Int. Adv. Otol. 2010; 6:(2) 195-200

ORIGINAL ARTICLE

Efficiency of Bioflavonoids in the Prevention of Experimental Myringosclerosis

Ali E. Ilknur, Rıza Dundar, Sinan Basoglu, Sevinc Inan, Sinem Aktas, Hale Aslan, Yilmaz Ozkul, Sedat Ozturkcan, Huseyin Katilmis

Department of Otorhinolaryngology and Head and Neck Surgery, İzmir Atatürk Research and Training Hospital, Ministry of Health, Izmir, Turkey, (Aİ, RD, SB, SA, HA, SO, HK)
Department of Histology and Embryology, Celal Bayar University, Faculty of Medicine, Manisa, Turkey, (SI)

Objectives/Hypothesis: It has been noted that some materials with anti-inflammatory and antioxidant effects decrease sclerotic lesions in experimental myringosclerosis. Our purpose in this study is to investigate the effect of micronized purified flavonoid fraction (MPFF), an antioxidant with anti-inflammatory effects, in experimental myringosclerosis in guinea pigs.

Materials and Methods: Two study groups were formed. The first group was administered 100mg/kg/day MPFF by catheter for five days before myringotomy and 10 days after myringotomy, while the second group was administered distilled water by the same method, before and after myringotomy. On the 15th day of the study, after the tympanic membranes were examined otomicroscopically for myringosclerosis, they were removed by dissection together with the bone annulus, for histochemical and immunohistochemical examinations.

Results: In the MPFF group, the otomicroscopical sclerosis score, inflammation score and tympanic membrane thickness were significantly less than those in the untreated group ($p<0.05$). It was also determined that the immunoactivity of the anti-VEGF, anti-TGF-beta, anti-eNOS, anti-iNOS, and anti-IL1-beta primary antibodies, which are known to have an important role in angiogenesis and inflammation, significantly decreased in the MPFF group ($p<0.05$).

Conclusion: In conclusion, this study shows that orally administered MPFF can be efficient in the prevention of experimental myringosclerosis in guinea pigs.

Submitted : 19 November 2009 *Revised : 21 January 2010* *Accepted : 20 March 2010*

Introduction

Tympanosclerosis and myringosclerosis (MS) is a condition characterized with clinically calcareous plaque formation as a result of the thickening of the collagen fibrils and the accumulation of calcium phosphate crystals in the middle ear mucosa and in the tympanic membrane after a period of inflammation [1]. Although the aetiology and pathogenesis of tympanosclerosis cannot be defined exactly, among the causes proposed are infection, trauma, myringotomy, ventilation tube application, immunological reaction and genetic susceptibility [2-4].

Despite the fact that tympanosclerosis can be treated surgically, a similar process accompanying hyalinization and calcification is generally repeated after the surgery. Thus, studies performed to determine a treatment method in order to prevent the formation of this period show that some materials with anti-inflammatory and antioxidant effects decrease sclerotic lesions in experimental MS [5-8].

Micronized purified flavonoid fractions (MPFF) prevent the synthesis of free oxygen radicals causing tissue defects during inflammation and thus decrease the damage [9]. At the same time, by decreasing the serum complement (C1q, C3) level which is effective on vascular permeability and chemotaxis and by inhibiting the synthesis of inflammatory mediators which have vasodilatation effects such as PGE2, PGF2 and TXB2, MPFFs decrease the inflammatory response [10-11].

The aim of this study is to investigate the effect of MPFF in the experimental MS model in guinea pigs.

Materials and Methods

The study was performed in the Animal Laboratory of İzmir Atatürk Training and Research Hospital after receiving the approval of the Ethics Council of Izmir Atatürk Training and Research Hospital. The trial was performed on 10 healthy female guinea pigs whose weights varied between 300-400 grams. They were kept under standard laboratory and cage conditions. In

Corresponding address:
Ali Ekber Ilknur
Ilıca mah. No: 43/6 Narlıdere, İzmir, Türkiye
Phone: +90 232 2385806; E-mail: ekber68@yahoo.com

addition, a guinea pig was used for histopathological comparison. The animals were separated into two groups; a 5-animal study group (1st group) and a 5-animal control group (2nd group). First, 50 mg/kg Ketamine (Ketalar, Eczacibasi, Warner Lambert, Istanbul, Turkey) and 5 mg/kg Xylasine (Basilazin, aniMedica GmbH, Senden-Bösensell, Germany) anaesthesia were intraperitoneally administered to the animals in both groups. Then, perforation was achieved through myringotomy on the posterior-superior quadrant of the right and left tympanic membranes under an otomicroscope. The first group received Daflon 500 tb (S 5682, Servier, France) including two separate MPFF (%90 Diosmin, %10 hesperidin), which started orogastrically with a 100mg/kg/day dose 5 days before myringotomy and continued for 10 days after the myringotomy whereas the second group was administered distilled water before and after myringotomy in the same way.

Otomicroscopy

On the 15th day of the study, the tympanic membranes were examined otomicroscopically in terms of MS. In this examination, a 4-score scale was used for MS. Otomicroscopical findings were scored as follows: if there was no visible sclerotic lesion, this was scored as (0). If the sclerotic lesion was only on the malleus arm or adjacent to it, this was scored as (+). If the sclerotic lesion was adjacent to the malleus arm and on the upper frontal part of the pars tensa, this was scored as (++). If the sclerotic lesion was adjacent to the malleus and continued through the annulus, this was scored as (+++) [5].

Histopathology

After otomicroscopical examination, for histochemical and immunohistochemical examination, the subjects were sacrificed with a high dose (80 mg/kg) of intraperitoneal thiopental sodium injection, and their tympanic membranes together with the bone annulus were dissected under the otomicroscope.

After the tympanic membrane samples were embedded in 10% formalin for 24-48 hours, they were put in fresh EDTA solution every other day for 3 weeks in order to decalcify the bony tissue. After sufficient tissue smoothness was obtained, the samples were washed under running water, dehydrated with gradually graded ethyl alcohol series and then embedded in paraffin and cleared with xylene.

Histopathological findings were evaluated by a doctor who was blinded to the groups. For histological evaluation, sections 5 µm in thickness were taken from the blocks and deparaffinised for an hour in xylene and for one night in an 60 °C incubator and then were stained in accordance with the hematoxilene-eosine (Surgipath, 01562E, 01602E, Peterborough, UK) histochemistry staining procedure by applying dehydration process with gradually degraded ethyl alcohol series.

The 5-µm- section moved from the same blocks to the poly-lysine covered laminas was prepared for indirect immunohistochemical staining by using the avidin-biotin peroxidase method. The preparations to be immunohistochemically stained were kept in a 60 °C incubator an overnight and were left in xylene for 1 hour and the deparafinisation process was completed. The sections became ready for the distilled water stage after passing through the gradually degraded ethyl alcohol series. The sections were embedded in Dako Pen (Dako, Glostrup, Denmark) and then the sections treated with trypsin were washed with PBS (Phosphate Buffer Solution), and 3% hydrogen peroxidase was applied in order to inhibit peroxidase activity in the tissue. The sections washed with PBS were kept in a ‘’non-immune blocking’’ solution for an hour and then incubated in a +4°C moist environment for 18 hours together with anti-VEGF and anti-TGF-beta, anti-e-NOS, anti-iNOS and anti IL1-beta, primary antibodies (in 1/100 dilutions). An avidine-biotine peroxidase system was used as a secondary kit. The sections washed with PBS were incubated for 30 minutes with a biotine-marked hydrogen peroxidase secondary antibody. The sections washed with PBS were treated with streptavidine for 30 minutes. To observe the immunoreactivity, sections treated with DAB (diaminobenzidine) were mounted with mounting medium after having been counterstained with Mayer’s hematoxylin.

In order to test whether the immunoreactivities were specific, one section from each block was separated for control staining, and IgG, similar to primary antibodies, was used without primary antibodies in the identical manner.

The sections stained with histochemical and immunohistochemical methods were examined with an Olympus BX40 (Olympus, Tokyo, Japan) light microscope. The tympanic membrane thicknesses of the sections taken from the sections stained with HE

were measured with an ocular micrometer. The inflammation score, the presence of leukocyte with polymorphic nuclei and the vein widths were evaluated and scored as 1 (normal structure), 2(mild inflammation) and 3 (severe inflammation). The immunohistochemistry staining strengths were semi-quantitatively evaluated as + (light), ++ (moderate), +++ (severe).

The statistical evaluation was performed with a Mann-Whitney U test and those with p values less than 0.05 were accepted as statistically significant.

Results

Otomicroscopical findings

The tympanic membranes were otomicroscopically examined in terms of MS, and the otomicroscopical findings are shown in Table 1. In this examination, it was observed that MS development was less in the MPFF group than in the untreated group (p =0.018).

Histochemical Findings

The sections evaluated with hematoxylene-eosine (HE) staining were examined by indirect immunohistochemical staining with anti-VEGF, anti-TGF-beta, anti-e-NOS, anti-iNOS and anti-IL1-beta primary antibodies.

The evaluation of the sections obtained from the normal tympanic membrane and stained with HE under the light microscope for histopathological comparison revealed that the tympanic membrane had the normal histological structure, the external ear epithelia was located in the outer side, the fibroblasts were in their normal structures and they were composed of the fibrous connective tissue layer and the middle ear mucosa inside (Figure 1). The inflammation score was evaluated as 1 (normal structure) in both samples. When the sections obtained from the myringotomy group and stained with HE under the light microscope were evaluated, separations in the membrane structure, an increase in its thickness, thickening in the lamina propria, an increase in active fibroblasts and collagen fibrils, vasodilatation and inflammatory cell infiltration in the sub-epithelial layer were observed (Figure 2) and the inflammation score was determined as 3 on average. The evaluation of the sections obtained from the myringotomy and MPFF group and stained with HE under the light microscope showed that the membrane thickness decreased, the active fibroblasts and collagen fibrils decreased, vasodilatation decreased, inflammatory

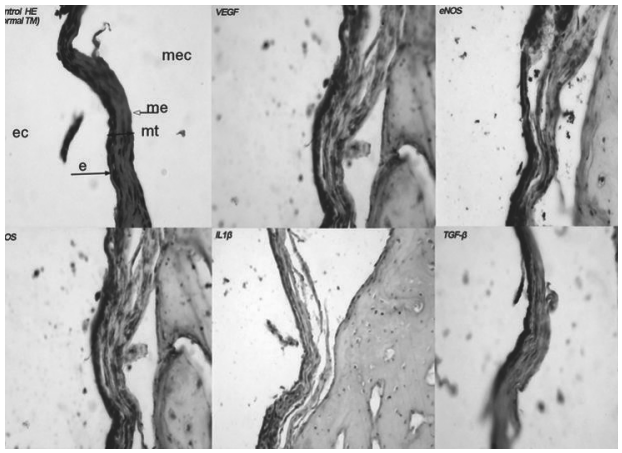

Figure 1. Light microscopic images of normal tympanic membrane (X 400). In the evaluation of the sections stained with hematoxylene-eosine, it was observed that the tympanic membrane had the normal histological structure, that the external ear epithelia was located in the outer side, and that he tympanic membrane was composed of the fibrous connective tissue layer in which the fibroblasts had normal structures and of the middle ear mucosa inside. Immunohistochemical staining strengths for anti-VEGF, anti-TGF-beta, anti-eNOS, anti-iNOS and anti-IL1-beta were + (mild). Mec=middle ear cavity; ec=external ear; me=mucosal epihelium; mt=membrane thickness; e=epitehelium.

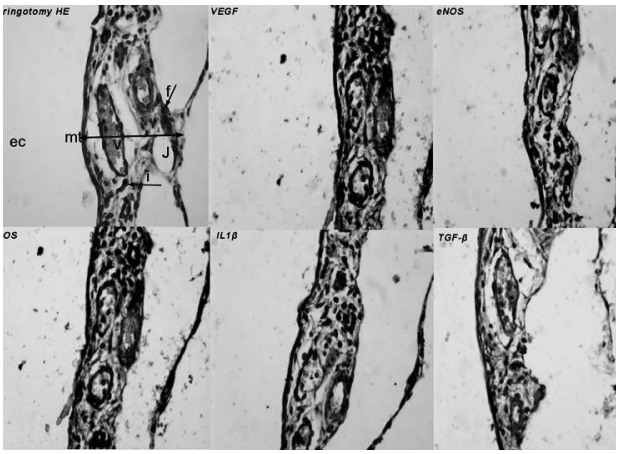

Figure 2. Light microscopic images of the sections of tympanic membrane obtained from the myringotomy group (X 400). In the evaluation of the sections stained with hematoxylene-eosine, separations in the membrane structure, an increase in its thickness, thickening in the lamina propria, an increase in active fibroblasts and collagen fibrils, vasodilatation and inflammatory cell infiltration in the sub-epithelial layer were observed. The immune staining strengths for the anti-VEGF, anti-TGF-beta, anti-eNOS, anti-iNOS, and anti-IL1-beta were ++ (moderate), +++ (severe). ec=external canal; mt=membrane thickness; V= vascularization; J= junctional degeneration; f=fibroblast proliferation; i= inflammatory cells.

cells decreased compared to those in the myringotomy group (Figure 3) and the inflammation score was determined as 2 on average. The inflammation scores

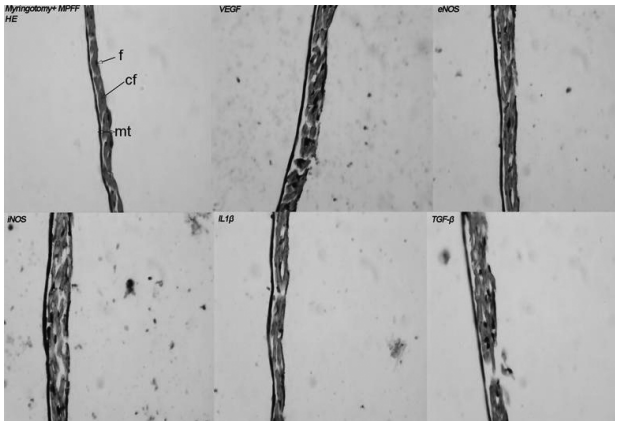

Figure 3. Light microscopic images of the sections of tympanic membrane obtained from the myringotomy and MPFF group (X 400). In the evaluation of the sections stained with hematoxylene-eosine, it was observed that the membrane thickness, the active fibroblasts and collagen fibrils, vasodilatation and inflammatory cells decreased compared to those in the myringotomy group. The immune staining strengths for anti-VEGF, anti-TGF-beta, anti-eNOS, anti-iNOS, and anti-IL1-beta were + (mild), ++ (moderate). Cf= collagen fibers; f=fibroblast; mt=membrane thickness

obtained from the myringotomy group and the MPFF group after myringotomy are shown in Table 2. A statistically significant difference was determined (p = 0.044) when inflammation scores between the MPFF group and the untreated group were compared.

The thicknesses of the tympanic membranes obtained from the normal tympanic membrane used for histopathological comparison were determined as 0.035 mm and 0.036 mm. The tympanic membrane thicknesses obtained from the myringotomy group and the MPFF group after myringotomy are shown in Table 3. A statistically significant difference was determined (p = 0.000) when the tympanic membrane thicknesses between the MPFF group and the untreated group were compared.

Immunohistochemical Findings

The evaluation of the normal tympanic membranes used in histopathological comparison and the tympanic membranes obtained from the myringotomy, myringotomy and MPFF groups by indirect immunohistochemical method shows that different strengths of staining appeared in the epithelial and endothelial cells of the veins and the fibroblasts. The immune staining (Figures 1-3) strengths related to the distribution of the anti-VEGF, anti-TGF-beta, anti-eNOS, anti-iNOS, and anti-IL1-beta primary antibodies in the tympanic membrane, which play an important role in angiogenesis and inflammation, are shown in Table 4.

When the tympanic membranes obtained from the myringotomy, and myringotomy and MPFF groups

Table 1. Otomicroscopical Findings

		Numerical distribution of otomicroscopical sclerosis scores			
Group	**Number of subjects (n)**	**0**	**+**	**++**	**+++**
Myringotomy+ MPFF	10	1	6	2	1
Myringotomy	10		2	3	5

MPFF= micronized purified flavonoid fraction

Table 2. Histopathological inflammation scores

		Numerical distribution of inflammation scores		
Group	**Number of subjects (n)**	**1 (Normal)**	**2 (Moderate)**	**3 (Severe)**
Myringotomy+ MPFF	10	2	6	2
Myringotomy	10		4	6

MPFF= micronized purified flavonoid fraction

Table 3. The thicknesses of the tympanic membranes

	Thicknesses of the tympanic membranes of subjects (mm)									
Group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Myringotomy+ MPFF	0.058	0.056	0.055	0,050	0.050	0.049	0,047	0.058	0.072	0.070
Myringotomy	0.104	0.103	0.102	0.102	0.100	0.100	0.092	0.090	0.080	0.095

MPFF= micronized purified flavonoid fraction

and evaluated by the indirect immunohistochemical method were compared, it was observed that the immunoreactivity was significantly decreased in the MPFF group, and the statistical values are given in Table 5.

Discussion

So far, some drugs with anti-inflammatory and antioxidant effects used to prevent tympanosclerosis and MS formation have been researched in several empirical studies. In one of the studies performed to examine the effect of glucocorticoids in MS development, it was found that, in rats, intraperitoneally applied dexamethsone decreased MS, an inflammatory process [5]. In an empirical study performed with different application doses of fenspiride, an anti-inflammatory drug, it was determined that fenspiride decreased the MS development [6]. In a study performed with caffeic acid phenethyl ester, an anti-inflammatory and antioxidant drug, it was determined that sclerosis development was histopathologically less in the treatment group than in the control group and the tympanic membrane thickness was thinner in the caffeic acid phenethyl ester applied group than in the untreated group [7]. It was determined that intraperitoneally applied L-carnitine prevented MS development in myringotomy-performed rats and fibroblastic development was less than in the untreated group, and the tympanic membrane thickness is thinner in the caffeic acid phenethyl ester applied group than in the control group. And it was stated that intraperitoneally applied L-carnitine can be used to prevent MS development in cases to undergo the ventilation tube of L-carnitine or myringotomy application [8].

Although, there are not any studies indicating that MPFFs are used in MS models in the literature, there are some studies on anti-inflammatory and antioxidant effects of MPFFs. It has been shown that hesperidin, an MPFF, is effective in MS formation and inhibits the inducible isoforms of COX-2 and NOS and this can be related to the antioxidant, anti-inflammatory and anti-carcinogenic effects of hesperidin [12]. Infection is also one of the important factors of MS and TS development. It was shown that the systemic and topical application of MPFF heals the injuries of the infected wounds developed in the guinea pigs better and faster and that this effect might be due to the anti-inflammatory antioxidant effects of MPFF and its ability to decrease oedema or to regulate microcirculation on the wound [13]. In one study performed on the ischemia/reperfusion models developed in rats, it was shown that MPFF is an important agent which protects the tissues from ischemic defects by decreasing free oxygen formation

Table 4. Immunoreactivity scores

Immunohistochemistry	Numerical distribution of immunoreactivity scores in the groups								
	Normal tympanic membranes (n=2)			Myringotomy (n=10)			Myringotomy+MPFF (n=10)		
	+	++	+++	+	++	+++	+	++	+++
VEGF	2				1	9	2	8	
TGF-beta	2				9	1	9	1	
e-NOS	2				9	1	8	2	
i-NOS	2				1	9	3	7	
IL-1 β	2				9	1	9	1	

MPFF= micronized purified flavonoid fraction

Table 5. Statistical comparison of immunoreactivity scores

	Myringotomy group (n=10)	Myringotomy+MPFF group (n=10)	
Immunohistochemistry	Mean± Standard deviation	Mean± Standard deviation	p Value
VEGF	2.900±0.316	1.800±0.422	.000
TGF-beta	2.100±0.316	1.100±0.316	.000
e-NOS	2.100±0.316	1.200±0.422	.000
i-NOS	2.900±0.316	1.700±0.483	.000
IL-1 β	2.100±0.316	1.100±0.316	.000

MPFF= micronized purified flavonoid fraction

due to ischemia and by decreasing the leukocyte infiltration in the perivascular area [14]. A clinical study showed that plasma VEGF levels were higher in chronic venous disease patients with skin findings than in patients with no skin findings, and that after MPFF treatment, the plasma VEGF level decreased in chronic venous disease patients with skin findings [15].

In our study performed on guinea pigs, it was observed that the sclerosis score, inflammation score and membrane thicknesses decreased in the myringotomy group which underwent myringotomy and to which MPFF was administered orally compared to the group which only underwent myringotomy. It was also determined that VEGF, TGF-beta, eNOS, iNOS, ve IL1-beta, which have an important role in angiogenesis and inflammation, showed less immunoreactivity in the MPFF group than in the untreated group. As a result, we concluded that per os MPFF can be effective in the prevention of MS developed experimentally in guinea pigs.

Acknowledgments

We thank Hülya Ellidokuz, MD, for her assistance with the statistical analysis.

References

1. Bhaya MH, Schachern PA, Morizono T, Paparella MM. Pathogenesis of tympanosclerosis. Otolaryngol Head Neck Surg 1993; 109:413-20.

2. 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.

3. Tos M, Stangerup SE. Hearing loss in tympanosclerosis caused by grommets. Arch Otolaryngol Head Neck Surg 1989; 115:931-5.

4. Koç A, Uneri C. Genetic predisposition for tympanosclerotic degeneration. Eur Arch Otorhinolaryngol 2002; 259:180-3.

5. Mattsson C, Stierna P, Hellström S. Treatment with dexamethasone arrests the development of myringosclerosis after myringotomy. Am J Otol 2000; 21:804-8.

6. 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.

7. Song JJ, Kwon SK, Cho CG, Park SW. The effect of caffeic acid phenethyl ester on the prevention of experimentally induced myringosclerosis. Int J Pediatr Otorhinolaryngol 2007; 71:1287-91.

8. Akbaş Y, Pata YS, Görür K, Polat G, Polat A, Ozcan C, Unal M. The effect of L-carnitine on the prevention of experimentally induced myringosclerosis in rats. Hear Res 2003; 184:107-12.

9. Wilmsen PK, Spada DS, Salvador M. Antioxidant activity of the flavonoid hesperidin in chemical and biological systems. J Agric Food Chem 2005; 53:4757-61.

10. Di Perri T, Auteri A. Action of S 5682 on the complement system. In vitro and in vivo study. Int Angiol 1988; 7:11-5.

11. Damon M, Flandre O, Michel F, Perdrix L, Labrid C, Crastes de Paulet A. Effect of chronic treatment with a purified flavonoid fraction on inflammatory granuloma in the rat. Study of prostaglandin E2 and F2 alpha and thromboxane B2 release and histological changes. Arzneimittelforschung 1987; 37:1149-53.

12. Sakata K, Hirose Y, Qiao Z, Tanaka T, Mori H. Inhibition of inducible isoforms of cyclooxygenase and nitric oxide synthase by flavonoid hesperidin in mouse macrophage cell line. Cancer Lett 2003; 199:139-45.

13. Hasanoglu A, Ara C, Ozen S, Kali K, Senol M, Ertas E. Efficacy of micronized flavonoid fraction in healing of clean and infected wounds. Int J Angiol 2001; 10:41-4.

14. Unlü A, Sucu N, Tamer L, Coskun B, Yücebilgiç G, Ercan B, et al. Effects of Daflon on oxidative stress induced by hindlimb ischemia/reperfusion. Pharmacol Res 2003; 48:11-5.

15. Shoab SS, Scurr JH, Coleridge-Smith PD. Plasma VEGF as a marker of therapy in patients with chronic venous disease treated with oral micronised flavonoid fraction - a pilot study. Eur J Vasc Endovasc Surg 1999; 18:334-8.