The Journal of Laryngology & Otology
November 2000, Vol. **114,** pp. 832–836

Protective role of superoxide dismutase in rat eustachian tubal mucosa against acute otitis media induced by upper respiratory tract infection

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Abstract

Superoxide dismutase has been known to play a role as an anti-oxidative system against oxidative injury during acute inflammation. To investigate the role of superoxide dismutase in eustachian tubal mucosa during acute otitis media (AOM), an animal model was made. Sprague-Dawley rats were inoculated with *Streptococcus pneumoniae* through the nasal cavity following development of virus-induced upper respiratory infection. The animals were divided into three groups according to their tympanic cavity conditions following bacterial inoculation; inoculated animals with no resultant AOM (no-AOM), animals with resultant AOM (AOM) and animals with resolving otitis media (recovery). The changes of superoxide dismutase in each tubal mucosa were compared with that of the normal control using immunohistochemistry and immunoblotting methods. On Western blot, there were little changes of optical density and surface area in no-AOM (213.5 ± 22.4, 13.2 ± 0.8 mm^2) and recovery group (219.3 ± 18.7, 14.8 ± 0.7 mm^2) compared to the normal control (223.5 ± 26.2, 16.7 ± 0.4 mm^2). However, a marked decrease was found in the AOM model (167.6 ± 19.3, 6.5 ± 0.9 mm^2). These findings suggest that superoxide dismutase may play a role in protecting tubal mucosa from free radical injury during AOM.

Key words: Antioxidants; Eustachian Tube; Otitis Media; Respiratory Tract Infections

Introduction

The superoxide anions (O_2^-) are oxygen metabolites generated by leukocytes and other phagocytes whenever necessary.[1,2] These free oxygen radicals have protective role directed to the destruction of invading foreign cells.[3] On the other hand they also have adverse cytotoxic effects to the host cells when they are released and may contribute to the pathogenesis of some diseases. They cause tissue damage by chemical modification of cellular proteins, carbohydrates, nucleotides and lipids.[4,5] To abolish this host cell cytotoxic damage by inappropriately generated free radicals, superoxide anion should be catalyzed by a scavenging enzyme, superoxide dismutase.[6] Biochemical and cytochemical assays have revealed that superoxide dismutase is present in many organs and tissues as an anti-oxidative system against oxidative injury and is reported to be localized in the upper respiratory epithelium such as the bronchioles, columnar epithelium of the alveoli, and the olfactory epithelium.[7–9] Superoxide dismutase is also known to be present in middle-ear mucosa and protects mucosa against free radical injury. Parks *et al.*[10] reported the existence of superoxide dismutase in the guinea pig middle-ear mucosa, and that its amount was decreased in AOM following direct inoculation of bacteria into the bulla. Thereby they postulated that superoxide dismutase plays an epithelial protective role in AOM. However, the AOM model made by direct inoculation of bacteria into the middle-ear cavity does not correlate well with the known pathogenesis of AOM, which usually develops following upper respiratory infection (URI).[11] In URI, viruses inhibit the ciliary motion, ventiltaroy function, and leukocyte function of the tubal epithelia, hence inviting bacterial entrance into the middle-ear cavity and the development of AOM.[12] The tubal mucosa and respiratory epithelium share basic properties such as homeostasis of air-filled cavities and mucociliary clearance toward the pharynx. Therefore we believed that studies on this free radical scavenging enzyme in tubal mucosa may help towards a better understanding of the pathophysiology and oxygen-related antimicrobial system of AOM and that this observation supports the hypothesis that superoxide

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Accepted for publication: 22 June 2000.

https://doi.org/10.1258/0022215001904374 Published online by Cambridge University Press

dismutase deficiency could contribute to the frequent development of AOM.

This study was attempted to evaluate the existence of superoxide dismutase in tubal mucosa and compared quantitative changes of superoxide dismutase in mucosal extracts obtained from different periods of AOM induced after URI.

Materials and methods

Fifty healthy pathogen-free Sprague-Dawley rats weighing 200–300 **g** were purchased from the National Institute of Safety Research, Ministry of Health and Welfare (Korea) and used as experimental animals.

Experimental AOM model

After confirming that both ears of 40 rats were disease-free otoscopically, URI was induced by instillation of 0.2 ml (6×10^6 CFU) of influenza virus A Johannesburg/45/90 (Dept. Respiratory Virus, National Institute of Safety Research Ministry of Health and Welfare, Korea) into the nasal cavity. Animals having URI symptoms were selected based on daily evaluation of symptoms, such as nasal discharge and conjunctival infection. The right nasal cavity was packed with cotton wool soaked in 100 μl of a 10^8/ml suspension of non-typed, non-encapsulated *Streptococcus pneumoniae* the day after the appearance of URI symptoms. Then the animals were examined otoscopically on a daily basis for presence of AOM. Ordinary cultures were performed from the middle-ear contents and nasal secretions.

The same amount (0.2 ml) of sterile normal saline was put into the nasal cavities of 10 animals and were used as a control group.

All 40 animals developed URI after viral instillation. Among them, 25 showed AOM signs such as purulent otorrhoea, injection or bulging of the tympanic membrane following bacterial inoculation and 15 animals were free of AOM. Among the 25 AOM animals, nine animals recovered spontaneously after five days and the remaining 16 were sacrificed on the fifth day of AOM.

Immunohistochemistry

The experimental animals were sacrificed by intraperitoneal injection of urethane (25 per cent, w/v, 6–8 ml/kg, Sigma, USA). Perfusion fixation was performed through the heart with lactated Ringer's solution followed by four per cent paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, Sigma, USA). The bulla was removed en bloc and was postfixed in the same solution overnight. These specimens were decalcified for 24 hours in Plank-Rynchlo's solution (7 g aluminum chloride, 8.5 M hydrochloric acid, 5 ml formic acid, 100 ml distilled water) and cytoprotection was performed by immersion in 30 per cent sucrose solution (Sigma, USA) overnight. Serial frozen sections of 15 μm thickness were cut on a cryostat (Reichert-Jung, Germany), treated with 0.3 per cent (H_2O_2 for 15 minutes to inactivate endogenous peroxidase activity, and then washed for five minutes in 0.2 M phosphate buffered solution (PBS) three times. The sections were treated for 30 minutes in normal goat serum (Vector, USA) to block non-specific bindings, then treated overnight with 1:1,000 sheep anti-ZnCu superoxide dismutase antibody (Biodesign, USA) at 4°C. After washing with PBS, the frozen sections treated with primary antibody were incubated with secondary antibody (biotin-labelled anti-sheep anti-superoxide dismutase, Vector Lab, USA) for one hour and then stained immunohistochemically by the avidin-biotin-peroxidase complex method. The sections were treated with 0.05 per cent diaminobenzidine (DAB, Sigma, USA) for detection of immunoreactivity and then counterstained with alcian blue to differentiate goblet cells from epithelial cells. After dehydration and mounting, the sections were examined under the light microscope (Olympus BH-2, Japan). The immunostaining control was performed by omission of the primary antibodies and replacement with normal goat serum.

Western blot

The experimental animals were divided into four groups and the tubal mucosae obtained from them were studied: normal control (n = 10) inoculated animals with resultant otitis media (AOM, n = 16), inoculated animals with no resultant otitis media (no-AOM, n = 15), and inoculated animals with resolving otitis media (recovery, n = 9).

Post-fixation and decalcification of bulla, the tubal mucosae (50–100 mg) were carefully peeled out under a dissecting microscope. Specimens were placed in 1.5 ml TRI reagent (Molecular Research Center, USA), then homogenized (Wheaton, USA) and sonificated (Teitec, Japan). The proteins were precipitated through a treatment with chloroform-isopropanol and were obtained as a protein pellet after a treatment with ethanol. The pellet was resolved in one per cent sodium dodecyl sulphate (SDS, Sigma, USA) and protein concentration was determined by the method of Bradford.[13] The protein extracts (40 μg volume protein/well) obtained from each group were run simultaneously on 10 per cent SDS-PAGE gel using a vertical electrophoresis system (Novex, USA) to separate the proteins by their molecular weight. A standard marker protein and 0.1 μg pure superoxide dismutase (Sigma, USA) was also run simultaneously. The proteins were then transferred electrophoretically to a nitrocellulose membrane. The membrane was hybridized with the same antibody used in the immunohistochemical staining and detected with the Western-Light chemi-luminescent detection system (Tropix, USA). After developing and washing, all the blots were photographed and scanned. Using an NIH image analyzer (release -9), the surface area and optical density of blots were calculated and compared with each other statistically by the Student's *t*-test.

https://doi.org/10.1258/0022215001904374 Published online by Cambridge University Press

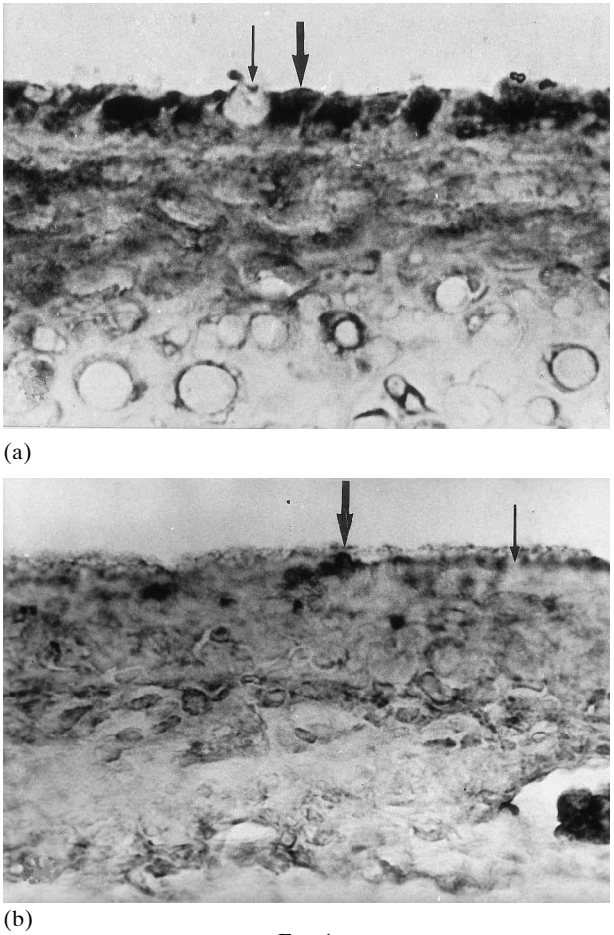
TABLE I
ORGANISMS CULTURED FROM EAR DISCHARGE

Organisms	*n*
Streptococcus pneumoniae	10
Escherichia coli	9
Enterobacter cloaca	7
Pseudomonas putida	6
Staphylococcus aureus	4
Stenotrophomonas maltophilia	4
No growth	11

Results

All 40 animals receiving influenza virus presented URI symptoms. Among them, 25 animals showed signs of AOM after nasal inoculation of *Streptococcus pneumoniae*. The same organisms were cultured from the middle-ear contents in 10 animals. *E. coli*, *Enterobacter cloaca* and *Staphylococcus aureus* were also cultured and these organisms were consistent with normal flora cultured from the nasal cavity (Table I).

Immunopositive reactions of superoxide dismutase were detected mainly in the epithelial cells of tubal mucosa in both normal and AOM tubal mucosa. However, the immunoreactivities were decreased and less evenly distributed in AOM epithelium than in normal mucosa. In AOM mucosa, the goblet cells were hypertrophied and increased in numbers and showed negative immunoreactivity. The submucosa was infiltrated with inflammatory cells, markedly oedematous and exhibited weak immunoreactivity as did the normal submucosa (Figure 1).



(a)

(b)

FIG. 1

Superoxide dismutase is diffusely stained (large arrow) in the cytoplasm of columnar cells of the normal eustachian tube (a), however the immunoreactivities are poorly observed in the infected mucosa (b). Note the goblet cells (small arrow) show no immunoreactivities (×400).

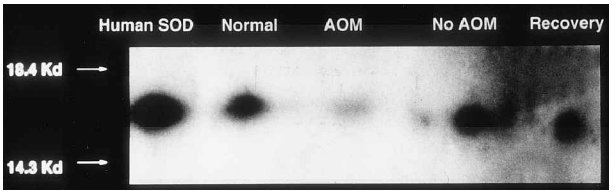


FIG. 2

Western blot of 40 μg of normal mucosa crude protein (second lane) demonstrating a denser band than 40 μg of infected mucosa crude protein in the third lane. The first lane contains pure superoxide dismutase. The bands of mucosae obtained from animals did not develop AOM (no-AOM, in fourth lane) and recovered form AOM (recovery, in fifth lane) show similar width and density to those of normal mucosa.

Western blotting demonstrated a single band at 16 kD in the epithelium of all mucosal extracts. The 40 μg protein band of the normal mucosa was darker and wider than the other bands when running simultaneously, whereas the 40 μg protein band from the AOM mucosa was barely visible (Figure 2). The optical density and surface area of bands calculated from the no-AOM and recovery groups were close to those of the normal control ($p<0.05$, Table II).

Discussion

Free radicals are generated by leukocytes and other phagocytes when the need arises for the destruction of invading pathogens and other foreign bodies.[14] Although they are necessary components of immune-mediated cytotoxicity, they may also leak into the surrounding host tissue and cause local tissue damage with lipid peroxidation[6] and this may contribute to the pathogenesis of some diseases.[15–18] Evidence that the middle-ear epithelium is damaged by free radicals has been provided by several authors.[19,20] Myeloperoxidase, also an oxidative by-product of activated neutrophils, has recently been shown to be elevated in the middle-ear effusion of an experimental animal method of *Streptococcus* otitis media.[21]

Our study attempted to confirm the presence and distribution of superoxide dismutase in the eustachian tube epithelium of rats and to compare the amount of superoxide dismutase with the mucosae in various pathologic conditions; inoculated animals with resultant otitis media, inoculated animals with no resultant otitis media and inoculated animals with resolving otitis media.

Among the 25 rats, which developed AOM, *Streptococcus pneumoniae* was the most commonly cultured organism (10/25). However, the other organisms turned out to be identical with those cultured from normal nasal cavity. These culture results were consistent with other reports[11,12] that

https://doi.org/10.1258/0022215001904374 Published online by Cambridge University Press

TABLE II
OPTICAL DENSITIES AND SURFACE AREAS OF THE FOUR DIFFERENT MUCOSAL EXTRACTS RUNNING WITH 40 μg OF PROTEINS

	Normal	AOM*	no-AOM	Recovery
Optical density	223.5 ± 26.2	167.6 ± 19.3	213.5 ± 22.4	219.3 ± 18.7
Surface area (mm^2)	16.7 ± 0.4	6.5 ± 0.9	13.2 ± 0.8	14.8 ± 0.7

AOM = inoculated animal with resultant acute otitis media; no-AOM = inoculated animal with no resultant otitis media; recovery= inoculated animal with resolving otitis media.
*The optical density and surface area of the AOM was significantly decreased ($p<0.05$) compared with those of the other groups.

the previous viral infection facilitates bacterial entry into the eustachian tube by damaging ciliary movement. From the fact that the normal flora were cultured from the middle ear with AOM, a presumption can be made that the viral infection has induced pathogenic conversion of normal flora of the nasal cavity.

As shown in the results of immunohistochemical staining and immunoblotting, the immunoreactivities and amounts of superoxide dismutase were markedly decreased in the tubal mucosa of AOM, whereas in resistant and resolved mucosae the immunoreactivities and total amounts were close to those of the normal control. The decrease of superoxide dismutase in the infected mucosa may implicate that more superoxide dismutases were exhausted to scavenge the free radicals released during the inflammatory process. However, in this study we could not clarify whether the deficiency of superoxide dismutase in AOM animals was related to genetic deficiency or a temporary reduction due to marked consumption.

Ovesen and Borglum[22] reported in their study comparing superoxide dismutase levels in the middle-ear fluid from children undergoing ventilating tube insertion for OME, that a more successful outcome of the disease could be expected in children who had detectable concentrations of superoxide dismutase than in those who had no detectable superoxide dismutase. They hypothesized that the difference in the superoxide dismutase concentrations in the middle-ear effusions was due to the presence or absence of free radical-producing phagocytes.

The epithelial cells are capable of increasing their generation of superoxide dismutase if they are exposed to high oxygen concentrations during an ischaemic state, influx of excessive amounts of free radical may cause tissue damage. The oxygen tension in the normal middle ear is a quarter to a third of that in the atmosphere. Obstruction of the eustachian tube due to inflammation will further decrease the oxygen tension in the middle ear, that will initiate the release of phagocyte-stimulated cytotoxic oxygen-derived free radicals into the extracellular space, ultimately causing tissue damage.

By considering the fact that the amount of superoxide dismutase was decreased by only a small amount in the resistant and resolved mucosae, we presume that this remaining superoxide dismutase has contributed to the maintenance and restoration of tubal patency to permit oxygen entrance. Therefore, we can postulate that a significant decrease in the amount of superoxide dismutase can make the host tissue more susceptible to free radical injury.

Acknowledgements

We would like to express our gratitude to Dr Jee-Hee Kim in the Department of Respiratory Virus, National Institute of Safety Research, Ministry of Health and Welfare for providing experimental animals and viruses. This research was supported by a grant from the Medical Science Research Center, Korea University.

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Dr H. K. Suh takes responsibility for the integrity of the content of the paper.
This research was supported by a grant from the Medical Science Research Centre, Korea University.
