

Reduction in Superoxide Dismutase Expression in the Epithelial Mucosa of Eosinophilic Chronic Rhinosinusitis with Nasal Polyps

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Key Words

Chronic rhinosinusitis · Eosinophils · Nasal polyp · Superoxide dismutase

Abstract

Background: Eosinophils generate large amounts of oxidant species. The eosinophil-dominant type of chronic rhinosinusitis (CRS) with nasal polyps is related to more extensive disease and a decreased likelihood of surgical success. Superoxide dismutase (SOD) is the first-line and only antioxidant enzyme that converts superoxide to hydrogen peroxide.

Methods: The patients with CRS with nasal polyps were divided into eosinophilic and noneosinophilic groups. The expression of three isoforms of SOD, intracellular copper-zinc SOD (CuZnSOD), mitochondrial manganese SOD (MnSOD) and extracellular SOD (ECSOD), were examined by enzyme activity assay, immunohistochemistry and quantitative real-time RT-PCR sampled by laser capture microdissection. **Results:** SOD activity in the eosinophilic and noneosinophilic groups was significantly reduced compared to that of the control groups. Immunostaining of both CuZnSOD and MnSOD in the eosinophilic group was significantly decreased compared with that in the noneosinophilic and control groups. CuZnSOD mRNA of the eosinophilic group was significantly decreased compared with that of the control

group, whereas MnSOD mRNA in the eosinophilic group was significantly decreased compared with that in the noneosinophilic and control groups. Neither immunoreactivity nor mRNA of ECSOD was different among the three groups. The degree of epithelial damage and disease severity were inversely correlated with CuZnSOD and MnSOD immunoreactivity. **Conclusions:** The reduction in SOD activity and the downregulation of the SOD message are suggested to be related to eosinophil recruitment and epithelial damage of CRS with nasal polyps.

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Introduction

Chronic rhinosinusitis (CRS) is defined as persistent inflammation of the nasal and paranasal cavity mucosa lasting ≥ 3 months [1]. Based on an epidemiological study, CRS affects 12.5% of the population in the United States [2]. The prevalence and medical costs of CRS are increasing and have become important social issues.

The histomorphological pattern of CRS with nasal polyps is characterized by the predominance of eosinophils and mixed mononuclear cells, and the relative paucity of neutrophils. CRS with nasal polyps associated with mucosal infiltration of eosinophils may be regarded as eo-

sinophilic CRS due to the distinctive feature of tissue eosinophilia [3], which is more refractory to surgical treatment and is frequently associated with bronchial asthma. The histopathological features of asthma, including tissue eosinophils, epithelial damage and basement membrane thickening of the lower airways, are also observed in sinonasal specimens with CRS [4]. Epithelial damage in asthma is thought to be a consequence of ongoing inflammation, bronchoconstriction and host susceptibility factors [5]. We found a significant correlation between basement membrane thickness and eosinophilic infiltration into both the epithelial and subepithelial layers of the nasal polyps of CRS [6].

Eosinophils have some cytotoxic mediators such as eosinophilic peroxidase, which can be produced during oxidative stress, resulting in severe damage to the epithelia [7]. The role of oxidative stress in the pathogenesis of nasal polyps has been recognized recently. High levels of malondialdehyde, one of the metabolites of free radical-mediated lipid peroxidation, were observed in nasal polyps [8, 9]. Moreover, free radical levels in nasal polyps were associated with nasal polyp severity [10, 11]. The overproduction of free radicals and decrements in the antioxidant system can both cause tissue injury. We previously demonstrated that heme oxygenase-1, a cytoprotective enzyme against oxidant, is reduced in epithelial cells in CRS with eosinophilic infiltration [12].

Superoxide dismutase (SOD) is the most important antioxidant enzyme system against free radicals and the decomposition of superoxide radicals into H_2O_2 in the human lung. SODs are composed of three isoforms of intracellular copper-zinc SOD (CuZnSOD), mitochondrial manganese SOD (MnSOD) and extracellular SOD (ECSOD). Previous studies regarding the measurement of SOD activities in nasal polyps are still controversial. Karlidag et al. [13] and Cannady et al. [14] reported that the enzyme activity of SOD was lower in the nasal polyps than in the normal inferior turbinate. In contrast, SOD activity has been reported to be unchanged in the nasal polyps compared with the normal middle turbinate. Cheng et al. [15] reported that the expression profiles of SOD isoforms could be determined by reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay and Western blotting, and found that the expression of both CuZnSOD and ECSOD was higher in polyp tissue. However, previous reports did not focus on eosinophilic infiltration into nasal polyps. Herein, we examined the enzymatic activity, localization of the protein and expression of the message of SOD from the viewpoint of eosinophilic inflammation. Fur-

thermore, we analyzed the relationship between SOD expression and epithelial damage. This study will provide a helpful key to determining whether SOD would be a protective factor for eosinophilic CRS and a new tool for ameliorating eosinophilic CRS.

Patients and Methods

Patients

Cases of CRS with nasal polyps were diagnosed based on the criteria of the European position paper [16]. The disease extent on computed tomographic (CT) scans was categorized according to Lund and Mackay [17]. None of the patients were treated with systemic corticosteroids or other immune-modulating drugs for at least 1 month before surgery. All patients gave their written informed consent, and the study was approved by the Ethics Committee of the Juntendo University Faculty of Medicine.

Sampling of Tissue Specimens

Tissue specimens were obtained from nasal polyps located in the middle meatus. Control samples were obtained from normal mucosal membranes of the sphenoid sinus removed during surgery for pituitary adenoma. A part of each sample was fixed in 10% formalin embedded in paraffin wax, processed routinely and stained with hematoxylin-eosin. The patients were classified into 2 groups according to our recent report [18]. The eosinophilic and noneosinophilic groups were defined as eosinophil counts of the nasal polyp of more and less than 100/microscopic field (magnification: $\times 400$) using 3 fields, respectively. Eosinophils were quantified in the foci of the densest cellular infiltrate. The length of epithelial sloughing was expressed as a percentage of the total epithelial length. The residual sample tissue of the nasal polyps was used for the enzyme activity assay, immunohistochemistry and quantitative real-time RT-PCR. However, due to the amount of the samples or the conditions of preparation, these three series of experiments could not be performed using samples from all the patients.

SOD Activity

The tissue samples were homogenized using a bead-based homogenizer. After centrifugation at 78,000 g for 60 min at 4°C, the upper clear layer was taken. The total protein concentration was measured by a Pierce 660-nm protein assay reagent (Thermo Scientific Pierce, Japan). SOD activity was assayed using an SOD assay kit (WST; Dojindo Laboratories, Japan) according to the manufacturer's instructions.

Immunohistochemistry

Nasal polyps were fixed in 10% formalin, embedded in paraffin wax, processed routinely and then prepared as routine semithin sections (3.5 μm). The primary antibodies were CuZnSOD 1:200, MnSOD 1:500 and ECSOD 1:2,000. Rabbit anti-human CuZnSOD and MnSOD was obtained from Stressgen Bioreagents (USA). Rabbit anti-human ECSOD was obtained from Abnova (USA). The sections were stained by a Ventana iVIEW DAB detection kit using a Ventana (USA) automated stainer (NexES IHC). The sections treated with control rabbit and mouse IgG1 served as negative controls.

Table 1. Clinical and epidemiologic profiles of the study groups

	Eosinophilic (n = 31)	Noneosinophilic (n = 28)	Control (n = 20)	p value
Age, years	49.5±12.3	49.8±18.8	60.9±17.0	
Gender, male/female	21/10	18/10	10/10	
Symptomatic score	11.8±2.43	8.91±2.21		<0.01
CT score	19.7±5.08	12.7±4.61		<0.001
Peripheral blood eosinophils, n/μl	640.1±303.9	143.0±118.2		<0.001
Tissue eosinophils, n	325.7±198.7	23.2±24.4		<0.001

Laser Capture Microdissection

Frozen sections, microdissected (8 μm) on a laser capture microdissection system (Leica DMLA, Leica Microsystems, Germany), were immediately fixed in 99.5% ethanol. At least 500 epithelial cells were microdissected from cryosections.

Quantitative Real-Time RT-PCR

Total epithelial RNA was extracted from the microdissected samples using an RNeasy micro kit (Qiagen, Germany). cDNA was synthesized from total epithelial RNA with the PrimerScript II first-strand cDNA synthesis kit (TAKARA Bio, Japan). Real-time RT-PCR was performed for CuZnSOD, MnSOD and ECSOD using a 7500 fast real-time PCR system (Applied Biosystems, USA) and TaqMan gene expression assays (assay identification Nos. Hs00533490_m1, Hs00167309_m1 and Hs00162090_m1) according to the manufacturer's specifications. Data analysis was done on Applied Biosystems 2.0.1 software. All results were normalized to β-actin to compensate for differences in the amount of cDNA (assay identification No. Hs99999903_m1).

Statistical Analyses

Data were expressed as means ± SD. Student's t test was used for age, gender, symptomatic score, CT score, and eosinophils in the serum and tissue. One-way ANOVA was used for SOD activity, immunohistochemistry and quantitative real-time RT-PCR. Pearson's correlation coefficient was applied to assess the relationships among the rate of SOD-positive epithelial cells, CT score and degree of epithelial damage. Statistical analysis was performed using State Mate IV for Windows. * $p < 0.05$ was considered as statistically significant.

Results

Clinical Background

Clinical and epidemiologic data are shown in table 1. There were no significant differences in age or gender among the eosinophilic, noneosinophilic and control groups. Four clinical parameters such as symptomatic score, CT score, blood eosinophils and tissue eosinophils were significantly higher in the eosinophilic group than in the noneosinophilic group.

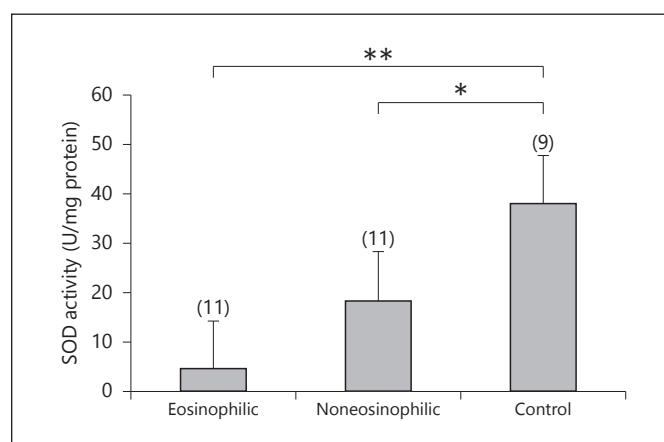


Fig. 1. Decreased activity of SOD in eosinophilic and noneosinophilic CRS with nasal polyps compared with the control group. Numbers of samples are given in parentheses; * $p < 0.05$; ** $p < 0.01$.

SOD Activity

SOD activity was examined in the eosinophilic and noneosinophilic CRS with nasal polyps as well as control sinus mucosa (fig. 1). SOD activity of the eosinophilic and noneosinophilic groups was significantly lower than that of the control group. The eosinophilic group tended to show reduced SOD activity compared with the noneosinophilic group, but no significant difference was found.

Immunohistochemical Study

The labeling localization and distribution of the three SOD isoforms were determined using immunohistochemistry (fig. 2). CuZnSOD immunoreactivity was mainly observed in the epithelium, fibroblasts and vascular endothelium in normal sinus mucosa. In the epithelium, CuZnSOD was expressed most prominently in ciliated epithelial cells. MnSOD was moderately to highly expressed in epithelial cells and vascular endothelium.

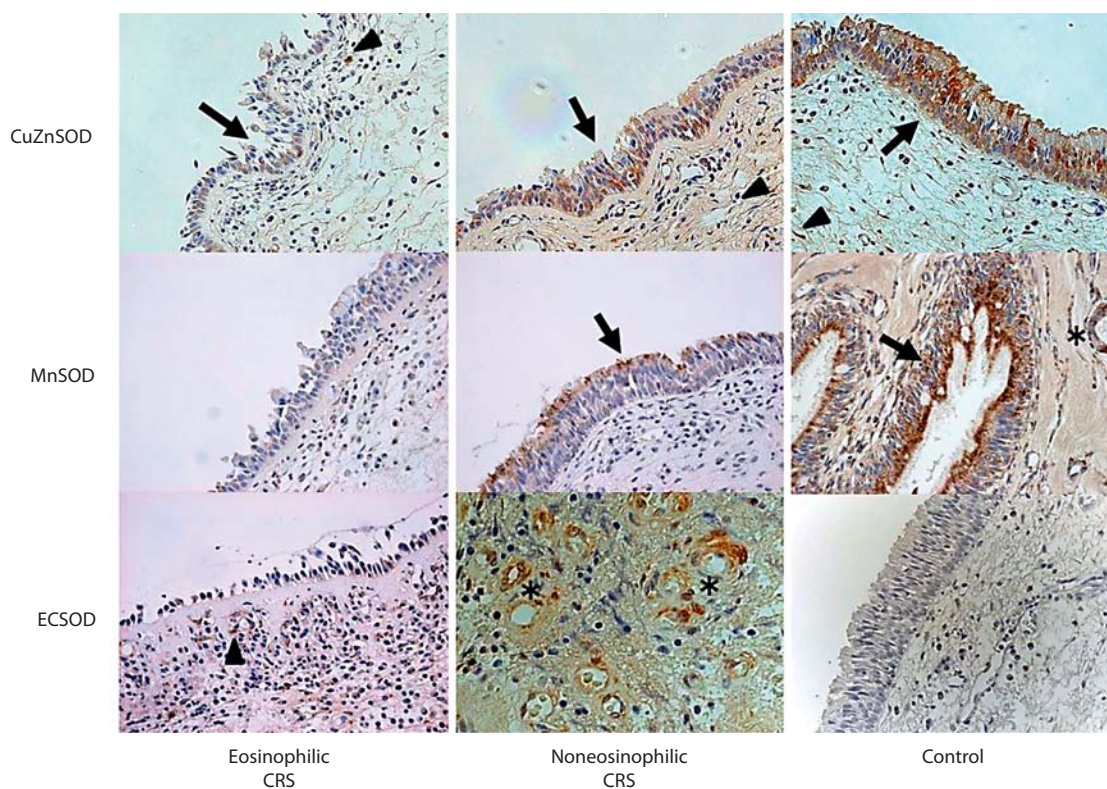


Fig. 2. Histochemical localization and distribution of SOD in nasal polyps. Arrows indicate epithelial cells; arrow heads fibroblast, and asterisks indicate vascular endothelium.

On the other hand, weak expression of ECSOD was observed in fibroblasts and vascular endothelium as well as epithelial cells. Immunoreactivity for SOD isoforms was semiquantitatively assessed in epithelial cells (fig. 2, 3). A significant reduction in immunopositivity for CuZnSOD and MnSOD in eosinophilic CRS was recognized in comparison with the noneosinophilic and control groups (fig. 3a, b). In contrast, there was no difference in ECSOD among the three groups (fig. 3c).

Real-Time Quantitative PCR

In order to obtain selective sampling of the cells enriched with target molecules, we applied laser capture microdissection to histological sections of nasal polyps. Information on the mRNA expression of SOD in epithelial cells by real-time quantitative PCR is shown in figure 4. The CuZnSOD mRNA levels in eosinophilic CRS were significantly decreased compared with those of the controls (fig. 4a). Although not statistically significant, the CuZnSOD mRNA levels in the eosinophilic group tended to be lower than those of the noneosinophilic group. A significant downregulation in the MnSOD message was

seen in the eosinophilic group compared with those of the other two groups (fig. 4b). ECSOD mRNA in epithelial cells showed no difference among the three groups (fig. 4c).

Relevance of SOD Expression to Disease Severity and Epithelial Damage

CT scores were significantly and inversely correlated with the reduction in SOD activity in CRS with nasal polyps. The correlation between immunopositivity for CuZnSOD and MnSOD with the degree of epithelial damage was evaluated using all samples from CRS with nasal polyps. The epithelial damage had a significantly inverse correlation with the rate of both CuZnSOD- and MnSOD-positive epithelial cells (fig. 5).

Discussion

The present study first and clearly demonstrated that eosinophilic infiltration and resultant tissue damage in CRS with nasal polyps is characterized by a disruption

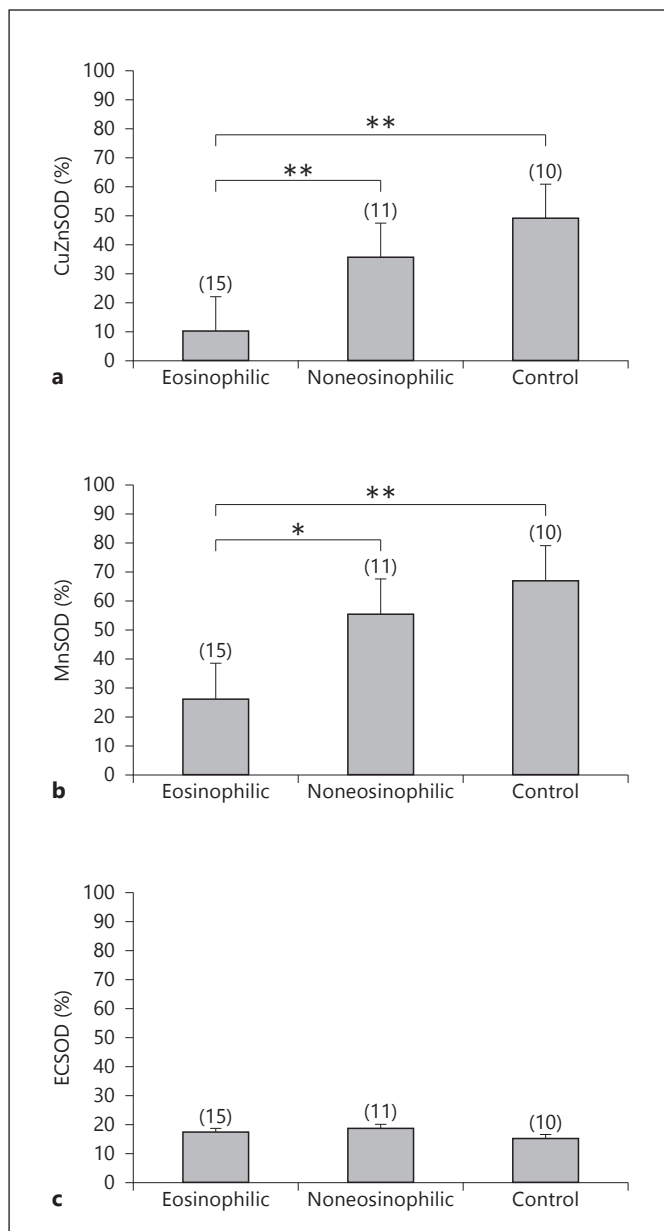


Fig. 3. The positive rate for SOD immunoreactivity in the epithelium. Numbers of samples are given in parentheses; * $p < 0.05$; ** $p < 0.01$.

of the SOD scavenger system for oxidative stress. The results can be summarized as follows: (i) SOD activity of the CRS groups was significantly decreased compared with that of the controls; (ii) immunostaining of both CuZnSOD and MnSOD of the eosinophilic group was significantly decreased compared with that of the noneosinophilic and control groups; (iii) CuZnSOD mRNA of the eosinophilic group was significantly decreased

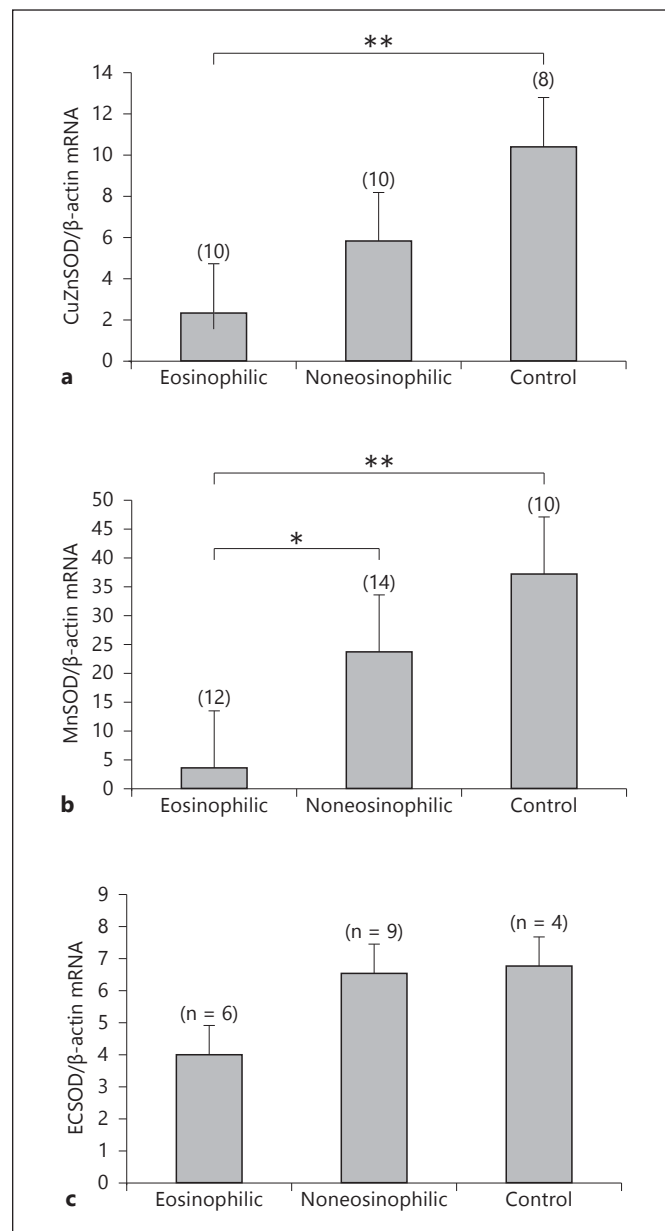


Fig. 4. Measurement of SOD mRNA by real-time RT-PCR. Numbers of samples are given in parentheses; * $p < 0.05$; ** $p < 0.01$.

compared with that of the control group; (iv) MnSOD mRNA of the eosinophilic group was significantly decreased compared with those of the noneosinophilic and control groups; (v) neither immunoreactivity nor mRNA of ECSOD differed among the three groups, and (vi) the degree of epithelial damage and CT scores inversely correlated with CuZnSOD and MnSOD immunoreactivity.

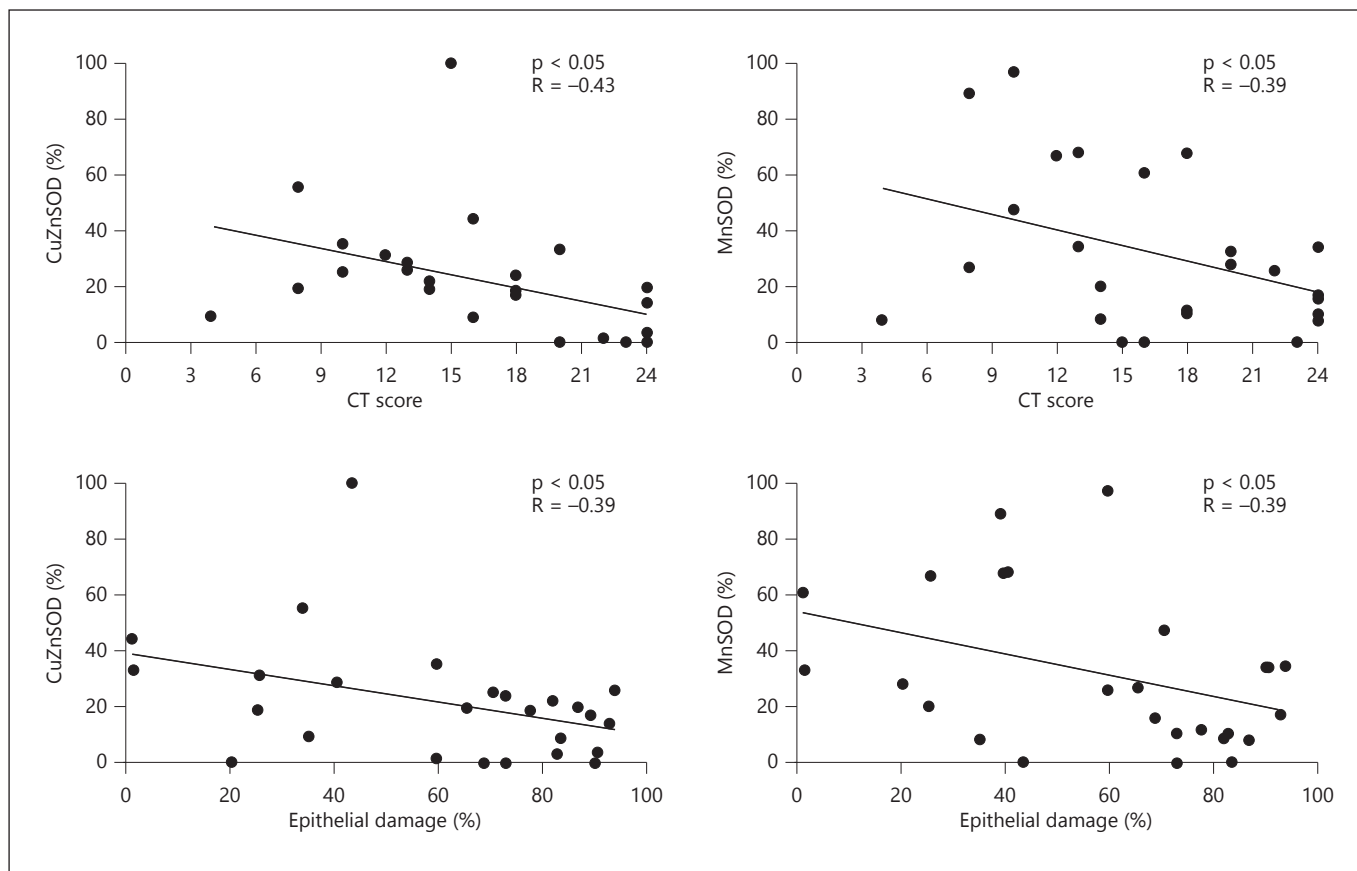


Fig. 5. Relevance of CT scores and epithelial damage to SOD activity.

A category of eosinophil-based inflammation in CRS with nasal polyps is thought to be related to more extensive disease and a decreased likelihood of surgical success [19]. Activated eosinophils can generate superoxides (O_2^-) via the membrane-associated NADPH-dependent complex. Subsequently, dismutation of O_2^- gives hydrogen peroxide (H_2O_2). O_2^- and H_2O_2 per se are moderate oxidants; however, both species are critical for the formation of potent cytotoxic radicals in biological systems that possess eosinophils. Eosinophil peroxidase catalyzes the oxidation of halides by H_2O_2 to form hypohalous acids. Hypohalous acid production is important in the host defense against infectious agents, which is one the exacerbating factors of eosinophilic CRS with nasal polyps [20]. However, during this reaction, the hydroxyl radical, which is a powerful and indiscriminate oxidant, is also produced. The oxidative injury caused by eosinophils can be substantial because the cells possess several times greater capacity to generate O_2^- and H_2O_2 than neutrophils [21], and the content of eosinophil peroxidase in

eosinophils is 2–4 times higher than the amount of myeloperoxidase in neutrophils [22]. Thus, eosinophils infiltrating into the sinonasal mucosa are suggested to play a potential role in the pathogenesis of CRS with nasal polyps, particularly during exacerbations.

SOD is one of the essential first-line antioxidant enzymes, since catalytically converted O_2^- produced by eosinophils forms H_2O_2 . The present finding that CuZnSOD expression in the nasal polyps characterized by a large amount of infiltrating eosinophils was reduced is well consistent with what is found in asthmatic patients [23]. However, the expressed forms of MnSOD are different between the upper and lower respiratory airways. The former show decreased expression of MnSOD in eosinophilic CRS compared with controls, whereas in the latter no difference was detected between asthmatic and control subjects [23].

The homeostasis of cellular functions during oxidative stress depends on the rapid induction of protective antioxidant enzymes. Naturally occurring antioxidants exist

to protect cells and tissue from the continuous production of reactive oxygen species. However, high levels of reactive species overwhelm the antioxidant defenses, resulting in loss of barrier function, increased permeability or destruction of epithelial cells [24]. In asthma, the lower SOD activity is a consequence of the increased oxidative stress, and thus is a sensitive marker of the airway redox status and asthma severity [25]. Similarly, the reduction in SOD activity in CRS with nasal polyps may influence the extent and severity of disease related to tissue eosinophilia.

The decreased SOD activity and protein may be brought about by (i) depletion of the enzyme due to consumption, (ii) inactivation of the enzyme, and (iii) effects of transcriptional regulation or gene mutation. It has been shown that oxidative modification/inactivation of MnSOD was present in asthmatic airway epithelial cells [26], indicating functional impairment in SOD activity due to oxidative processes. Although the promoter of CuZnSOD has been shown to be induced by oxidants and

metal ions [27], most studies have shown that there was no upregulation of lung CuZnSOD induced by cytokines or oxidant stress in vivo or in vitro [28, 29]. In contrast, oxidants and cytokines generally cause MnSOD induction [30]. No experimental data have been reported in the upper airway, so far. We first demonstrated that mRNA of both CuZnSOD and MnSOD was downregulated in the eosinophilic CRS with nasal polyps. Our study suggests that there are differences in the expression patterns of SOD between the upper and lower airways. The impairment in oxidant-decomposing mechanisms in eosinophilic CRS with nasal polyps might involve genetic and environmental factors, which will require further research for clarification.

In conclusion, a disrupted balance between the oxidant generation mediated by recruited eosinophils and the oxidant defense system of SOD is suggested to play an important role in the formation and exacerbation of CRS with nasal polyps.

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