International Journal of Pediatric Otorhinolaryngology 78 (2014) 1019–1025

Contents lists available at ScienceDirect

International Journal of Pediatric Otorhinolaryngology

journal homepage: www.elsevier.com/locate/ijporl

Inhibitory effect of N-acetyl cysteine and ascorbic acid on the development of myringosclerosis: An experimental study

Rıza Dündar [a], Sevinç İnan [b], Nuray Bayar Muluk [c,*], Cemal Cingi [d], Ali Ekber İlknur [e], Hüseyin Katılmış [f]

[a] Kızıltepe State Hospital, ENT Department, Mardin, Turkey
[b] Celal Bayar University, Faculty of Medicine, Histology and Embryology Department, Manisa, Turkey
[c] Kırıkkale University, Faculty of Medicine, ENT Department, Kırıkkale, Turkey
[d] Osmangazi University, Medical Faculty, Department of Otorhinolaryngology, Eskisehir, Turkey
[e] Urla State Hospital, ENT Department, İzmir, Turkey
[f] İzmir Katip Çelebi University, Training and Research Hospital, ENT Clinics, İzmir, Turkey

ARTICLE INFO

Article history:
Received 26 October 2013
Received in revised form 14 March 2014
Accepted 29 March 2014
Available online 12 April 2014

Keywords:
Ascorbic acid
Inducible nitric oxide synthase (iNOS)
Interleukin 1-beta (IL1-β)
Myringosclerosis
N-acetyl cysteine (NAC)
Vascular endothelial growth factor (VEGF)

ABSTRACT

Objectives: This study investigated the effects of ascorbic acid and N-acetyl cysteine (NAC) antioxidants on the development of myringosclerosis (MS) in an experimental model.
Methods: Myringotomies were performed in the ears of 15 guinea pigs, and Spongostan® pieces were placed on the perforated regions of the tympanic membrane. The subjects were divided randomly into three groups and treated with three different solutions on the Spongostan—group 1: (control, 0.9% saline), group 2 (ascorbic acid), and group 3 (NAC). On day 15 after treatment, specimens from the tympanic membranes were obtained and examined via light microscopy. Sclerosis and inflammation scores and the tympanic membrane thicknesses were evaluated. Immunohistochemical methods were used to evaluate the expression of VEGF, TGF-β, iNOS, and IL1-β in all groups.
Results: Lower sclerosis and inflammation scores and reduced tympanic membrane thicknesses were observed in groups treated with NAC or ascorbic acid compared with the control group. Immunohistochemical studies revealed significantly less expression of VEGF, TGF-β, and iNOS in groups 2 and 3 compared with group 1. Additionally, IL1-β expression was significantly less in group 3 than in group 1. Compared with group 1, group 2 animals exhibited reduced inflammation in the lamina propria, fewer active fibroblasts, less leukocyte infiltration, and decreased thickness of the vessels; group 3 animals exhibited decreased numbers of active fibroblasts and collagen fibers in the lamina propria.
Conclusions: Inflammation scores, cellular infiltration, and expression of VEGF, TGF-β, and iNOS were reduced by ascorbic acid and/or NAC treatments, thereby decreasing MS development. Decreased expression of IL1-β was observed only in animals treated with NAC.

1. Introduction

Myringosclerosis (MS) is a condition that develops after a middle ear infection or traumatic intervention. In this condition, hyalinization and calcification of the collagen layer develop in certain areas of the tympanic membrane (TM) with increased collagen fibers due to progressive fibroblast infiltration, hyaline degeneration, and extracellular calcium deposition within the lamina propria [1–3]. MS is characterized by the development of calcified plaques on the TM [4,5]. The etiology of MS includes inflammatory disease, intratympanic membrane bleeding after tube insertion, traumatic tube insertion, excessive aspiration of middle ear fluid, and increased production of oxygen-derived free radicals within the middle ear after myringotomy [5–9].

Tos et al. [10] reported that decreased ear membrane mobility after insertion of a ventilation tube (VT) promotes hyalinization and calcification in the collagen layer, resulting in MS. While most cases of MS remain asymptomatic, the clinical condition in cases with symptoms can range from mild to severe hearing loss associated with large sclerotic plaques [11]. MS develops due to free oxygen radicals secondary to hyperoxic medium and

* Corresponding author at: Birlik Mahallesi, Zirvekent 2. Etap Sitesi, C-3 blok, No.: 62/43, 06610 Çankaya/Ankara, Turkey. Tel.: +90 312 4964073/532 7182441; fax: +90 312 4964073.
E-mail addresses: nbayarmuluk@yahoo.com, nurayb@hotmail.com (N.B. Muluk).

http://dx.doi.org/10.1016/j.ijporl.2014.03.029
0165-5876/

mechanical injury within the middle ear. Several studies have reported that agents with antioxidant characteristics inhibited the development of MS [3,11,12]. Aydoğan et al. [1] reported that oral administration of coenzyme Q10 did not reduce MS formation in myringotomized rats.

Spratley et al. [12] studied the effects of topical ascorbic acid applied on Gelfoam cups to the TMs in 12 Sprague-Dawley rats that were myringotomized bilaterally. They reported that the extent of sclerotic lesions was significantly reduced in the ascorbic acid-treated group compared to saline-treated or untreated animals, and concluded that topical ascorbic acid reduced the occurrence of MS following TM perforations.

In the present experimental study, we investigated the protective effects of known anti-oxidant agents, ascorbic acid and N-acetyl cysteine (NAC), compared with saline solution, on the development of MS. MS was evaluated using light microscopy. Saline solution was used as a control because TM thicknesses were found to be similar in untreated and saline-treated rats after myringotomy [13].

2. Materials and methods

This study was conducted at Izmir Ataturk Training and Research Hospital and followed the principles of the Declaration of Helsinki [14]. Adaptation and care of the animals and the experimental study were performed at the same center.

2.1. Animal subjects

Fifteen female guinea pigs (weighing 350–400 g) were used in this study. Approval for the study was obtained from the Committee for Ethical Issues of Izmir Ataturk Training and Research Hospital (date: 2008, number: 4551710). All animal procedures were performed in accordance with the approved protocol. All interventions to the animals were conducted under aseptic conditions.

2.2. Methods

During the experimental procedure, the guinea pigs were sedated using 10 mg/kg xylazine and 30 mg/kg intraperitoneal injection of ketamine. Myringotomies were performed in the upper posterior quadrant of the TM (2 mm in length) in both ears of each guinea pig. Pieces of Spongostan® were placed on the perforated region of the TM.

The subjects were randomly divided into three groups with five animals in each group. Three different solutions were applied onto the Spongostan® (three drops twice a day for 10 days) as follows: group 1 received saline (0.9% NaCl) solution ($n = 5$, 10 ears); group 2 received ascorbic acid (0.28 mmol/L in sterile water) [12] ($n = 5$, 10 ears); and group 3 received NAC (1.2 mg/mL) [15] ($n = 5$, 10 ears).

On day 15 of the study, the TMs were examined otomicroscopically, and the findings were scored according to the development of MS: (0) no visible sclerotic lesion; (+) sclerotic lesion only on the malleus arm or adjacent to it; (++) sclerotic lesion adjacent to the malleus arm and on the upper frontal part of the pars tensa; or (+++) sclerotic lesion adjacent to the malleus and continuing through the annulus.

2.3. Histological examination procedure

Rats were sacrificed using 80 mg/kg pentothal 15 days after treatments began. Immediately after death, the temporal bones were removed, and the otic bullas were excised and placed in fixative (10% formalin). The temporal bones were decalcified in 5% formic acid [16], deparaffinized, and dehydrated by immersion into xylene twice for 10 min. Following dehydration in an ascending series of ethanol (70, 80, 96, 100%), tissue samples were cleared in xylene and embedded in paraffin. The preparations were transferred into citrate-based antigen retrieval solution to detect vascular endothelial growth gactor (VEGF), transforming growth factor beta (TGF-β), inducible nitric oxide synthase (iNOS), and interleukin 1-beta (IL1-β) antigens. All slides were microwaved twice for 5 min in a microwave oven (750 W). Using the Shandon Sequeza Tm manual staining device for standardization, the classical streptavidine avidin-biotin-peroxidase (Strept. AB-Peroxidase) method and diaminobenzidine (DAB) chromogen (20 min) were applied for immunohistochemical analysis using four antibodies. Non-immune mouse serum served as a negative control and Mayer's hematoxylin was used as the counterstain. Cytoplasmic staining was considered evidence of positivity. A minimum of 3–4 fields for each sample were examined and scored by an observer blinded to the treatment of the animals [17]. Slides were examined using light microscopy with an Olympus BX40 microscope, and photos were taken with an Olympus DP-70 digital camera.

The inflammation process was evaluated by observing the cellular density, the existence of polymorphonuclear leukocytes, and the vascular diameter. Samples were scored as having (1) normal structure, (2) moderate inflammation, or (3) severe inflammation.

Immunohistochemical staining properties were evaluated semi-quantitatively by examiners who were blinded to the treatment. Antigen (+) cells were assessed by counting a total of 100 cells in 3–4 fields of high magnification (400×), and the mean expressions of antigen were calculated. Scoring was performed using a scale of 0–3: (0) represented negative staining, 0% cell count; (+) mild staining, <5% cell count; (++): moderate staining, 5–50% cell count; and (+++) severe, >50% cell count [18].

2.4. Statistical analysis

The statistical package for SPSS (Version 16.0) was used for statistical analyses. Kruskal–Wallis variance analysis was used to analyze differences between groups. When statistically significant results were obtained, pairwise comparisons were performed using the Mann–Whitney U Test with Bonferrroni correction to detect the value that had caused a difference. A p-value < 0.05 was considered to indicate statistical significance.

3. Results

3.1. Otomicroscopical sclerosis scores (OSS)

Table 1 and Fig. 1 presents the OSS values for groups 1–3. Significant differences were detected between OSS values ($p = 0.004$): OSS values in groups 2 and 3 were significantly lower than those in group 1 ($p^{\text{groups 1 and 2}} = 0.014$, $p^{\text{groups 1–3}} = 0.002$). No significant difference was detected between groups 2 and 3 ($p^{\text{groups 2 and 3}} = 0.318$).

3.2. Inflammation score (IS)

Table 2 and Fig. 1 presents the IS values in groups 1–3. Significant differences were detected between IS values ($p = 0.003$): IS values in groups 2 and 3 were significantly lower than those in group 1 ($p^{\text{groups1 and 2}} = 0.014$, $p^{\text{groups 1–3}} = 0.002$). No significant differences were observed between groups 2 and 3 ($p^{\text{groups 2 and 3}} = p = 0.313$).

3.3. TM thickness

Table 3 lists TM thicknesses in groups 1–3. Significant differences were detected between TM thicknesses ($p = 0.000$):