Protective Effect of Melatonin on Experimental Otitis Media with Effusion in Guinea Pigs

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Abstract. The aims of this study were: (a) to assess whether the increased oxidative stress in otitis media with effusion (OME) induced in guinea pigs by histamine injection into the middle ear cavity is reflected by lipid peroxidation in erythrocytes, plasma, and middle ear effusion fluid; (b) to survey the alterations of oxidant and antioxidant enzyme activities in experimental OME; and (c) to determine the effects of melatonin and methylprednisolone on this oxidative stress. Malondialdehyde (MDA) level, erythrocyte total (enzymatic plus non-enzymatic) superoxide scavenger activity (TSSA), non-enzymatic superoxide scavenger activity (NSSA), superoxide dismutase (SOD), catalase (CAT), and xanthine oxidase (XO) activities were measured in 4 groups of 7 guinea pigs at 3 hr after injection of 0.1 ml of histamine (or saline) into the middle ear. Group I was the control group, Group II was an experimental group with OME induced by histamine, Group III was a melatonin-pretreated OME group, and Group IV was a methylprednisolone-pretreated OME group. In erythrocyte, plasma, and middle ear effusion samples, MDA levels were significantly increased in guinea pigs with OME (Group II), compared to controls (Group I); erythrocyte TSSA and SOD activities were lower and erythrocyte XO activity was increased in guinea pigs with OME (Group II) compared to controls (Group I). No significant differences were found in erythrocyte NSSA and CAT activities. In Group III, pretreatment of guinea pigs with ip melatonin at 1 hr prior to histamine induction of OME decreased the erythrocyte, plasma, and effusion MDA levels, compared to Group II; erythrocyte TSSA and SOD activities were lower and erythrocyte XO activity was increased in guinea pigs with OME (Group II) compared to controls (Group I). No significant differences were found in erythrocyte NSSA and CAT activities. In Group III, pretreatment of guinea pigs with ip melatonin at 1 hr prior to histamine induction of OME increased the erythrocyte, plasma, and effusion MDA levels, compared to Group II; erythrocyte TSSA and SOD activities were lower and erythrocyte XO activity was increased in guinea pigs with OME (Group II) compared to controls (Group I). In Group IV, pretreatment of guinea pigs with ip methylprednisolone at 1 hr prior to histamine induction of OME decreased the plasma and effusion MDA levels and increased the erythrocyte TSSA and SOD activities, compared to Group II. These results suggest that reactive oxygen species (ROS) play a role in histamine-induced OME. Pretreatment with ip melatonin or methylprednisolone both decrease the ROS generated by experimental OME, but melatonin appears to be more effective than methylprednisolone. (received 11 March 2004; accepted 27 March 2004)

Keywords: malondialdehyde, otitis media with effusion, oxidative stress, melatonin, methylprednisolone

Introduction

Otitis media with effusion (OME) is a very common disease, especially in childhood and infancy. It is characterized by non-purulent fluid in the middle ear and fluctuating conductive hearing loss. OME is an inflammatory response of the middle ear caused by multiple factors such as viral or bacterial infection, eustachian tube dysfunction, or allergy. Inflammatory mediators seem to play a major role in OME. The pathogenesis of OME is not fully understood and is being intensively studied [1]. The therapeutic use of tympanostomy tubes has risks and financial costs, so effective medical treatments are needed. Development of new treatments for OME depends upon the elucidation of its pathogenesis.
Reactive oxygen species (ROS) have been implicated in a numerous diseases. Increased production or inadequate removal of ROS results in oxidative stress that causes metabolic disturbances and damages macromolecules. Lipid peroxidation involves the oxidative conversion of polyunsaturated fatty acids to products such as malondialdehyde (MDA), which is an end product of lipid peroxidation and serves as an index of oxidative damage. MDA is cytotoxic and has inhibitory actions on protective enzymes [2-5].

Melatonin is a direct antioxidant and a strong scavenger of ROS [5,6]. It enhances the expression of mRNA for superoxide dismutase and the activities of glutathione peroxidase (GSH-Px), glutathione reductase, and glucose-6-phosphate dehydrogenase (all of which are anti-oxidative enzymes), thereby augmenting its anti-oxidative effects. Methylprednisolone prevents lipid peroxidation, acting as a platelet activating factor (PAF) and macrophage inhibiting factor (MIF) inhibitor [6,7]. Although methylprednisolone is used in patients to treat OME, adverse effects may be encountered.

To our knowledge, there is no previous study that simultaneously investigates oxidative stress in erythrocytes, plasma, and effusion fluid in OME. Moreover, there is no previous trial of melatonin as an anti-oxidant in experimental OME. In this study, we investigated the role of ROS in pathogenesis of OME and tested melatonin and methylprednisolone for protective effects on oxidative stress of guinea pigs with experimental OME. In this study, we measured erythrocyte total (enzymatic plus non enzymatic) superoxide scavenger activity (TSSA), non-enzymatic superoxide scavenger activity (NSSA), superoxide dismutase (SOD), catalase (CAT), xanthine oxidase (XO) activities, and MDA levels, which serve as an index of lipid peroxidation.

Material and Methods

Animals. This study was performed on 28 guinea pigs weighing 600-700 g. All animals received humane care in compliance with the guidelines of Atatürk University Research Council. The guinea pigs were fed laboratory chow and water ad libitum. Absence of middle ear disease was established by otoscopy and tympanometry. Histamine dihydrochloride (Sigma Chemical Co, St Louis, MO) was dissolved in saline (1 mg/ml) and adjusted to pH 7.4 with NaOH. Melatonin (Sigma) was dissolved in ethanol and diluted with isotonic NaCl.

The animals were divided into 4 groups of 7 guinea pigs; group I was the control group, group II was the histamine-treated OME group, group III received combined treatment with histamine and melatonin, and group IV received combined treatment with histamine and methylprednisolone. Groups I and II received ip injection (0.5 ml) of isotonic NaCl. Group III received ip injection (0.5 ml) of melatonin (10 mg/kg). Group IV received ip injection (0.5 ml) of methylprednisolone (10 mg/kg). One hr later, the guinea pigs were anesthetized and the controls (group I) received an injection of isotonic NaCl solution (0.1 ml) via a 27-gauge needle through the right tympanic membrane into the middle ear cavity [8]. Groups II, III, and IV received an injection of histamine solution (0.1 ml) via a 27-gauge needle through the right tympanic membrane into the middle ear cavity. Three hr later [8] the animals were re-anesthetized and blood was withdrawn by cardiac puncture after thoracotomy. The blood samples were collected in Vacutainer tubes with potassium-EDTA as anticoagulant. Isotonic NaCl (0.1 ml) was injected into the right middle ears of the guinea pigs and then withdrawn.

Biochemical measurements. Erythrocytes were sedimented and then hemolysed by 50-fold dilution with deionized water. After centrifugation, the hemolysed supernatant was used for the analyses. Hemoglobin (Hb) concentrations of the samples were measured by a GEN-S hematology analyzer. The hemolysed samples were stored at -80°C prior to biochemical determinations. TSSA and NSSA assays, which are indicators of erythrocyte antioxidant capacity, were performed in the hemolysed samples before and after addition of trichloroacetic acid (TCA, 20%) according to Durak et al [9]. In the TSSA assay, a xanthine-xanthine oxidase complex produced superoxide radicals that reacted with nitroblue tetrazolium (NBT) to form a formazan compound. The TSSA activity was measured at 560 nm by detecting the inhibition of this reaction. By
using a blank study in which all reagents were present except supernatant sample and by determining the absorbance of sample and blank, the TSSA activity was calculated. The activity measurements were also made in TCA-treated fractions, prepared by treating part of the sample with 20% (w/v) TCA solution to remove all enzymes and centrifuging at 5,000 x g for 30 min. NSSA activity was assayed in the supernatant fraction. SOD activity was calculated as the difference between TSSA and NSSA [10]. CAT activity was measured in hemolysates according to Aebi [10]. Briefly, H2O2 was used as a substrate and the decrease in H2O2 concentration at 20°C in phosphate buffer was followed by spectrophotometry at 240 nm. One unit of CAT activity was defined as the amount of enzyme that degrades 1 µmol H2O2 per min. XO activity was determined by uric acid formation based on spectrophotometry at 293 nm, as described by Hashimoto [11]. The MDA assay involved spectrophotometry of the pink color complex formed by reaction of MDA with thiobarbituric acid [12]. The total thiobarbituric acid-reactive substances (TBARS) were expressed as MDA, using a molar extinction coefficient for MDA of 1.56 x 10^5 cm^-1•M^-1. Results were expressed as U/mg Hb for TSSA, NSSA, and SOD; as IU/mg Hb for CAT, as mIU/g Hb for XO, and as nmol/g Hb for MDA. One unit of TSSA, NSSAs and SOD was defined as the amount of enzyme protein that caused 50% inhibition of the NBT reduction rate.

**Statistics.** Results were expressed as mean ± SD. The data were analyzed by one-way ANOVA using the SPSS program (version 10.0). The LSD (least significant difference) multiple range test was used to compare mean values. A p value <0.05 was considered significant.

**Results**

As listed in Table 1, MDA levels in the samples of plasma, erythrocytes, and effusion fluid from guinea pigs in Group II (experimental OME, histamine-treated) were significantly higher than in controls (Group I). In Group III, melatonin prevented the increased levels of MDA and was highly effective for protection against lipid peroxidation. In plasma, erythrocytes, and effusion fluid, the MDA levels in Group III (melatonin group) were significantly lower than in Group II. Only in plasma was there statistically significant reduction of the MDA level of Group IV (methylprednisolone group) compared to Group II (experimental OME, histamine-treated). In plasma, erythrocytes, and effusion fluid, the MDA levels in Group III (melatonin group) were significantly lower than in Group II (experimental OME, histamine-treated). Only in plasma was there statistically significant reduction of the MDA level of Group IV (methylprednisolone group) compared to Group II (experimental OME, histamine group).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Units of measurement</th>
<th>Sample type</th>
<th>Group I (Controls, n = 7)</th>
<th>Group II (Experimental OME group, n = 7)</th>
<th>Group III (OME guinea pigs, melatonin-pretreated, n = 7)</th>
<th>Group IV (OME guinea pigs, methylprednisolone-pretreated, n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>nmol/g Hb</td>
<td>erythrocytes</td>
<td>2.1±0.7</td>
<td>3.3±0.7 b</td>
<td>2.07±0.5 e</td>
<td>3.02±0.5 a,b</td>
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<tr>
<td>MDA</td>
<td>nmol/ml</td>
<td>plasma</td>
<td>5.2±0.5</td>
<td>8.6±0.6 c</td>
<td>6.4±1.4 e</td>
<td>6.9±1.9 a,d</td>
</tr>
<tr>
<td>MDA</td>
<td>nmol/ml</td>
<td>effusion fluid</td>
<td>18.1±0.7</td>
<td>25.6±1.9 c</td>
<td>16.4±0.7 a,f</td>
<td>24.3±1.4 c,h</td>
</tr>
</tbody>
</table>

* a p<0.05, b p<0.01, c p<0.001 vs control group (Group I).
  d p<0.05, e p<0.01, f p<0.001 vs experimental OME group (Group II).
  g p<0.05, h p<0.001 vs melatonin-pretreated group (Group III).
prednisolone group), only TSSA and SOD activities were significantly increased, compared to Group II.

**Discussion**

ROS and lipid peroxides have been implicated in the pathogenesis of aging and in numerous diseases, eg, diabetes mellitus, cancer, and rheumatoid arthritis [13-19]. ROS have been implicated in the pathogenesis of OME and acute otitis media (AOM) [20-21]. To prevent damage caused by ROS, multiple defense systems, collectively termed anti-oxidants, are present in erythrocytes and in other organs and tissues. A combination of enzymatic and non-enzymatic defense mechanisms protects cells against oxidative injury. The scavenging of ROS is aided by anti-oxidant enzymes, such as SOD, GSH-Px, and CAT. Anti-oxidant substances such as vitamin E, melatonin, and glutathione protect cells against oxidative stress by non-enzymatic mechanisms [22].

Lipid peroxides (LPO) and MDA are important indicators of lipid peroxidation. Parks et al [23] reported that LPO is elevated in the middle ear fluid of guinea pigs infected with pneumococci. Doner et al [24] reported that MDA levels of erythrocytes and infected middle ear mucosa are significantly higher in animals with OME, compared to controls. Aktan et al [3], using the histamine-induced OME model, found that erythrocyte MDA level was significantly higher than the control group. In the present study, we found that erythrocyte, plasma and effusion MDA levels in the experimental OME group (Group II) were significantly higher than in the controls (Group I). The erythrocyte, plasma and effusion MDA levels in the melatonin-treated group (Group III) were significantly lower than in Group II. There was a statistically significant difference in the MDA levels of melatonin-treated group (Group III) and the methylprednisolone-treated group (Group IV) in erythrocyte and effusion fluid, but not in plasma. Thus, the present study showed that histamine-induced damage in OME is associated with an increase in MDA levels in guinea pigs and that pretreatment with melatonin, inhibits the increase of MDA level. MDA levels in melatonin-treated group were close to the normal MDA levels in TABLE 2. Superoxide dismutase (SOD), xanthine oxidase (XO), catalase (CAT), total superoxide scavenger activity (TSSA), and non-enzymatic superoxide scavenger activity (NSAA) (means ± SD) in erythrocyte samples from control guinea pigs (Group I) and those with histamine-induced otitis media with effusion (OME), Groups II, III, and IV).

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<th>Sample type</th>
<th>Group I (Controls, n =7)</th>
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<th>Group IV (OME guinea pigs, methylprednisolone-pretreated, n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>U/mg Hb</td>
<td>erythrocytes</td>
<td>678.5±26.4</td>
<td>473.8±55.7 c</td>
<td>601.3±35.9 b,f</td>
<td>538.4±62.49 c,d,g</td>
</tr>
<tr>
<td>XO</td>
<td>mIU/g Hb</td>
<td>erythrocytes</td>
<td>5.2±0.4</td>
<td>6.8±0.8 b</td>
<td>4.3±0.8 f</td>
<td>6.5±0.9 a,i</td>
</tr>
<tr>
<td>CAT</td>
<td>IU/mg Hb</td>
<td>erythrocytes</td>
<td>93.4±23.0</td>
<td>74.2±25.5</td>
<td>128.7±51.5 a,e</td>
<td>76.0±25.8 h</td>
</tr>
<tr>
<td>TSSA</td>
<td>U/mg Hb</td>
<td>erythrocytes</td>
<td>1152.3±35.1</td>
<td>891.6±65.7 c</td>
<td>1041.6±45.1 c,f</td>
<td>971.8±44.2 c,e</td>
</tr>
<tr>
<td>NSSA</td>
<td>U/mg Hb</td>
<td>erythrocytes</td>
<td>473.4±27.7</td>
<td>417.9±37.2 b</td>
<td>440.5±25.6</td>
<td>434.2±28.1 a</td>
</tr>
</tbody>
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\(a p<0.05, \ b p<0.01, \ c p<0.001 vs control group (Group I).\)
\(d p<0.05, \ e p<0.01, \ f p<0.001 vs experimental OME group (Group II).\)
\(g p<0.05, \ h p<0.01, \ i p<0.001 vs melatonin-pretreated group (Group III).\)

and Haddad [21] reported that LPO is elevated in the middle ear fluid of guinea pigs infected with pneumococci. Doner et al [24] reported that MDA levels of erythrocytes and infected middle ear mucosa are significantly higher in animals with OME, compared to controls. Aktan et al [3], using the histamine-induced OME model, found that erythrocyte MDA level was significantly higher than the control group. In the present study, we found that erythrocyte, plasma and effusion MDA levels in the experimental OME group (Group II) were significantly higher than in the controls (Group I). The erythrocyte, plasma and effusion MDA levels in the melatonin-treated group (Group III) were significantly lower than in Group II. There was a statistically significant difference in the MDA levels of melatonin-treated group (Group III) and the methylprednisolone-treated group (Group IV) in erythrocyte and effusion fluid, but not in plasma. Thus, the present study showed that histamine-induced damage in OME is associated with an increase in MDA levels in guinea pigs and that pretreatment with melatonin, inhibits the increase of MDA level. MDA levels in melatonin-treated group were close to the normal MDA levels in
controls. These results revealed that melatonin clearly decreased the lipid peroxidation in OME model in guinea pigs. This effect may be achieved by melatonin's activity as a scavenger for free radicals. There was also a decrease in the plasma MDA level in the methylprednisolone-treated group (Group IV), but no significant decreases in the effusion and erythrocyte MDA levels.

The role of SOD in otitis media is not clearly understood. Sigemi et al [20] reported that plasma SOD levels in patients with otitis media were significantly elevated. Aktan et al [3] reported that SOD and TSSA activities in guinea pigs with OME were higher than in controls. Parks et al [25] showed that the levels of superoxide dismutase were depleted in the infected versus normal middle ear mucosa of guinea pigs. They suggested that since superoxide dismutase specifically quenches aberrant superoxide radicals, a decrease in this enzyme activity might render tissue more susceptible to free radicals. In an investigation of oxidative stress in rats exposed to radiation, Buyukokuroglu et al [22] reported that lipid peroxidation was increased in erythrocytes, and SOD and GSH-Px activities were decreased. We found that erythrocyte SOD and TSSA activities were decreased in guinea pigs with OME. This is in agreement with Parks's and Buyukokuroglu's studies but it differs from Sigemi's and Aktan's studies. CAT plays a major role in the acquisition of tolerance oxidative stress in the adaptive response of cells [3,26]. XO functions in purine and the production of (O•⁻), which is potentially toxic to cellular structures [27]. Erythrocytes cannot synthesize XO de novo. Blood flow increases in inflamed regions and XO released by injured cells may possibly be absorbed by erythrocytes. This may account for the increased XO activity in erythrocytes of guinea pigs with OME [3]. Similarly, we found that erythrocyte XO activity in guinea pigs with experimental OME was significantly higher than in the controls. In the melatonin-treated group, there were significant increases in the SOD, CAT and TSSA levels, compared to the experimental OME group. There were significant increases in SOD and TSSA levels in the methylprednisolone group, compared to the experimental OME group. The increases of both SOD and TSSA in the melatonin-treated group were higher than in the methylpred-nisolone-treated group. While there was an obvious decrease of erythrocyte XO activity in the melatonin-treated group, there was not such a significant decrease in the methylprednisolone-treated group. In this study, we found a significant difference between the effects of melatonin and methylprednisolone treatments in terms of erythrocyte SOD, CAT, TSSA, and XO activities but not of NSSA activities.

In vitro studies of human peripheral blood mononuclear cells have shown that melatonin inhibits the production of tumour necrosis factor (TNF) [28]. Sacco et al [29] found that melatonin inhibits TNF production through its antioxidant activity. Lin et al [30] reported that TNF is involved in the pathogenesis of experimental OME. Thus, the present authors speculate that melatonin may be effective in OME treatment.

It is important to understand the role of free radicals in middle ear fluid in the pathogenesis of otitis media for two reasons. First, middle ear fluid bathes the mucosa, and free radical activity within the fluid may reflect free radical damage to the mucosa. Second, middle ear fluid is available in human studies, while the middle ear mucosa is not. Therefore, studies of middle ear fluid are relevant to investigations of the role of free radicals in the pathogenesis of human otitis media [21].

In conclusion, this study showed that erythrocyte, plasma and effusion MDA levels are increased in this model of experimental OME, suggesting that lipid peroxidation may be a cause of cell and tissue damage in OME. By decreasing lipid peroxidation and increasing antioxidant enzyme activities, melatonin reduced oxidative stress in OME. Therefore, melatonin might be a new treatment for OME and other otorhinolaryngological diseases associated with oxidative stress. The therapeutic effect of methylprednisolone was less than that of melatonin. Since melatonin has no known side effects, the authors envision that melatonin therapy may be preferable to methylprednisolone in otorhinolaryngological diseases associated with oxidative stress. However, further studies on this subject are obviously needed.

References


