Antioxidant Role of Selenium in Rats with Experimental Acute Otitis Media

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Abstract The aim of this prospective experimental animal study was to determine whether selenium had a protective effect on oxidative stress in rats with acute otitis media, by measuring the alterations of antioxidant parameters and lipid peroxidation on days 4 and 10 after inoculation into the middle ear. *Streptococcus pneumoniae* was inoculated into the middle ear cavities of 32 rats in animal laboratory of a tertiary medical center. Group 1 served as the control group and the animals were administered 1.5 ml/day saline. Group 2 received 0.2 mg/kg/day oral selenium for 10 days. The blood samples of each group were obtained on post-inoculation days 4 and 10. The levels of thiobarbituric acid reactive substances, albumin,

total *sulphydryl*, superoxide *dismutase* and glutathione peroxidase were investigated. Day 10 level of thiobarbituric acid reactive substance in group 2 was lower than the day 4 level of the same substance in the control group. Although glutathione peroxidase and superoxide dismutase levels significantly decreased starting from 4th day until 10th day in group 1, their levels increased in group 2. Day 10 levels of albumin and total *sulphydryl* in group 1 were significantly higher than day 4 levels in group 2. We found that selenium supplementation for 10 days decreased thiobarbituric acid reactive substances and increased glutathione peroxidase and superoxide dismutase levels when compared to the control group. We believe that selenium supplementation may be beneficial to prevent the clinical sequelae and recurrence of otitis media.

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Introduction

Acute otitis media (AOM) is one of the most frequent and painful diseases in childhood and its recurrence is an important problem [1]. The sequelae and morbidity of AOM are significant public health concerns [2]. AOM is also of great importance due to its possible intracranial and/or extracranial complications [1].

Free oxygen radicals are produced in a number of physiologic conditions and neutralized by enzymatic and non-enzymatic defense systems, including enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx), and nonenzymatic components such as beta-carotene, retinol, alfa-tocopherol, ascorbic acid, reduced glutathione, albumin, ceruloplasmin, transferrin and total

sulphydryl group. When the balance between free oxygen radical production and antioxidant defense mechanisms is disturbed, the levels of free oxygen radicals increase, finally leading to tissue damage by chemically modifying cellular lipids, proteins, carbohydrates, and nucleotides [3].

Free oxygen radicals have been implicated in the pathogenesis of AOM because of two sources of free radicals, namely neutrophils and bacteria [4, 5]. In AOM, middle ear mucosa shows evidence of significant free radical-mediated damage. Free radical-mediated damage of the mucosa may delay recovery, and may predispose to persistent middle ear effusion, recurrent infection, or both [4].

It was reported that free radical damage to the middle ear mucosa may occur despite appropriate antibiotic therapy in AOM, since nonviable bacteria and/or bacterial components released during death of the pathogens induce lipid peroxidation [4]. Possibly, antioxidant and anti-inflammatory agents can reduce free radical-induced inflammatory changes. Therapy with combined antibiotics and antioxidant agents may be beneficial for relieving the clinical sequelae of otitis media. To our knowledge, there is a lack of reports that focused on the effect of antioxidants in AOM.

Selenium, an essential trace element, is a structural component of several enzymes with physiological antioxidant properties including GPx. It is regarded as an antioxidant agent due to its effects on the reduction oxidative stress [6]. Dietary selenium is an essential micronutrient that plays a role for the maintenance of optimal immune responses. Some studies have suggested that selenium supplementation might enhance immunity, including both humoral and cell-mediated responses. Most of the available data suggest that selenium status affects resistance to bacterial, parasitic and fungal infections, depending on the microorganism involved [7].

To our knowledge, there are no available data investigating the antioxidant role of selenium in AOM. One of the primary aims of the study is to determine whether selenium has a protective effect on the oxidative stress in rats with *Streptococcus pneumoniae* type 3-induced AOM by measuring the alterations of antioxidant parameters and lipid peroxidation on post-inoculation days 4 and 10. For this reason, we measured serum thiobarbituric acid reactive substances (TBARS), albumin, total sulphydryl levels and erythrocyte SOD and GPx activities in the blood samples of rats with experimental AOM on days 4 and 10 after bacterial inoculation.

Materials and Methods

Experimental Design

The experimental design of this study was approved by the Ethics Research Committee of Animal Experiments in

Ministry of Health Ankara Training and Research Hospital (protocol number: 1/03 Ankara, Turkey). This study was complied with experimental ethical principles and animal protection laws according to the rules and regulations in Turkey, and was performed in the Animal Laboratory of Ankara Training and Research Hospital. All animals' care and procedures were performed humanely. The animals were kept in ordinary cages at a temperature of 20 to 22°C with free access to food and water, and were subjected to a 12-h artificial light/dark cycle. They were given pellets (2700 ME kcal/kg, 23% HP) and water ad libitum, and used after 1 week of quarantine and acclimatization.

Thirty-two male, healthy, Wistar albino rats with a mean weight of 220 g were used in this study. None of them had external or middle ear infection or tympanic membrane adhesion, perforation or retraction under otomicroscopic examination. They were anesthetized by ketamine hydrochloride (50 mg/kg, intramuscular). All procedures were performed under sterile conditions. Under otomicroscopic view (Opmi 1, Zeiss, Germany), 0.05 ml of *S. pneumoniae* type 3 solution, consisting of 10^7 to 10^9 pneumococci per milliliter, was inoculated into the right middle ear cavity via transtympanic route. The animals were initially examined with otomicroscopy 48 h after inoculation, and the presence of AOM was verified. Then, they were randomly divided into two groups, each group consisting of 16 rats. Group 1 served as the control group and was treated with 1.5 ml/day saline orally for 10 days. Group 2 was treated with selenium (0.2 mg/kg/day, GNC 100 µg tablet) through a nasogastric tube for 10 days [8]. In each group, half of the rats were sacrificed painlessly using high dose pentobarbital (80 mg/kg, intraperitoneal injection) on days 4 and 10 after bacterial inoculation. Before sacrifice, they were anesthetized and blood samples were collected for biochemical analysis. The temporal bones of the animals were removed and the middle ear mucosa was carefully peeled using a forceps under the dissecting microscope. They were prepared for histopathological examination. Levels of TBARS, albumin and total sulphydryl groups as well as erythrocyte SOD and GPx activities were investigated in the venous blood sample. Two groups were compared for biochemical and histopathological parameters obtained 4 and 10 days after bacterial inoculation.

Histopathological Evaluation

The specimens were stained with hematoxylin–eosin and were examined under the light microscope. Histopathological findings obtained on days 4 and 10 in group 1 (saline) were compared to those in group 2 (selenium). The examiners were unaware of the groups. Inflammation and the integrity of the epithelium was scored in the histopathological analysis [9]. The severity of inflammation was

assessed on a scale where grading was determined as none (0), minimal (1): rare individual inflammatory cells in the mucosa and submucosa; mild (2): mild infiltration of individual inflammatory cells or their clusters; moderate (3): dense infiltration of inflammatory cells; and severe (4): inflammatory infiltration dense enough to obscure the normal architecture of the mucosa and submucosa. The integrity of middle ear epithelium was assessed on a scale where complete integrity was scored as (0), focal epithelial loss without vacuolization or metaplasia was scored as (1), focal epithelial loss and vacuolization was scored as (2), and focal epithelial loss and epithelial metaplasia was scored as (3).

Biochemical Analysis

Blood samples were obtained via venipuncture of the vena cava. Blood was collected in evacuated gel separator tubes, ethylenediaminetetraacetic acid containing tubes and heparinized syringes, and centrifuged at +4°C and 1600×g for 15 min. Approximately 500–1,000 µl of the samples were taken and used for measurements of albumin, thiobarbituric acid reactive substances and total sulphhydryl groups. Ethylenediaminetetraacetic acid samples were used for SOD and GPx enzyme measurements, and heparinized syringes were used for determining the hemoglobin levels.

GPx (E.C.1-11.1.9)

GPx enzyme activity in the erythrocyte hemolysates was determined with commercial Randox kit according to the method described by Paglia and Valentine [10]. The activity of GPx was expressed as units per gram of hemoglobin (U/g Hb).

SOD (E.C.1.15-1.1)

SOD enzyme activity in the erythrocyte hemolysates was determined with the commercial Randox kit. The activity of SOD was expressed as units per gram of hemoglobin (U/g Hb).

TBARS

The fluorometric method for measuring TBARS was based on the reaction between malondialdehyde and thiobarbituric acid levels as described by Wasowicz et al. [11]. Hitachi F-2500 spectrofluorometry apparatus was used. TBARS levels were expressed in µmol/l.

Serum Total Sulphydryl Groups

Total thiol groups in serum was estimated according to the method of Sedlak and Lindsay [12]. The molar extinction

coefficients of 13,100 at 412 nm was used to estimate the total thiol contents, and the values were expressed in µmol/l.

Serum Albumin

Serum albumin levels were determined with Olympus AU 2700 autoanalyzer with original Olympus albumin kit.

Hemoglobin

Hemoglobin measurements were carried out with Roche cobas b 221 blood gas system.

Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) 11.5 software (SPSS Inc., Chicago, IL, United States). Normal distribution of continuous variables was tested using Shapiro–Wilk test. Normally distributed variables were shown as mean ± SD. Median (minimum – maximum) was used when the variables were not normally distributed or if they were ordinal variables. The differences of the means were compared using Student's t test. Mann–Whitney U test was applied for comparisons of the medians. For all possible multiple comparisons, the Bonferroni correction was applied in order to control Type I error. *P* value less than 0.025 was considered as statistically significant.

Results

We observed that the rats that received selenium appeared clinically healthier and more active than the control group throughout the study. Two animals in group 1 (saline) died of generalized infection on the 3rd day after inoculation. One blood sample from group 2 (selenium) could not be evaluated because of coagulation.

The biochemical parameters obtained on days 4 and 10 in group 1 (saline) and group 2 (selenium) are presented in Table 1.

TBARS Results

TBARS level in group 2 (selenium) on day 10 was lower than the level obtained on day 10 in group 1 (saline) although this difference was statistically insignificant (*P* = 0.073). On the other hand, TBARS level was lower on day 10 when compared to the day 4 value in group 2 (selenium). This difference was close to level of significance (*P* = 0.029).

Table 1 The results obtained on post-inoculation days 4 and 10 in the groups and the *P* values

Parameters	Group 1 (saline)	Group 2 (selenium)	<i>P</i> values ^a
GPx (U/g Hb) ^b			
4th day	21.6 (16.8–35.4)	20.5 (15.8–22.0)	0.536
10th day	14.5 (13.3–19.0)	21.2 (15.0–23.7)	0.017
<i>P</i> values ^c	<0.001	0.694	
SOD (U/g Hb) ^d			
4th day	5817.3 ± 1276.8	3977.4 ± 1106.5	0.010
10th day	4178.1 ± 891.7	4817.0 ± 972.3	0.224
<i>P</i> values ^c	0.017	0.145	
TBARS (μmol/l) ^b			
4th day	2.2 (1.8–3.5)	2.2 (1.8–2.6)	0.779
10th day	2.1 (1.8–3.1)	1.9 (1.7–2.1)	0.073
<i>P</i> values ^c	0.805	0.029	
Albumin (g/l) ^d			
4th day	22.3 ± 1.9	24.0 ± 2.4	0.152
10th day	28.0 ± 1.4	23.3 ± 1.4	<0.001
<i>P</i> values ^c	<0.001	0.500	
T-SH (μmol/l) ^d			
4th day	190.7 ± 44.0	222.2 ± 24.4	0.104
10th day	305.0 ± 54.0	235.8 ± 31.1	0.012
<i>P</i> values ^c	<0.001	0.361	

GPx glutathione peroxidase, SOD superoxide dismutase, TBARS thiobarbituric acid reactive substances, T-SH serum total sulphydryl groups

^a Comparisons between groups

^b Values are expressed median (min – max)

^c Comparisons between days. *P* < 0.025 statistically significant

^d Values are expressed mean ± SD

GPx Results

Although GPx enzyme activity did not show a significant difference between the groups on day 4 after bacterial inoculation, it significantly increased in group 2 (selenium) when compared to group 1 (saline) on day 10 (*P* = 0.017). When days 4 and 10 of the group 2 were compared, its level increased on day 10 (*P* = 0.694). Comparison of the levels on days 4 and 10 in group 1 (saline) showed that GPx enzyme activity significantly decreased on day 10 (*P* < 0.001).

SOD Results

On day 4, SOD activity was significantly higher in the group 1 (saline) when compared to day 10 value in same group and day 4 value in group 2 (selenium) (*P* = 0.017, *P* = 0.010, respectively). We found that SOD activity on day 10 in group 2 (selenium) was higher than the activity measured on day 4 in the same group and on day 10 in group 1 (saline) (*P* = 0.145, *P* = 0.224, respectively).

Albumin and Serum Total Sulphydryl Groups Results

In the saline group, albumin and serum total sulphydryl groups levels were significantly higher on day 10 when compared to the levels on day 4 in the same group (*P* < 0.001 for both). In addition, these parameters were significantly higher than those obtained on day 10 in group 2 (selenium) (*P* < 0.001 for albumin, *P* < 0.012 for serum total sulphydryl groups). Moreover, the level of serum total sulphydryl groups was higher on day 10 when compared to the value obtained on day 4 in group 2 (selenium) (*P* < 0.361).

Histopathological Evaluation

Histopathological evaluation did not reveal any statistically significant differences between the groups or the days studied. The inflammatory changes and epithelial integrity in the middle ear mucosa were similar in both groups (Figs. 1, 2). Histopathological data of the groups are presented in Table 2.

Discussion

The most important mechanism of tissue damage caused by free oxygen radicals is the production of lipid peroxides in the cell membrane [3]. Lipid peroxidation decreases both the fluidity and the barrier function of the membranes, resulting in disturbances in structural organization, enzymatic inhibition, and possible death of the cell. In addition, lipid peroxides are able to inhibit protein synthesis, block macrophage function, and alter chemotactic activity.

Fig. 1 Middle ear mucosa prepared from group 1 shows unimpaired epithelium with squamous metaplasia, marked submucosal edema and inflammatory cell infiltration (hematoxylin and eosin, original magnification ×200)

Fig. 2 Middle ear mucosa prepared from group 2 shows an epithelium with squamous metaplasia and minimal inflammatory cell infiltration (hematoxylin and eosin, original magnification $\times 200$)

Table 2 The results of the histopathological evaluation on days 4 and 10 after bacterial inoculation

Parameters	Group 1(saline)	Group 2 (selenium)	<i>P</i> values ^a
Inflammation			
4th day	2 (1–2)	2 (1–3)	0.463
10th day	2 (1–3)	2 (1–3)	0.535
<i>P</i> values ^b	0.383	0.694	
Epithelial integrity			
4th day	1 (1–3)	3 (0–3)	0.232
10th day	1 (0–3)	1 (0–3)	0.318
<i>P</i> values ^b	0.902	0.121	

^a Comparisons between group 1 (saline) and group 2 (selenium)

^b Comparisons between days 4 and 10 after inoculation. *P* < 0.025 statistically significant

Malondialdehyde, which is an end product of lipid peroxidation, is usually measured as TBARS in the studies [2]. The level of malondialdehyde is determined to consider it as a sign of tissue damage [13].

The most important enzymatic antioxidants are SOD and GPx [3, 9]. SOD catalyzes the dismutation of the highly reactive superoxide anion into H_2O_2 (first step of antioxidant pathway). H_2O_2 itself is not a free radical, but it undergoes auto-oxidation to form an extremely reactive hydroxyl radical. H_2O_2 is cleared from the environment by catalase or GPx through conversion to water (second step of the antioxidant pathway) [14]. GPx is an important enzyme in the elimination of H_2O_2 and lipid hydroperoxides [13]. SOD and GPx have been perceived as performing similar functions to cope with oxidative stress [15].

Selenium is incorporated into crucial antioxidant selenoenzymes such as the GPx [7]. There are available data focusing on positive relationship between GPx activity and

selenium [16, 17]. GPx displays different tissue distribution, and the activity and expression of GPx are regulated by the selenium status of tissues. Over-expressed GPx decreases reactive oxygen species levels [17].

SOD is a ubiquitous and important intracellular enzyme induced by high oxygen tension. It plays a key role in the protection of various tissues against oxidative damage [1]. The role of SOD in otitis media is not clearly understood [18]. Some studies reported that selenium did not induce any significant amelioration in total SOD activity [16]. Contrary to these studies, some studies suggested that selenium affected SOD activity [17]. Our data are in agreement with the latter investigations.

Albumin represents the major and predominant antioxidant in the plasma. A large proportion of total serum antioxidant properties can be attributed to albumin [19]. In addition, total sulphydryl groups are among the most important biological antioxidants found in the plasma. Sulphydryl groups represent the most expendable source of antioxidants in the plasma and are preferentially consumed when the plasma is exposed to free radicals [20]. We found that the levels of albumin and total sulphydryl groups significantly increased on day 10 in group 1 (saline) when compared to the levels on day 4, as well as the levels obtained on the same day in the group 2 (selenium). However, the level of total sulphydryl groups increased on day 10 in group 2 (selenium). It might be speculated that plasma levels of albumin and total sulphydryl groups were relatively higher in group 1 (saline) due to water deprivation induced by more severe inflammation.

It has been reported that oxygen radicals play a role in the pathogenesis of experimental otitis media [3, 18]. Lipid peroxidation may be the possible mechanism of cell injury in otitis media [2]. Parks et al. [2] reported that both lipid hydroperoxide and malondialdehyde were significantly elevated in the middle ear mucosa of guinea pigs infected with pneumococci when compared to normal middle ear mucosa. Takoudes and Haddad Jr. [5] reported that purulent effusions could elevate the lipid hydroperoxide levels more than other types of effusions. Oxygen radicals are over-produced, tissue damage and inflammation are enhanced [18].

Cemek et al. [1] investigated the antioxidant status in children with AOM and tonsillitis. They found that SOD and GPx activities were significantly elevated in the study groups when compared to healthy control group [1].

There is a paucity of data on the role of antioxidant agents in the prevention of oxidative tissue damage in AOM. As far as we know, Aladag et al. [9] found that vitamin A had a therapeutic effect in experimentally induced AOM. They reported that GPx and SOD activities increased and the malondialdehyde level decreased in rats receiving antibiotics and vitamin A, when compared to the

control rats. Moreover, it was reported that vitamin A supported epithelial integrity as shown by the histopathological evaluation [9]. Except for epithelial integrity, our results were in agreement with this study. We did not find significant histopathological differences between the groups.

We determined that erythrocyte TBARS level was lower on day 10 when compared to the level on day 4 in group 2 (selenium). However, total sulphydryl groups level, SOD and GPx enzyme activities were increased at the same time. When group 1 (saline) and group 2 (selenium) were compared on day 10, SOD and GPx enzyme activities in group 2 (selenium) were higher than group 1. This difference was statistically significant for GPx enzyme activity in group 2 (selenium). This result suggests that GPx enzyme activity increases with selenium treatment for 10 days, while this activity was lower following selenium supplementation for 4 days. The effect of selenium supplementation on GPx activity appeared after 10 days. The time courses for GPx and SOD in group 2 (selenium) were almost identical. The increase of GPx by selenium supplementation was also confirmed in the experimental AOM.

We have hypothesized that longer selenium supplementation would cause statistically significantly increased GPx between the days 4 and 10 in group 2 (selenium). Wu et al. found that SOD and GPx enzyme activities recovered to normal values following selenium supplementation for a month in the aorta of selenium deficient rats, and suggested that GPx might function as one of indicators for the selenium content [17]. Schnabel et al. [16] similarly reported that GPx enzyme activity increased after 6 weeks of 200 µmg selenium supplementation in patients with coronary artery disease when compared to placebo. These investigations also support our hypothesis.

Interestingly, although SOD and GPx enzyme activities increased on day 10 when compared to day 4 in group 2 (selenium), these values decreased in group 1 (saline) on the same days. We observed that selenium supplementation elevated not only GPx enzyme activity, but also SOD activity. The elevation of SOD activity was not statistically significant.

Conclusion

It has been reported that oxygen radicals play a role in the pathogenesis of AOM. Oxygen radical-mediated damage to the mucosa may delay recovery and lead to recurrent infections and chronic ear disease. In this study, we found that selenium supplementation for 10 days decreased TBARS levels and increased GPx and SOD values when compared to the control group in rats with experimental AOM. We believe that selenium supplementation may be

beneficial to prevent the clinical sequelae and recurrence of otitis media. Further clinical studies are needed to elucidate the effect of selenium on AOM. We hope that this piece of work would provide a new treatment philosophy in treatment of AOM.

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Conflict of interest The authors declare that they have no conflict of interest.

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