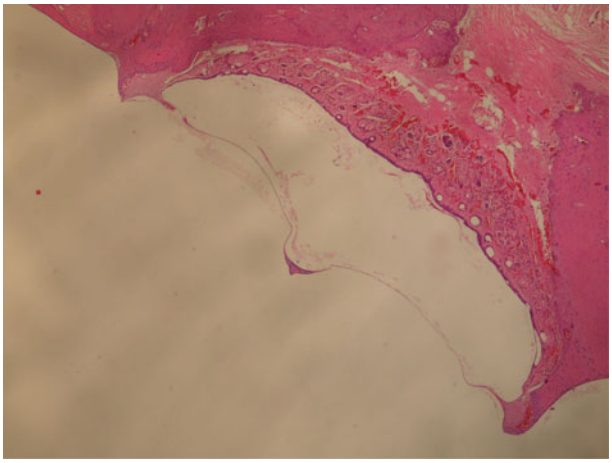

FIG. 2
Photomicrograph of tympanic membrane specimen from group one (non-myringotomised) rat, showing a normal tympanic membrane, with inner mucosal layer and thin lamina propria and without inflammatory cells or angioneogenesis. (H&E; original magnification ×40)

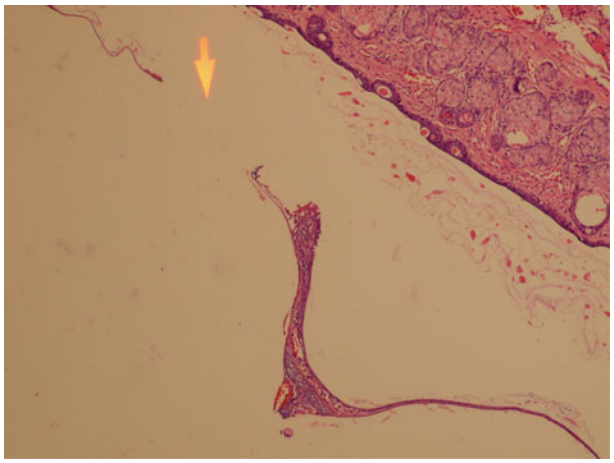

FIG. 3
Photomicrograph of tympanic membrane specimen from group two rat, showing perforation edges (arrow) with increased lamina propria thickness due to inflammatory cell infiltrate. (H&E; original magnification ×40)

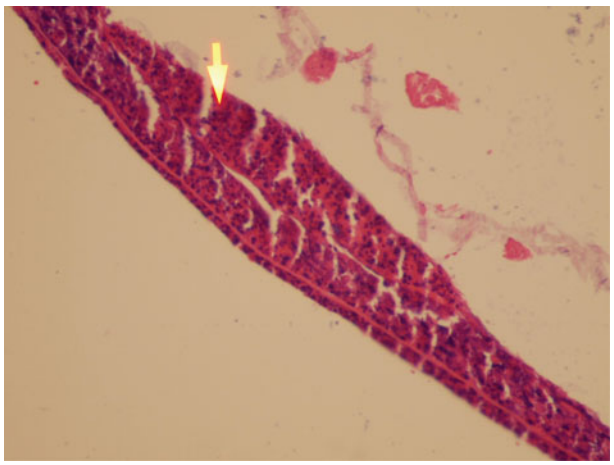

FIG. 4
Higher magnification photomicrograph of the same tissue section shown in Figure 2, showing a myringotomised tympanic membrane with dense infiltration of inflammatory cells, especially neutrophils. (arrow)

https://doi.org/10.1017/S002221511000263X Published online by Cambridge University Press

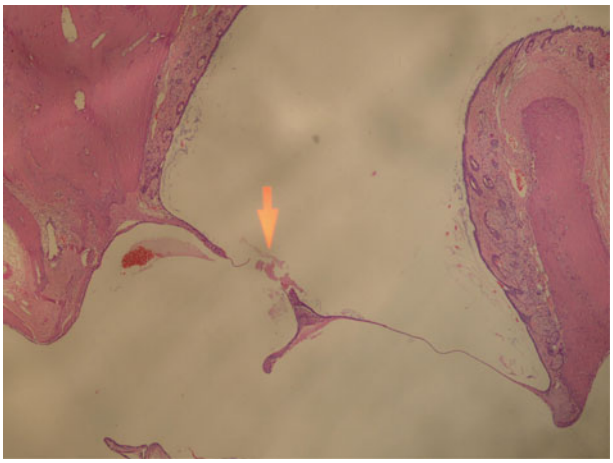

FIG. 5

Photomicrograph of tympanic membrane specimen from group three rat, showing slight inflammatory cell infiltration and minimally increased lamina propria thickness. (arrow)

In group three, normal or slightly increased lamina-propria thickness was found. Conversely, moderately increased laminapropria thickness was significantly high in group two ($p < 0.01$).

Table II compares the histological changes observed in the three groups.

Discussion

Our study findings indicated that oral administration of pomegranate extract before and after myringotomy resulted in a reduction in reactive oxygen species levels and acute inflammation in the tympanic membranes of myringotomised rats, compared with the tympanic membranes of myringotomised rats not thus treated. To the best of our knowledge, the current study represents the first published report evaluating the effectiveness of pomegranate extract in reducing acute tympanic membrane inflammation following myringotomy.

TABLE II
GRADED HISTOLOGICAL CHANGES

Grade	Grp 2 (*n* (%))	Grp 3 (*n* (%))
Infl cell density		
1	0 (0)	4 (40)
2	0 (0)	3 (30)
3	1 (10)	1 (10)
4	9 (90)	2 (20)
LP thickness		
1	0 (0)	5 (50)
2	0 (0)	3 (30)
3	8 (80)	2 (20)
4	2 (20)	0 (0)
TM vessel density		
1	0 (0)	7 (70)
2	0 (0)	2 (20)
3	9 (90)	1 (10)
4	1 (10)	0 (0)

Grade 1 = normal; 2 = slightly increased; 3 = moderately increased; 4 = severely increased. Grp = group; infl = inflammatory; LP = lamina propria; TM = tympanic membrane

The oxygen concentration in the middle-ear cavity is approximately 5.5–12.1 per cent, much lower than that of ambient air.[15] Myringotomy permits passage of ambient air into the middle-ear cavity, resulting in relative hyperoxia.[16] This hyperoxia increases the formation of reactive oxygen species in mitochondria and endoplasmic reticulum. A previous study showed that myringotomy is associated with increased levels of reactive oxygen species.[5] Increased reactive oxygen species and impaired antioxidant defence mechanisms have been postulated to be causative factors in inflammatory disease.[17] Increased production of reactive oxygen species may also be the first stage in the accumulation and aggregation of calcium and phosphorus, forming sclerotic deposits and eventually causing myringosclerosis.[4]

Pomegranates can easily be obtained. They are rich in antioxidants of the polyphenolic class, which includes tannins and anthocyanins.[13] These antioxidants are more potent, on a molar basis, than many other antioxidants, including vitamins C and E, coenzyme Q-10, and α-lipoic acid. The antioxidant level in pomegranates has been found to be higher than that in other fruits such as blueberries, cranberries, oranges and grapes.[18] Anthocyanins have been shown to be effective inhibitors of lipid peroxidation, inducible nitric oxide synthase (iNOS) and therefore of nitrous oxide.[19]

A previous study has reported increased nitrous oxide levels in tympanic membrane samples taken on the first day after myringotomy. [20] Nitrous oxide may play a role in the inflammatory response of the middle ear and also in the formation of myringosclerosis.[21] As demonstrated recently, ascorbic acid and gingko biloba extract can scavenge nitrous oxide and inhibit its production by (iNOS) inducible nitrous oxide synthase.[7,22] In the current study, rats receiving oral pomegranate extract before and after myringotomy showed significantly lower levels of reactive oxygen species and acute inflammation, compared with myringotomised rats not treated with pomegranate extract. This may be due to a direct effect of pomegranate extract in reducing nitrous oxide levels, or to an effect on inducible nitrous oxide synthase resulting in increased nitrous oxide scavenging.

The level of reactive oxygen species in the tympanic membrane is known to increase following myringotomy.[6] Furthermore, in injured tissue free radical scavengers have been shown to prevent the concentration of reactive oxygen species from increasing.[3] Using chemiluminescence analysis, Polat *et al.* showed that systemic application of vitamin E decreases the concentration of reactive oxygen species in the middle-ear mucosa and tympanic membrane.[6] To the best of our knowledge, the current study is the first to show that oral administration of pomegranate extract causes a significant decrease in the level of reactive oxygen species in the tympanic membrane, as shown by chemiluminescence analysis.

https://doi.org/10.1017/S002221511000263X Published online by Cambridge University Press

Following tympanic membrane perforation, the wound healing process starts immediately, with the proliferation and migration of inflammatory cells.[23] Inflammatory cells are thought to be involved in the tissue formation and remodelling phases, in addition to their known role in cleaning the area around the wound. Schiff *et al.* believe that myringosclerosis may be triggered by exposure of damaged collagen to an intense inflammatory cell infiltrate.[24] During myringosclerosis, angioneogenesis occurs along the handle of the malleus and the annulus region to enable increased blood flow, which increases myringosclerotic plaque formation.[25] In the current study, we observed decreased inflammatory cell density, lamina propria thickness and tympanic membrane vascular density in myringotomised rats treated with pomegranate extract, compared with myringotomised rats not thus treated. These findings suggest that oral administration of pomegranate extract may reduce acute tympanic membrane inflammation, and may also decrease the formation of myringosclerosis, following myringotomy.

Polat *et al.* observed that topical vitamin E application to perforated rat tympanic membrane was associated with a significant reduction in reactive oxygen species levels and myringosclerotic deposits.[6] Oral application of vitamin E has also been found to decrease reactive oxygen species levels, although the observed reduction was lower than that following topical application.[6]

- **This study examined the effect of oral pomegranate extract on the inflammatory effect of myringotomy in rats**
- **Reactive oxygen species was measured by chemiluminescence and inflammation assessed by histological study, 48 hours post-myringotomy**
- **Reactive oxygen species levels, lamina propria thickness and inflammatory infiltrate density were significantly decreased in rats receiving pomegranate extract**
- **Oral pomegranate extract treatment decreases reactive oxygen species and acute inflammation in the rat tympanic membrane following myringotomy**

In the current study, we treated rats with oral pomegranate extract before and after myringotomy, and found lower levels of tympanic membrane reactive oxygen species, compared with non-treated myringotomised rats.

Conclusion

To the best of our knowledge, this study is the first to evaluate the effect of oral pomegranate extract on acute tympanic membrane inflammation due to myringotomy. Our findings indicate that pomegranate extract may be useful in this clinical setting. Small dietary changes may help reduce the complications of myringotomy. However, further studies on indications and dosages are needed before clinical application becomes possible.

References

1 Mattsson C, Magnuson K, Hellström S. Myringotomy: a prerequisite for the development of myringosclerosis. *Laryngoscope* 1998;**108**:102–6
2 Riley DN, Herberger S, McBride G. Myringotomy and ventilation tube insertion: a ten year follow up. *J Laryngol Otol* 1997;**111**:257–61
3 Mattsson C, Magnuson K, Hellström S. Myringosclerosis caused by increased oxygen concentration in traumatized tympanic membranes. Experimental study. *Ann Otol Rhinol Laryngol* 1995;**104**:625–32
4 Mattsson C, Johansson C, Hellström S. Myringosclerosis develops within 9 hours of myringotomy. *ORL J Otorhinolarygol Relat Spec* 1999;**61**:31–6
5 Mattsson C, Marklund SL, Hellström S. Application of oxygen free radical scavengers to diminish the occurrence of myringosclerosis. *Ann Otol Rhinol Laryngol* 1997;**106**:513–18
6 Polat S, Oztürk O, Uneri C, Yüksel M, Haklar G, Bozkurt S. Determination of reactive oxygen species in myringotomized tympanic membranes: effect of vitamin E treatment. *Laryngoscope* 2004;**114**:720–5
7 Spratley JE, Hellström SO, Mattsson CK, Pais-Clemente. Topical ascorbic acid reduces myringosclerosis in perforated tympanic membranes. *Ann Otol Rhinol Laryngol* 2001;**110**:585–91
8 Akbaş Y, Pata YS, Görür K, Polat G, Polat A, Ozcan C. The effect of L-carnitine on the prevention of experimentally induced myringosclerosis in rats. *Hear Res* 2003;**184**:107–12
9 Ozcan C, Gorur K, Cinel L, Talas DU, Unal M, Cinel. The inhibitory effect of topical N- acetylcysteine application on myringosclerosis in perforated rat tympanic membrane. *Int J Pediatr Otorhinolaryngol* 2002;**63**:179–84
10 Esmaillzadeh A, Tahbaz F, Gaieni I. Cholesterol-lowering effect of concentrated pomegranate juice consumption in type II diabetic patients with hyperlipidemia. *Int J Vitam Nutr Res* 2006; **76**:147–51
11 de Nigris F, Williams-Ignarro S, Sica V, Lerman LO, D'Armiento FP, Byrns RE *et al*. Effects of a pomegranate fruit extract rich in punicalagin on oxidation-sensitive genes and eNOS activity at sites of perturbed shear stress and atherogenesis. *Cardiovasc Res* 2007;**73**:414–23
12 Tugcu V, Kemahli E, Ozbek E, Arinci YV, Uhri M, Erturkuner P *et al*. Protective effect of a potent antioxidant, pomegranate juice, in the kidney of rats with nephrolithiasis induced by ethylene glycol. *J Endourol* 2008;**22**:2723–31
13 Aviram M, Dornfeld L. Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. *Atherosclerosis* 2001;**158**:195–8
14 Kaplan M, Hayek T, Raz A, Coleman R, Dornfeld L, Vaya J *et al*. Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid peroxidation, cellular cholesterol accumulation and development of atherosclerosis. *J Nutr* 2001; **131**:2082–9
15 Sade J, Luntz M. Dynamic measurements of gas composition in the middle ear. II: steady state value. *Acta Otolaryngol* 1993; **113**:353–7
16 Parks RR, Huang C-C, Haddad J. Evidence of oxygen radical injury in experimental otitis media. *Laryngoscope* 1994;**104**: 1389–92
17 Mattsson C, Hellström S. Inhibition of the development of myringosclerosis by local administration of fenspiride, an anti-inflammatory drug. *Eur Arch Otorhinolaryngol* 1997;**254**: 425–9
18 Aviram M, Dornfeld L, Kaplan M, Coleman R, Gaitini D, Nitecki S *et al*. Pomegranate juice flavonoids inhibit low-density lipoprotein oxidation and cardiovascular diseases: studies in atherosclerotic mice and in humans. *Drugs Exp Clin Res* 2002;**28**:49–62
19 Tsuda T, Horio F, Osawa T. Cyanidin 3-O-beta-D-glucoside suppresses nitric oxide production during a zymosan treatment in rats. *J Nutr Sci Vitaminol (Tokyo)* 2002;**48**:305–10

https://doi.org/10.1017/S002221511000263X Published online by Cambridge University Press

20 Görür K, Ozcan C, Polat A, Unal M, Tamer L, Cinel I *et al*. The anti-oxidant and anti-apoptotic activities of selenium in the prevention of myringosclerosis in rats. *J Laryngol Otol* 2002;**116**: 426–9
21 Forséni M, Bagger-Sjöbäck D, Hultcrantz M. A study of inflammatory mediators in the human tympanosclerotic middle ear. *Arch Otolaryngol Head Neck Surg* 2001;**127**:559–64
22 Kaptan ZK, Emir H, Gocmen H, Uzunkulaoglu H, Karakas A, Senes M *et al*. Ginkgo biloba, a free oxygen radical scavenger, affects inflammatory mediators to diminish the occurrence of experimental myringosclerosis. *Acta Otolaryngol* 2008;**17**: 1–6
23 Tahar Aissa J, Hultcrantz M. Acute tympanic membrane perforations and the early immunological response in rats. *Acta Otolaryngol* 2009;**23**:1–6
24 Schiff M, Poliquin JF, Cantanzaro A. Tympanosclerosis: a theory of pathogenesis. *Ann Otol Rhinol Laryngol Suppl* 1980;**89**:16–28
25 Hellström S, Spratley J, Eriksson PO, Pais-Clemente M. Tympanic membrane vessel revisited: a study in an animal model. *Otol Neurotol* 2003;**24**:494–9

Address for correspondence:
Dr Aysenur Meric,
Atakoy 9, Kisim A3A Blok D:36,
Bakirkoy, Istanbul, Turkey

Fax: +90 212 6217580
E-mail: aysenurmeric@yahoo.com

Dr A Meric takes responsibility for the integrity of the content of the paper
Competing interests: None declared