Evaluation of oxidative DNA damage and antioxidant defense in patients with nasal polyps

Malgorzata Mrowicka¹, Hanna Zielinska-Blizniewska², Jaroslaw Milonski², Jurek Olszewski², Ireneusz Majsterek¹

¹Department of Clinical Chemistry and Biochemistry, Medical University of Lodz, Hallera 1, Poland,

²Department of Otolaryngology and Laryngological Oncology, Medical University of Lodz, Zeromskiego 113, Poland

Objectives: The presence of inflammatory cells indicates the development of epithelial cell injury in nasal polyposis (NP) and the potential for production of high levels of reactive oxygen and nitrogen species. The aim of our study was to clarify the role of oxidative stress and antioxidant status in the deterioration accompanying NP.

Methods: Twenty patients (11 men) aged 47.2 ± 17.0 years with nasal polyps were included in the study. Twenty healthy subjects (7 men) aged 48.2 ± 15.3 years formed the control group. The erythrocyte activities of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and plasma nitric oxide (NO) concentrations were measured. An alkaline comet assay was used to determine the extent of blood lymphocyte DNA damage of oxidized purines as glycosylformamidoglycosylase (Fpg) sites, and oxidized pyrimidines as endonuclease III (Nth) sites.

Results: A significant increase of NO ($P < 0.05$) and non-significant decreases of SOD ($P > 0.05$), CAT ($P > 0.05$), and GPx ($P > 0.05$) were seen in NP patients compared to healthy controls. The level of blood lymphocyte oxidative DNA damage in NP patients was significantly higher compared to the control group ($P = 0.01$).

Discussion: The blood lymphocyte DNA damage level increased in patients with NP. Elevated DNA damage may be related to overproduction of reactive oxygen and nitrogen species and/or decreased antioxidant protection.

Keywords: Nasal polyps, DNA damage, Comet assay, Oxidative stress, Antioxidant enzymes

Introduction

The most severe form of chronic rhinosinusitis is nasal polyposis (NP). A diversity of inflammatory cells may be found inside the polypoid tissue or in the mucus. Eosinophils in the systemic circulation are activated to migrate from the peripheral vascular system into the nasal tissue in nasal polyps. The eosinophilic infiltration in the epithelial layer could play a role in the process of mucosal remodeling of chronic rhinosinusitis with nasal polyps.¹ Histological studies have implicated different inflammatory cells such as eosinophils, neutrophils, macrophages, lymphocytes, plasma cells, mast cells, and immune mediators such as various cytokines and chemokines, transforming growth factor-beta, and adhesion molecules in the

development of nasal polyps. The authors noted that the eosinophil plays a major role in the pathogenesis of chronic rhinosinusitis with or without nasal polyps.²

The toxic effects of the presence of inflammatory cells such as eosinophils, neutrophils, lymphocytes, or myofibroblasts may be associated with oxidative stress. The inflammatory response can lead to the recruitment of activated leukocytes, which may, in turn, give rise to a respiratory burst – an increased uptake of oxygen that causes the release of high quantities of reactive oxygen species (ROS) with subsequent DNA damage production.³ ROS are also produced by peroxisomal beta-oxidation of fatty acids, microsomal cytochrome P450 metabolism of xenobiotic compounds, arginine metabolism, and tissue-specific enzymes. Phagocytes recruited to the site of chronic infection abundantly generate reactive oxidants, such as nitric oxide (NO) and hypochlorite, to inactivate

Correspondence to: Malgorzata Mrowicka, Department of Clinical Chemistry and Biochemistry, Medical University of Lodz, Hallera 1, 90-647 Lodz, Poland. Email: malgorzata.mrowicka@umed.lodz.pl

the bacteria. These ROS can cause damage to host cells and DNA, and this chronic damage can contribute to the development of a tumor. Moreover, the reaction between NO and superoxide anions generates peroxynitrite, one of the most reactive oxidizing species, and the reaction between NO and protein thiols also can regulate protein function by altering conformation.⁴

Excessive levels of ROS or reactive nitrogen species (RNS) can damage cellular macromolecules, leading to lipid peroxidation and DNA, RNA, protein, and carbohydrate modification. Not all ROS damage DNA directly. For example, superoxide anions and hydrogen peroxide may initiate DNA damage by interaction with transition metal ion chelates. The hydroxyl radical is the damaging species with many different products formed from $\cdot\text{OH}$ attack on the bases in DNA. The principal oxidative DNA damage products include 8-hydroxyadenine, 8-hydroxyguanine, thymine glycol, and ring-opened lesions: 4,6-diamino-5-formamidopyrimidine or 2,6-diamino-4-hydroxy-5-formamidopyrimidine.³

The human body has a complex antioxidant defense system that includes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). These enzymes prevent the initiation of numerous free-radical chain reactions. Previous reports suggest that activities of antioxidant enzymes may be lower in patients with nasal polyps than in controls. Low SOD⁵ and GPx activities^{6,7} and an excessive level of plasma NO⁵ in NP patients compared to healthy subjects (HS) have been identified.

The aim of our study was to clarify the role of oxidative stress and antioxidant status accompanying NP. Therefore, we compared activities of erythrocytic antioxidant enzymes, SOD, CAT and GPx, and the concentration of plasma NO as oxidative stress markers, and levels of DNA damage in peripheral blood lymphocytes in patients with nasal polyps and HS.

Material and methods

In the present study, 40 patients were included. The patients were divided into two groups: Group I – 20 patients (mean age $\pm$ SD 47.2 $\pm$ 17.0 years) with chronic sinusitis and nasal polyps, Group II – 20 patients (mean age $\pm$ SD 48.2 $\pm$ 15.3 years) without chronic sinusitis and nasal polyps, nor allergy and bronchial asthma (control group). The patients were hospitalized in the Department of Otolaryngology and Laryngological Oncology Medical University of Lodz and qualified for functional endoscopic sinus surgery (FESS) due to chronic sinusitis and nasal polyps. The study methodology included subjective examination (medical interview), objective otorhinolaryngological examination, imaging tests (CT of

paranasal sinuses in three projections: frontal, sagittal, and lateral), patients referred for FESS due to EPOS 2005: adults, signs, and symptoms such as disorders of nasal patency, nasal discharge, olfactory disorders, headaches lasting longer than 8–12 weeks, concurrent changes in CT performed after a 4-week preservative therapy without any relapse of acute sinusitis. The patients did not undergo steroid therapy before endoscopic procedures. The polyps were evaluated on the four-point Johanson scale, which was also used by Modrzynski and Zawisza as it described the severity level of polyps. In the scale used by the authors, Stages 1 and 2 correspond with Stage 1 of the Johanson scale. In frontal rhinoscopy, the severity level in the studied patients was Stage II – 10 patients, Stage III – 10 patients.

The severity level of polyps in paranasal sinuses in CT (Kennedy criteria) in the studied patients was Stage II – 2 patients, Stage III – 15 patients, and Stage IV – 3 patients.

Blood count showed eosinophilia in 16 patients, each of them with diagnosed with chronic allergic rhinitis. Local eosinophilia was not observed.

About 5 ml EDTA anti-coagulated peripheral blood was taken in disposable syringes from patients in the Department of Otolaryngology and Laryngological Oncology, Medical University of Lodz. Prior to the specimen's collection, each patient was presented with the aim of the procedure, and informed consent obtained. The study was approved by the Bioethics Committee of Medical University in Lodz (RNN/40/09/KB 6.01.2009).

Blood sample preparation

Erythrocytes were separated from blood plasma by centrifugation (10 minutes, 710 $\times$ g) at 4°C and washed three times with 0.9% w/v NaCl before examination.

Peripheral blood lymphocytes from healthy donors and patients were isolated by centrifugation (15 minutes, 280 $\times$ g) in a density gradient of histopaque-1077 (Sigma, Poznan, Poland). Lymphocytes accounted for about 92% of leukocytes in the obtained cell suspensions as judged by the characteristic shape of their nucleus.

Hemoglobin assay

Hemoglobin (Hb) concentration in erythrocyte hemolysates was estimated at 540 nm using a spectrometer (UV/VIS Spectrometer Lambda 14P, Perkin Elmer, Überlingen, Germany) after conversion into cyanmethemoglobin with Drabkin reagent (Aqua-Med, Łódź, Poland).⁸ Hb concentration was needed to calculate the activity of antioxidant enzymes.

Catalase activity

CAT activity in erythrocytes was determined according to spectrophotometric procedure by Beers and Sizer⁹ and calculated as Bergmeyer units (BU/g Hb). CAT activity was measured at 25°C by recording H₂O₂ decomposition at 240 nm with a spectrometer (UV/VIS Spectrometer Lambda 14P, Perkin Elmer, USA). One BU of CAT activity is defined as the amount of enzyme decomposing 1 g of H₂O₂ per minute.

Glutathione peroxidase activity

GPx activity in erythrocytes was measured according to the spectrophotometric procedure of Little and O'Brien¹⁰ and presented as enzymatic units (U/g Hb). The difference in the rate of GPx reaction with glutathione and kumen in the sample was used for its activity determination by absorbance measurement with a spectrometer (UV/VIS Spectrometer Lambda 14P, Perkin Elmer, USA) at 412 nm. One unit of GPx activity is calculated as an amount of enzyme that causes a 10% decrease of the level of reduced glutathione within 1 minute at 25°C, pH 7.0.

Superoxide dismutase activity

SOD activity in erythrocytes was measured according to the procedure of Misra and Fridovich¹¹ and expressed in adrenaline units (U/g Hb/100 ml). The activity was determined at 37°C by the absorbance increase at 480 nm with a spectrometer (UV/VIS Spectrometer Lambda 14P, Perkin Elmer, USA) following the auto-oxidation of adrenaline inhibited by SOD. One unit of SOD activity is defined as the amount of enzyme inhibiting the adrenaline auto-oxidation by 50%.

Nitric oxide assay

Plasma NO concentration was evaluated by the Marletta¹² indirect method, which determines a relationship between concentrations of nitrites and nitrates and was expressed as $\mu\text{mol/l}$.

Comet assay

DNA damage of single- and double-strand breaks levels was measured by the single-cell gel electrophoresis method (comet assay), which was performed under alkaline conditions according to the procedure of Singh *et al.*,¹³ with modifications by Klaude *et al.*¹⁴ The final concentration of lymphocytes was adjusted to $1\text{--}3 \times 10^5$ cells/ml by adding RPMI-1640 medium (Sigma, Munich, Germany) to the single-cell suspension. Endogenous and exogenous DNA damage after lymphocyte incubation for 10 minutes at 4°C with 10 and 20 μM hydrogen peroxide at 4°C in growth medium was investigated. A suspension of cells in 0.75% low melting point agarose dissolved in phosphate-buffered saline was spread onto microscope

slides (Superior Marienfeld, Lauda-Königshofen, Germany) precoated with 0.5% w/v normal-melting agarose. The cells were then lysed for 1 hour at 4°C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% v/v Triton X-100, and 10 mM Tris, pH 10. After lysis, the slides were placed in an electrophoresis unit, and the DNA was allowed to unwind for 40 minutes in the electrophoretic solution consisting of 300 mM NaOH and 1 mM ethylenediaminetetraacetic acid (EDTA), pH > 13. Electrophoresis was conducted at 4°C (the temperature of the running buffer did not exceed 12°C) for 30 minutes at the electric field strength of 0.73 V/cm (30 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 $\mu\text{g/ml}$ DAPI (4',6-diamidino-2-phenylindole), and covered with cover slips. To prevent additional DNA damage, all the steps described previously were conducted under dimmed light or in the dark.

Endonuclease assay

Glycosyl-formamido-glycosylase (Fpg) and endonuclease III (Nth) induce DNA breaks at sites of oxidized purines and pyrimidines, respectively, which can be detected by the alkaline comet assay. Endogenous and exogenous oxidative DNA lesions after lymphocyte incubation for 10 minutes at 4°C with 10 μM hydrogen peroxide at 4°C in growth medium were investigated. According to the standard method of comet assay, slides after lysis were washed three times in an Fpg/Nth buffer comprising 40 mM HEPES-KOH, 0.1 mM KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0, and the agarose was covered with 25 ml of buffer or Nth as well as Fpg at 1 mg/ml in buffer, sealed with a cover glass, and incubated for 30 minutes at 37°C. Further steps were made as described previously.

Comet analysis

The specimens were observed at 200 $\times$ magnification in an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to a COHU 4910 video camera (Cohu, San Diego, CA, USA) equipped with a UV-1 filter block (an excitation filter of 359 nm and a barrier filter of 461 nm) and connected to a personal computer-based image analysis system Lucia-Comet v. 4.51 (Laboratory Imaging, Prague, Czech Republic). Two parallel tests with aliquots of the same sample were performed for a total of 100 cells, and the mean percentage of DNA in the comet tail (% tail-DNA) was calculated.

Statistical analysis

The values of the comet assay in this study were calculated for three separate experiments from each analyzed patient or control sample. The value from comet assay was expressed as mean percentage of DNA damage $\pm$ SEM. The activity of enzymes as well as the plasma

Figure 1 Mean activity of superoxide dismutase (SOD) calculated in (A) adrenaline units (U/g Hb/100 ml), (B) catalase (CAT) calculated in Bergmeyer units (BU/g Hb), (C) glutathione peroxidase calculated in enzymatic units (U/g Hb) and (D) nitric oxide concentration calculated as (mmol/l) measured in patients with nasal polyps (NP) and healthy subjects (HS). Each data point represents the mean $\pm$ SD. * $P < 0.05$ as compared with healthy subjects.

NO was expressed as mean value $\pm$ SD. Blinded replicate samples were used for quality control. If no significant differences between variations were found, as assessed by the Snedecor–Fisher test, the differences between means were evaluated by applying the Student's *t*-test. Otherwise, the Cochran–Cox test was used. The data were analyzed using the STATISTICA (StatSoft, Tulsa, OK, USA) statistical package.

Results

Antioxidant status

The antioxidant status between the study groups was assessed by measuring erythrocyte SOD, CAT, GPx activities, and plasma NO concentrations.

The SOD activity (U/gHb/100 ml) was not statistically significantly different in NP patients in comparison to controls (2671.1 ± 470.8 vs. 2747.2 ± 387.9 ; $P > 0.05$) (Fig. 1A). The activity of CAT (BU/gHb) was not significantly decreased in NP patients compared to control subjects (7.3 ± 1.0 vs. 7.9 ± 0.94 ; $P > 0.05$) (Fig. 1B). The GPx activity (U/gHb) in the examined group of patients with NP was non-statistically different compared to controls (53.4 ± 13.2 vs. 60.4 ± 9.16 ; $P > 0.05$) (Fig. 1C).

The NO level ($\mu\text{mol/l}$) was significantly increased in NP patients in comparison to healthy controls (3.5 ± 1.0 vs. 2.5 ± 0.9 ; $P < 0.05$) (Fig. 1D).

The mean levels of plasma NO, and SOD, CAT, GPx activities in the erythrocytes obtained from the patients are shown in Table 1.

Oxidative DNA damage

The mean percentage of DNA in the comet tail of lymphocytes from patients with NP and HS is shown in

Fig. 2. The mean level of endogenous and oxidative DNA damage was significantly higher in lymphocytes of NP patients compared to the control group ($P = 0.01$). The important endogenous processes leading to significant DNA damage are likely to be oxidation, methylation, deamination, and depurination. One of the major endogenous causes of DNA damage is that produced by ROS during aerobic metabolism. The level of DNA damage in the presence of Fpg and Nth glycosylase was higher in lymphocytes of NP patients (Fpg $P = 0.272$; Nth $P = 0.01$) compared to endogenous DNA damage in HS.

We found that the mean level of 10 μM hydrogen peroxide-induced DNA damage was higher in lymphocytes of NP patients than in HS ($P < 0.001$) and there was markedly higher endogenous and exogenous DNA damage after lymphocyte incubation with 20 μM hydrogen peroxide in the NP study group ($P < 0.001$) compared to controls. Oxidative DNA damage evoked by hydrogen peroxide (10 μM) revealed after treatment only with Nth enzymes was significantly higher in NP patients (Fpg $P = 0.084$; Nth $P = 0.005$) than in HS.

Table 1 Levels of plasma NO, and SOD, CAT, GPx activities in the erythrocytes, of patients with NP and HS

Variable	Patients with nasal polyps <i>n</i> = 20	Control group <i>n</i> = 20
NO ($\mu\text{mol/l}$)	$3.5 \pm 1.0^*$	2.5 ± 0.9
SOD (U/gHb/100 ml)	2671.1 ± 470.8	2747.2 ± 387.9
CAT (BU/gHb)	7.3 ± 1.0	7.9 ± 0.94
GPx (U/gHb)	53.4 ± 13.2	60.4 ± 9.16

* $P < 0.05$ as compared with the control group.

Figure 2 Mean percentage of DNA in the alkaline comet tail of lymphocytes from healthy subjects (HS) and NP patients (NP). The cells were (A) not treated or (B) treated with 10 mM H₂O₂ and (C) 20 mM H₂O₂ for 10 min at 4 °C with subsequent treatment with formamidopyrimidine-DNA glycosylase (Fpg) or endonuclease III (Nth) at 1 mg/mL. The black bars (control) present DNA strand breaks and alkaline labile sites, the light grey bars (Fpg) present oxidized purines and the dark grey bars (Nth) present oxidized pyrimidines. Each data point represents the mean $\pm$ SEM. *** P < 0.001, * P < 0.05 as compared with healthy subjects.

The mean level of 20 μ M hydrogen peroxide-induced DNA damage revealed after treatment with endonucleases Fpg and Nth was significantly increased in NP patients (Fpg P < 0.001; Nth P < 0.001) in comparison to healthy controls.

Discussion

The cause of NP is multifactorial. The most important factor in the development of NP is inflammation. The ROS can be generated through extracellular and intracellular effects of cytokines and inflammatory mediators. The main source of ROS *in vivo* is aerobic respiration but polymorphonuclear leukocytes also generate ROS as part of the inflammatory response. The presence of inflammatory cells such as eosinophils, neutrophils, lymphocytes, and macrophages indicate the development of epithelial cell injury in NP and, potentially, high levels of ROS and RNS.¹⁵ Oxidative mechanisms have been demonstrated to possess a potential role in the development of many chronic inflammation diseases, such as asthma, allergy, chronic rhinosinusitis, tonsillitis, rheumatoid arthritis, and ulcerative colitis.^{1,16–21}

The biologic effects of free-oxygen radicals are controlled *in vivo* by enzymatic and non-enzymatic

defense mechanisms such as SOD, which catalyzes dismutation of superoxide anions to hydrogen peroxide; CAT, which converts hydrogen peroxide into molecular oxygen and water; and seleno-dependent GPx, which catalyzes the degradation of H₂O₂ and hydroperoxides originating from unsaturated fatty acids at the expense of reduced glutathione.¹⁹ The oxidant–antioxidant balance was studied in plasma and erythrocytes of blood patients since this is considered to be an important pool of antioxidant defense systems in the body.

Our studies showed no significantly change in the activities of primary antioxidant enzymes such as erythrocyte SOD, CAT, and GPx, but significantly increased plasma NO concentrations in patients with NP compared to healthy subject without NP. Karlidag *et al.* reported that the activity of SOD in NP tissue and erythrocytes was lower in patients with NP than in a control group. Other authors documented that the expressions of SOD isoenzymes, SOD1 (CuZnSOD), and SOD3 (extracellular superoxide dismutase), were higher in polyp tissues in comparison to control tissues from subjects without NP.²² Taysi *et al.* also demonstrated abnormalities in lipid peroxidation and antioxidant enzymes in patients

with NP. However, these authors reported that CAT activity was higher in NP patients.

Dagli *et al.* showed that non-enzymatic antioxidants and antioxidant enzyme activities decreased, and the malondialdehyde (MDA) level, as marker of oxidative stress, increased in NP patients in comparison to the control group. Although acute severe asthma is known to increase oxidative stress and is associated with changes in antioxidant profiles. Jacobson *et al.*²³ documented increased MDA levels but no differences in plasma selenium levels or GPx activity in patients with asthma.

In addition to SOD, CAT and GPx activities we estimated the level of NO in plasma of patients with NP and control subjects. There were significant differences between NP patients compared to healthy controls. The end-products of NO metabolism are nitrite and nitrate. These biomarkers of nitro oxidation are relatively unreactive, tend to accumulate in biological fluids that generate NO, and are widely measured as a quantitative index of NO synthesis. NO and the end-products of metabolism may induce covalent modifications in proteins and other biomolecules and promote tissue injury. The interaction of RNS and ROS with DNA can result in damage to all four bases and to the deoxyribose molecule and also in an impairment of the genetic material in the cell nucleus. Moreover, the mechanism of development of NP may be associated with inflammation in which prolonged activation of inflammatory cells like macrophages, eosinophils, neutrophils, lymphocytes, and immune mediators such as transforming growth factor-beta, chemokines/cytokines, adhesion molecules, may lead to ROS and RNS production and endogenous oxidative stress that can damage DNA and contribute to the pathophysiology of NP. Our results suggest that inflammation may have induced the increased plasma NO concentration in patients with NP. Our results for plasma NO concentrations in NP patients are in agreement with those obtained in earlier studies.⁵ Moreover, Takeno *et al.* demonstrated that nasal epithelial cells of patients with allergic rhinitis produced higher levels of NO through the concomitant expression of different NO synthase isoforms. Giannessi *et al.*²⁴ reported that peroxynitrite plays pivotal role in the pathophysiology of nasal polyps. The authors speculated that peroxynitrite could influence the influx of eosinophils in the nasal mucosa. It was suggested that activated eosinophils up-regulated NO through inducible isoform of nitric oxide synthase expression, and have an intense respiratory burst, with formation of ROS such as superoxide anion, hydrogen peroxide, or hydroxyl radical. The inducible isoform of NO synthase is primarily expressed in epithelial cells and macrophages and is up-regulated by microbes and cytokines, permitting

NO production to increase markedly in response to infection and in inflammatory states. Another study revealed that chronic sinusitis is accompanied by a marker for peroxynitrite (3-nitotyrosine) and is probably caused by the action of eosinophil peroxidase, rather than by NO levels.¹⁵ Çekin *et al.*²⁵ observed decreased NO production in patient samples from nasal polyps-affected tissues compared to normal tissues. Kang *et al.*²⁶ also reported that the increased peroxynitrite may result from increased inducible NO synthase expression but is not related to decreased levels of CuZnSOD and MnSOD in patients with NP.

ROS generate a large number of oxidative modifications in DNA, including strand breaks and base oxidations. The molecular basis for increased risk is thought to be twofold: generation by inflammatory macrophages of ROS leads to DNA damage in the surrounding epithelial cells and enhanced proliferative signals mediated by cytokines released by inflammatory cells increases the number of cells at risk for mutation.

A number of inflammatory mediators including ROS cause tissue degeneration, particularly via vascular permeability and plasma exudation, thus initiating the inflammatory process. Increased ROS production may lead to an increase in oxidative damage to DNA, but also may decrease the expression/activity of the enzymes that prevent the persistence of such damage. Therefore, we wanted to assess the degree of DNA damage in patients with NP compared to healthy controls.

In the present study, we have shown that patients with NP have increased plasma NO levels and DNA damage in peripheral blood lymphocytes compared to HS. The lymphocytes of NP patients had significantly higher levels of basal and oxidative DNA damage. These findings may be a result of excessive production of inflammatory cytokines, which induces enzymes such as NADPH oxidase, NO synthase, eosinophil peroxidase, or myeloperoxidase. These enzymes, which produce ROS, may contribute to increased cancer risk in relation to oxidative DNA damage in inflammation. Moreover, we also reported that lymphocytes of NP patients were more susceptible to DNA damage induced by hydrogen peroxide in comparison to healthy controls.

In conclusion, our observations suggest that there is a relationship between the increased generation of oxidative stress and DNA damage seen in patients with nasal polyps, as compared to those seen in HS. The present study revealed that DNA damage may be related to enhanced RNS production, which may be contribute to pathogenesis of the disease in NP patients. In our opinion, further research is needed to explain the mechanism of this association and whether it is direct or indirect.

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Disclaimer statements

Contributors All authors contributed equally.

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