Allergy 2006: 61: 1290–1298

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DOI: 10.1111/j.1398-9995.2006.01228.x

Original article

Oxidative stress in nonallergic nasal polyps associated with bronchial hyperresponsiveness[1]

Background: Nasal polyposis (NP) is a chronic inflammatory disease of upper airway with unknown etiology. NP is frequently associated with asthma; the interaction between these comorbidities remains interesting. Oxidative stress has been implicated in the pathophysiology of NP and asthma. The aim of this study is to investigate the significance of oxidative stress in sinonasal microenvironments by evaluating its association with clinopathological parameters and its impacts on the pathogenesis of bronchial hyperresponsiveness (BHR) in NP.
Methods: Polyp biopsy specimens were obtained from 20 nonallergic patients; control mucosas were obtained from 20 volunteers. The levels of free radicals in the tissues and in blood were determined by a sensitive chemiluminescence (CL) method. NP patients were substratified into three subgroups, NP without BHR, NP with asymptomatic BHR, and NP with BHR and asthma by the results of provocative testing. Four histological characteristics of NP, inflammatory cells, eosinophil infiltration, edema and fibrosis were estimated and applied to correlate with the tissue-CL.
Results: The mean CL level in polyp-tissues, but not in blood, was higher than in the control specimens. In NP patients, tissue-CL was associated with endoscopy score; high tissue-CL levels were positively correlated with the abundance of inflammatory cells and eosinophils. Tissue-CL and endoscopy score were associated with BHR/asthma phenotype.
Conclusion: These results suggest an important role for oxidative stress in the pathophysiology of NP and a causal relation between oxidative stress and inflammatory cells, especially the eosinophils. Free radical levels in polyp-tissues associated with NP severity and with BHR/asthma phenotype in nonallergic NP patients.

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Key words: bronchial hyperresponsiveness; chemiluminescence; free radicals; nasal polyps; oxidative stress.

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Accepted for publication 6 July 2006

Nasal polyposis (NP) is an inflammatory chronic disease of the upper respiratory tract with unknown etiology. Inflammation is one of the most important factors involved in the development of NP (1–5). Clinical as well as experimental studies suggest that nasal polyp formation and growth may be initiated and perpetuated by both infectious and noninfectious inflammation (3). In several studies, inflammatory cells, including eosinophils, neutrophils, macrophages, and lymphocytes were found in high concentrations in NP tissue compared with normal tissue (1–4). Many studies have pointed out that histopathologic abnormities of NP, such as edema and inflammation, were closely related to the infiltration of inflammatory cells (2, 3, 6). Inflammatory cells produce free radicals (FRs) by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase during phagocytosis, which is the major source of biological reactive oxygen species (ROS) (7). The release of these toxic molecules into extracellular space directly contributes to inflammation (7). Over production of FRs and decrements in the antioxidant system can both cause tissue injury, a condition called oxidative stress (7). Free radical-mediated damage has been implicated in the pathogenesis of several disease processes, including arthritis, atherosclerosis, cataract, NP as well as asthma (7–12).

The role of oxidative stress in the pathogenesis of NP has been recognized only recently (8–11). Several studies reported that lipid peroxidation of cell membranes resulting from ROS may cause tissue damage and cell death (7). Malondialdehyde (MDA), one of the metabolites of FR-mediated lipid peroxidation, can be used as an indicator of FR levels. Doğru et al. and Dagli et al. demonstrated high levels of MDA in NP tissue, which supports the existence of cell injury (8, 9). Using nitrotyrosine immunohistochemical staining, Ruffoli et al. suggested that there exists progressive epithelium

[1]Part of this work was presented at 10th Asia–Oceania ORL Head and Neck Congress, Kuala Lumpur, Malaysia, 22–26 February 2004.

injury in polyp-tissues caused by peroxynitrite (11). In addition to the toxic effect, this reaction might decrease the concentration of NO, thereby diminishing its bioavailability (12).

Nasal polyposis is frequently associated with asthma (13). Both NP and asthma share similar pathophysiology; a causal relation has been speculated between NP and asthma (6, 14). Furthermore, in NP, asymptomatic bronchial hyperresponsiveness (BHR) is thought to precede the development of asthma. A revolutionary relation has been suggested between three stages of BHR in NP, isolated NP without BHR, NP with asymptomatic BHR, and NP with BHR and asthma (14, 15). Therefore, NP provides an ideal opportunity with its more easily accessible sources to explore the interaction between upper and lower airway diseases. The influence of oxidative stress has been well recognized in the pathophysiology of inflammatory respiratory diseases, especially in the lower respiratory tract (12). However, there are relatively fewer data collected from NP, a common chronic inflammatory disease of the upper airway. We hypothesized that a high concentration of inflammatory cells found in NP contributes to over production of FRs, which play a role not only in the presentation of NP, but also in the susceptibility of lower airway hyper-responsiveness. To investigate the oxidative stress in sinonasal microenvironments, we studied the generation of oxygen FRs in whole blood and in nasal tissues from nonallergic NP patients and control subjects using a sensitive chemiluminescence (CL) method (10, 16, 17). Besides, the significance of oxidative stress was examined by evaluating its association with clinopathological parameters and its impacts on the pathogenesis of BHR in NP.

Methods

Patients

This study involved 40 patients in the Department of Otolaryngology, China Medical University Hospital (CMUH); all patients underwent endoscopy, sinus CT scan, and spirometric measurements. Polyp specimens were taken from 20 patients with NP during endoscopic sinus surgery (ESS). Control mucosal specimens (mucosal punctates from lateral lamella of the middle turbinate) were acquired from 20 patients with no history of NP or asthma, when they underwent ESS for correction of anatomic variation, such as concha bullosa (18). All tissue specimens were harvested by the same investigator (Y.K.C.) using a 3 mm biopsy punch (Blakesley 'throughcut' forceps) in a same fashion. The diagnosis of NP was based on anterior rhinoscopy, endoscopy, and CT imaging. Nasal polyposis was defined as the presence of endoscopically visible bilateral polyps growing from the middle meatus with or without involvement of the nasal cavities and affecting more than one sinus according to CT scan. The presence of nasal symptoms associated with NP (obstruction, anosmia, sneezing, rhinorrhea, and itching) on the day of operation was scored from 0 to 3: 0 for no symptoms, 1 for mild symptoms, 2 for moderate symptoms, and 3 for severe symptoms, so that the maximal global nasal score was 15 (14).

Endoscopic physical findings were scored according to Lund and Kennedy (19). In endoscopy score system, the presence or absence and extent of nasal polyps, discharge, edema, crusting, and scaring were graded on a 3-point scale. The extent of nasal polyps was scored as 0 (absence of polyps), 1 (polyps within the middle meatus), and 2 (polyps beyond the middle meatus). The discharge was scored as 0 (no discharge), 1 (clear, thin discharge), and 2 (thick, purulent discharge). The edema, scarring, and crusting, was scored as 0 (absent), 1 (mild), and 2 (severe). Each side was graded separately, and the scores from each side were then added to determine the overall endoscopy score, so that the maximal endoscopy score was 12.

Findings on preoperative CT scans were graded according to the Lund–Mackay system. The mucosal abnormalities were graded as 0 (no abnormality), 1 (partial opacification), or 2 (total opacification) for each sinus group. The ostiomeatal complexes were scored bilaterally as 0 (not occluded) or 2 (occluded). The maximal CT grading score was 24 (19). Allergic status was evaluated based on the measurement of serum total IgE and an allergy screening test (Phadiatop, Pharmacia CAP System, Uppsala, Sweden) comprising a panel of 23 aeroallergens prevalent throughout the Taiwan, including five trees, five grasses, five weeds, four molds, dander from three animal species, and one species of mite (*Dermatophagoides pteronissinus*) (20, 21). Total serum IgE levels and Phadiatop were analyzed by the fluoroenzyme immunoassay (FEIA) method according to the manufacturer's instructions (Pharmacia CAP System). Subjects were considered nonallergic if they did not have Phadiatop positivity and had a serum IgE level < 100 IU/ml; meanwhile, subjects with seasonal or perennial allergic symptoms at the time of allergic evaluation were also excluded (Table 1) (18). Moreover, none of the subjects exhibited a common cold, nor had they received any form of antibiotics, antihistamines, or corticosteroids for at least 4 weeks prior to sampling. The study protocol was approved by the IRB of CMUH and informed consent was obtained from all patients.

Pulmonary function tests and methacholine challenge

All NP patients underwent methacholine inhalation challenge. The asthma severity was assessed according to the GINA classification (22). Exclusion criteria for patients with asthma were lower respiratory tract infection or asthmatic exacerbation in the previous 8 weeks, and treatment with inhaled or oral corticosteroids in the 3 months before the study. FEV_1 and maximal midexpiratory flow (FEF_{25-75}) were obtained from flow–volume curves through the use of a body plethysmograph (Eric Jaeger GmbH & Co., Würzburg, Germany) and expressed as percentage of predicted normal values (23). Methacholine challenge was performed by using a aerosol provocation system (APS Pro; Jaeger, Würzburg, Germany) with

Table 1. Demographic information of study subjects

	Nasal polyp ($n = 20$)	Control ($n = 20$)	*P*-value
Age (years)	37.5 ± 12.8	40.5 ± 13.6	NS
Gender (M/F)	16/8	13/7	NS
Allergic status	Nonallergic	Nonallergic	
Phadiatop	Negative	Negative	
Serum total IgE (IU/ml)	47.6 ± 16.3	52.5 ± 18.6	NS
Blood-CL	8.61 ± 0.63	8.04 ± 0.78	NS
Tissue-CL	3192.54 ± 137.00	1392.76 ± 85.24	<0.01

CL, chemiluminescence; NS, not significant; Unit of blood-CL, counts/WBC; unit of tissue-CL, counts/10 s/mg protein.

Table 2. Characteristics of patients with nasal polyps

	NP without BHR	NP with asymptomatic BHR	NP with AHR/asthma
Number of patients	8	6	6
Age (years)	36.8 ± 12.2	39.5 ± 13.1	38.5 ± 11.8
Gender (M/F)	5/3	3/3	4/2
Serum total IgE (IU/ml)	42.5 ± 13.5	45.7 ± 15.6	50.5 ± 17.3
Global nasal score	8.5 ± 0.8	9.0 ± 0.9	8.8 ± 1.1
Total endoscopy score	5.9 ± 0.4	7.3 ± 0.5*	9.3 ± 0.9**
CT grading score	12.5 ± 1.3	15.8 ± 1.3	16.5 ± 1.3*
FEV_1 (%)	110.2 ± 5.4	95.1 ± 6.7	95.2 ± 8.3
FEF_{25-75} (%)	102.7 ± 5.8	72.1 ± 11.4**	65.8 ± 8.8**
PC_{20} (mg/ml)	>16	5.3 ± 1.7**	2.9 ± 1.5**
Blood-CL (counts/WBC)	6.64 ± 0.74	12.14 ± 3.19	10.08 ± 2.03
Tissue-CL (counts/10 s/mg)	2656.43 ± 87.78	3298.78 ± 172.50*	3801.10 ± 201.55**

Results are expressed as mean ± SEM.
*$P < 0.05$, different from NP without BHR; **$P < 0.01$, different from NP without BHR.

methacholine concentrations ranging from 0.25 to 16 mg/ml. The provocative concentration (PC_{20}) was defined as the methacholine concentration necessary to decrease FEV_1 by 20% from baseline values. Asymptomatic BHR was defined as a methacholine $PC_{20} < 16$ mg/ml in the absence of symptoms or previous history suggestive of asthma (24). By the results of pulmonary function tests and provocative testing, the NP patients were then substratified into three subgroups according to their bronchial phenotype: that is, NP without BHR (or isolated NP, $n = 8$), NP with asymptomatic BHR ($n = 6$), and NP with BHR and asthma ($n = 6$). (Table 2). The BHR groups enclose both NP with asymptomatic BHR group and NP with BHR/asthma group.

Sample preparation

The mucosal punctate specimens from each NP patients, weighing approximately 50 mg on average, were then separated into two groups, one for analysis of FRs by the CL method, and the other by histological analysis. Tissue samples, for measuring FR level, were immediately frozen in liquid nitrogen for < 10 min after excision and kept until analysis. Preparation of tissue homogenate for CL was carried out by the following procedure (10, 16). The tissue was minced finely with scissors and suspended in 10 vol (v/w) of Tris-sucrose buffer (0.25 M sucrose in 20 mM Tris-HCl buffer containing 1 mM ethylenediaminetetraacetic acid at pH 7.4). Homogenization was done by a Polytron homogenizer (PT-10) at a setting of 5.5 for two 30-s periods at 0–4°C. The homogenate was centrifuged at 400 ***g*** in a refrigerated centrifuge at 4°C for 30 min. The supernatant was then immediately wrapped in aluminum foil and kept in ice box until CL testing which was done within 2 h.

Analysis of tissue free radicals with luminol-enhanced chemiluminescence (tissue-CL)

The measurement of luminol-amplified *t*-butyl hydroperoxide (TBHP)-initiated CL in tissue was similar to that describe previously with some modifications (10, 16). Briefly, 0.2 ml of luminol in phosphate buffered saline (PBS) buffer (5 mg/l, pH 7.4) was added to 0.4 ml of sample homogenate supernatant in a stainless cell. The sample mixture was then incubated at 37°C for 10 min. The CL was then measure in an absolutely dark chamber of the CL Analyzing System (Tohoku Electronic Industrial Co., Sendai, Japan). The photon detector (Model CLD-110), according to the manufacturer's specifications, is extremely sensitive and capable of detecting radiant energy as weak as 10^{-15} W. Proton emission from the sample was counted at 10-s intervals at 37°C under atmospheric conditions. At the 100-s time point, 0.1 ml of TBHP (Sigma Co., St Louis, MO, USA) in PBS (pH 7.4) was injected into the cell. The CL in the samples was continuously measured for a total of 1000 s. The total amount of CL was calculated by integrating the area under the curve and subtracting it from the background level, which was equivalent to the dark average (Fig. 1A). The assay was performed in triplicate for each patient sample and was expressed as CL counts/10 s/mg protein for the tissue-CL. A mean (±SEM, standard error mean) CL level of each sample was calculated (Fig. 1B).

Analysis of blood free radicals with luminol-enhanced chemiluminescence (blood-CL)

Whole blood samples were obtained with heparinized plastic syringes during the operation. The measurement of luminol-amplified zymosan-initiated CL in the whole blood was similar to that described formerly with some modifications (16, 17). Briefly, 0.2 ml of whole blood was mixed with 0.1 ml of PBS buffer (pH 7.4). The CL was then measured in an absolutely dark chamber of the CL analyzing system as described above. After 200 s, 1.0 ml of 1000 μM luminol in PBS was injected into the stainless steel cell and the CL in the blood sample was measured continuously. After 600 s, 0.2 ml of zymosan (Sigma) was added, and the CL count was measured for a total of 1020 s. The total amount of CL was calculated by integrating the area under the curve and subtracting it from the background levels, which was equivalent to the dark average. The assay was performed in triplicate for each sample and the production of CL per white blood cell (WBC) was calculated and expressed as CL counts/WBC. A mean (±SEM) CL level of each sample was calculated.

Histological studies

For histological examination under light microscopy, polyp specimens were fixed in 10% formalin, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin. Sections were coded and counted in a blinded, random fashion with an Olympus microscope with an eyepiece graticule at ×200 magnification. The graticule was oriented along the epithelial basement membrane (25). Histological changes were characterized by a scoring system according to Pasto et al. with some modifications (10). The abundance of inflammatory cells (*Ce*) was estimated as follows: 1 for a 'low' number of inflammatory cells, 2 for a 'moderate' number, and 3 for an 'intense' infiltration by inflammatory cells on the polyp surface (Fig. 2A). The percentage of eosinophils in the inflammatory cells (eosinophil infiltration, Eo) was estimated as follows: 1 for < 10% eosinophils, 2 for 10–50%, and 3 for more than 50% (Fig. 2B). In order to prevent underestimating the number of eosinophils, a second Giemsa stain was also used to illustrate the extent of the eosinophils in the areas of dense inflammation. The severity of edema (*Ed*) was scored as follows: 1 for minimal, 2 for moderate, and 3 for intensive edematous change (Fig. 2C). The abundance of fibrosis (*Fi*) was scored as follows: 1 for trace amounts of fibrous tissue or fibroblasts, 2 for moderate amounts, and 3 for fibrous sclerosis (Fig. 2D). Of each patient, at least two sections were stained, from which six to eight fields were evaluated by two independent

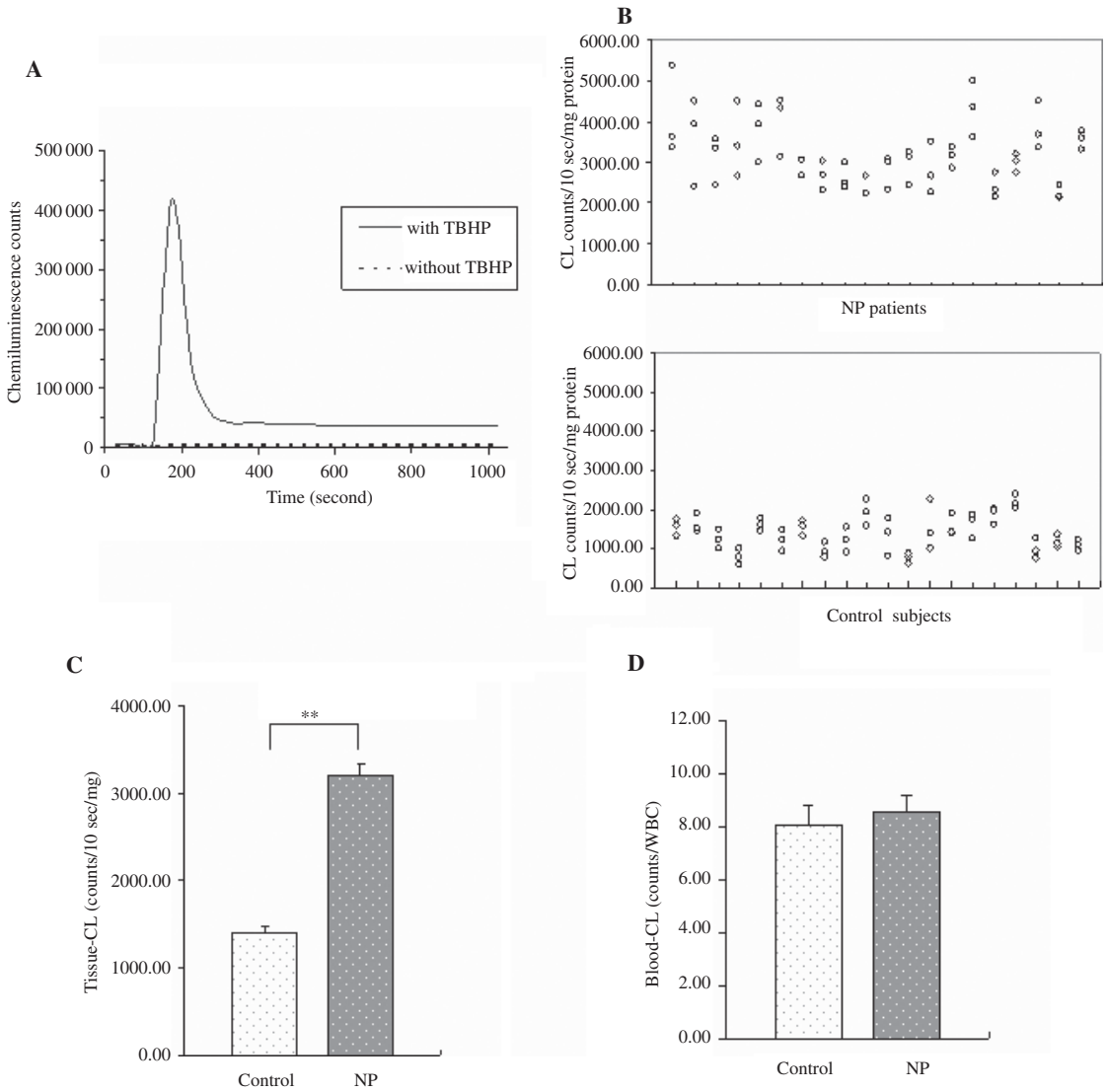

Figure 1. Estimating the levels of free radicals by luminol-elicited chemiluminescence in nasal biopsy specimens (tissue-CL) and in blood samples (blood-CL) from NP patients and controls. (A) A typical recording of a CL signal. Note a marked increase in the TBHP-initiated CL. (B) Reproducibility of tissue-CL from three different tissue specimens of each subjects. (C) Tissue-CL, $^{**}P < 0.01$. (D) Blood-CL.

physicians; subsequently, all histologic sections were reviewed jointly to arrive at a consensus opinion. The resulting scores of each patient were correlated with the tissue-CL (Table 3).

Statistical analysis

To compare the difference between the NP patients and controls, all data were tested for normality prior to the two-sample *t*-test by the SAS program. The scores of clinical parameters in the three groups of NP patients were compared through the use of use of a one-way ANOVA followed by a *post hoc* *t*-test for multiple comparisons. Correlations were done with use of linear regression analysis. A *p*-value of <0.05 was considered to be significant.

In order to avoid the effect of colinearity, correlation between luminol-CL counts and histological characteristics was evaluated by the cumulative logistic regression model (26). The response variables, denoted by Y_{1i} (*Ce*), Y_{2i} (*Eo*), Y_{3i} (*Ed*) and Y_{4i} (*Fi*), were ordinal variables with categories (1, 'low'; 2, 'moderate'; and 3, 'intense'). Both tissue- and blood-CL counts were regarded as explanatory variables (co-variates). Based on the forward selection procedure ($p < 0.05$ for entry), only the tissue-CL counts (X_i, continuous) were included in the model. The number of observations was twenty. The cumulative logit model (proportional odds) was given as follows:

$$\begin{aligned}&\text{logit}[P(Y_{ki} \geq j|X_i)]\\ &\quad = \log[P(Y_{ki} \geq j|X_i)/P(Y_{ki} < j|X_i)]\\ &\quad = \alpha_{kj} + \beta_k X_i \ \ (i = 1, 2, \ldots, 20; j = 1, 2; k = 1, 2, 3, 4) \qquad (1.1)\end{aligned}$$

Note that the parameter β_k in (1.1) is independent of *j*, which is a proportional odds model.

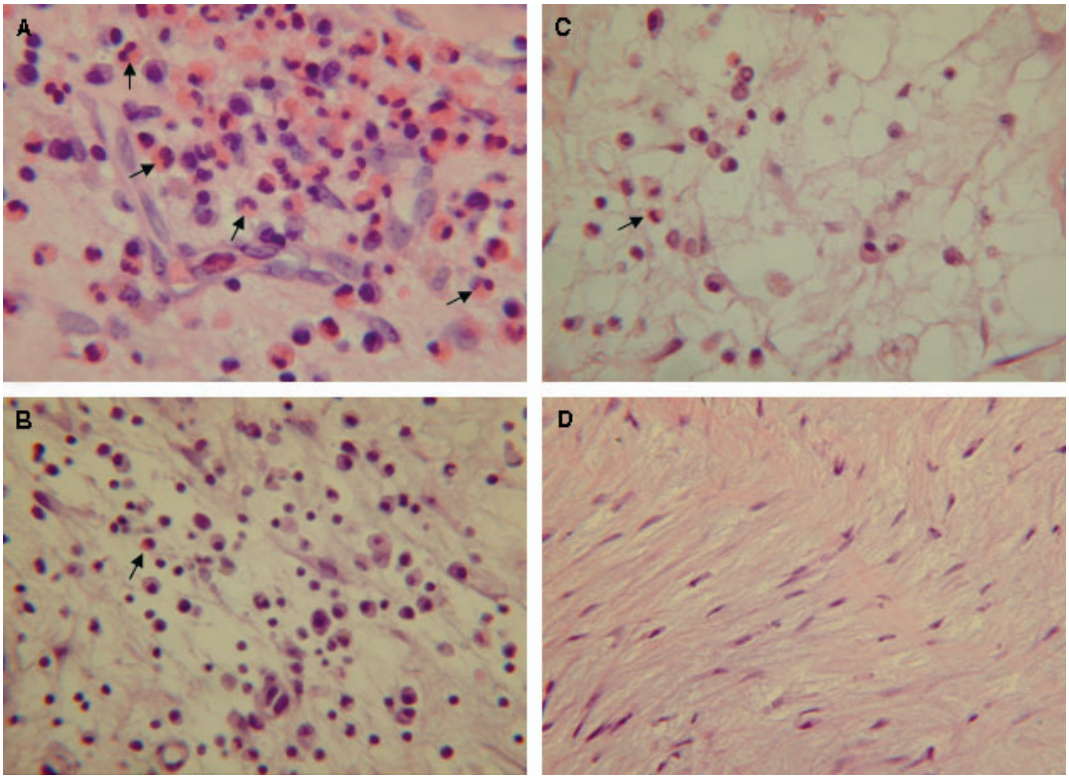

Figure 2. Representative pictures of histopathological characteristics of nasal polyps. (A) High cell abundance (*Ce*: score 3) and high percentage of eosinophil infiltration (*Eo*: 3, >50% eosinophils). Eosinophils (Eϕ) are characterized by a bi-lobar nucleus and the presence of red granules in the cytoplasm. (B) Moderate cell infiltration (*Ce*: 2) and low percentage of Eϕ (*Eo*: 1, <10% Eϕ). (C) Prominent edema (*Ed*: 3) and low cell infiltration (*Ce*: 1). (D) Low infiltration (*Ce*: 1 and *Eo*: 1) and abundant fibrosis (*Fi*: 3). (hematoxylin-eosin, H&E stain, original magnification: ×400 in all pictures).

Table 3. Distribution of histological characteristics of patients with nasal polyposis (NP)

	NP ($n = 20$)		
Histological characteristics	(1, +)	(2, ++)	(3, +++)
Abundance of cell infiltration (*Ce*)	5	8	7
Percentage of eosinophils in inflammatory cells (*Eo*)	5	9	6
Intensity of edema (*Ed*)	7	8	5
Intensity of fibrotic change (*Fi*)	8	9	3

Histological characteristics score included assessment of the abundance of inflammatory cells (*Ce*), percentage of eosinophils in inflammatory cells (*Eo*), intensity of edema (*Ed*), and intensity of fibrotic change (*Fi*) on a 3-point scale, mild (1, +), moderate (2, ++) and severe (3, +++).

Results

Characteristics of the study subjects

Table 1 lists the basic data of subjects in this work; all enrolled subjects were nonallergic. The NP group included patients with evident polyp formation, whose polyp score were no less one because of the definition (inclusion criteria). The clinic and pathophysiologic data of the NP patients are shown in Table 2. Eight patients did not respond to methacholine challenge, six exhibited BHR but did not have asthma symptoms, and six had asthma symptoms associated with BHR. There were no significant differences in serum total IgE and FEV_1 (%) among the three groups, whereas $FEF_{25–75}$ (%) and PC_{20} were significantly different in the two groups with BHR as compared with the NP without BHR group. The asthmatic subgroup included mainly patients with intermittent and mild asthma because of the steroid exclusion criteria. The normal values of FEV_1 (%) and $FEF_{25–75}$ (%) from healthy subjects ($n = 20$) were 112.3 ± 4.3 and 105.2 ± 4.8, respectively. There were no differences in FEV_1 (%) and $FEF_{25–75}$ (%) between the control group and NP without BHR group.

Chemiluminescent signals on free radicals from tissues (tissue-CL) but not from blood (blood-CL) were increased in NP patients

The mean levels of tissue-CL were significantly higher in NP tissues than in control specimens ($P < 0.001$). However, the results of blood-CL indicated that there was no significant difference between

the NP and control groups (Table 1 and Fig. 1C and D).

Total endoscopy score but not nasal score or CT score was correlated with tissue-CL in NP patients

In this work, the disease severity of NP was described by three clinical parameters, global nasal symptom score, total endoscopy score, and CT staging score. A positive correlation was observed between tissue-CL and endoscopy score ($r = 0.88$; $P < 0.0001$) but not with nasal score ($r = 0.39$; $P = 0.091$) or with CT score ($r = 0.43$; $P = 0.058$) (Fig. 3). In contrast, there were no correlations between blood-CL and the three scores (data not shown).

Tissue-CL and endoscopy score but not blood-CL were associated with the asthma phenotypes in NP patients

Tissue-CL and endoscopy score were significantly increased in the BHR/asthma group when compared with the BHR ($P < 0.05$) and the isolated NP groups ($P < 0.01$). There were also significant differences between the BHR and isolated NP group for tissue-CL and endoscopy score ($P < 0.05$). There were significant differences between the BHR/asthma and isolated NP group for CT score ($P < 0.05$). There was no variation of symptom score or blood-CL, whatever the group (Table 2 and Fig. 4).

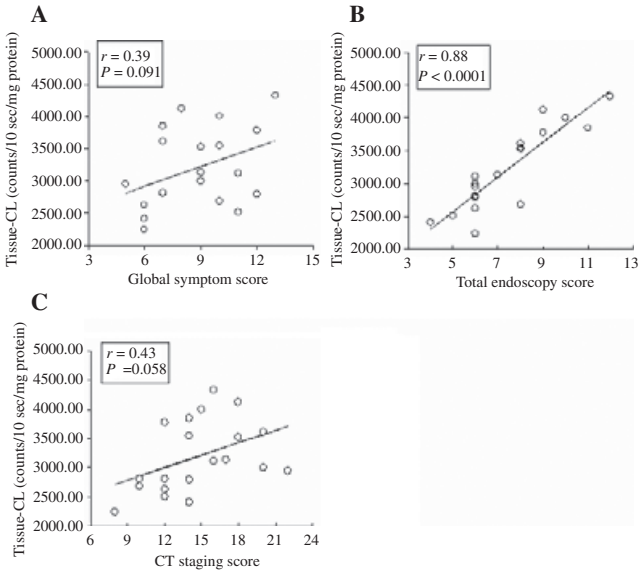

Figure 3. Relation between clinical parameters and chemiluminescent signals in polyp biopsy specimens (tissue-CL) from patients with NP. (A) Correlation between nasal score and tissue-CL. (B) Correlation between endoscopy score and tissue-CL. (C) Correlation between CT score and tissue-CL.

Table 4. Analysis of maximum likelihood estimates (MLE) for histological characteristics

Parameter	DF	Estimate	Standard error	Wald χ^2	Pr > χ^2
Y_{1i} (cells, *Ce*)†					
$\hat{\alpha}_{11}$	1	−7.0040	2.9711	5.5573	0.0184
$\hat{\alpha}_{12}$	1	−9.4882	3.3855	7.8543	0.0051
$\hat{\beta}_1$	1	0.00271	0.00101	7.2477	0.0071*
Y_{2i} (eosinophil, *Eo*)‡					
$\hat{\alpha}_{21}$	1	−19.2946	7.6601	6.3446	0.0118
$\hat{\alpha}_{22}$	1	−25.8044	10.0825	6.5501	0.0105
$\hat{\beta}_2$	1	0.00717	0.00278	6.6436	0.0100*
Y_{3i} (edema, *Ed*)§					
$\hat{\alpha}_{31}$	1	1.8669	2.3007	0.6585	0.4171
$\hat{\alpha}_{32}$	1	0.1277	2.2585	0.0032	0.9549
$\hat{\beta}_3$	1	−0.000383	0.000699	0.3005	0.5836
Y_{4i} (fibrosis, *Fi*)¶					
$\hat{\alpha}_{41}$	1	6.3297	2.8531	4.9219	0.0265
$\hat{\alpha}_{42}$	1	3.7199	2.5821	2.0755	0.1497
$\hat{\beta}_4$	1	−0.00181	0.000862	4.4233	0.0355*

Score test for the proportional odds model: †*P*-value = 0.715; concordant (%) = 80.2; ‡*P*-value = 0.0636; concordant (%) = 90.7; §*P*-value = 0.3525; concordant (%) = 55.0; ¶*P*-value = 0.3760; concordant (%) = 73.2.
D.f., degree of freedom; χ^2, chi-square; *$P < 0.05$.
Table 4 shows the results of forward selection, which included the maximum likelihood estimates (MLE) of the parameters (denoted by $\hat{\alpha}_{kj}$ and $\hat{\beta}_k$) and the corresponding estimated standard errors. Table 4 also lists the results obtained from testing the null hypothesis H_{0k}: $\beta_k = 0$ versus the alternative H_{ak}: $\beta_k \neq 0$ ($k = 1, 2, 3, 4$).

Tissue-CL correlated positively with the abundance of inflammatory cells and, to a greater extent, the percentage of eosinophil infiltration

Based on the Wald-test, the *P*-values for H_{01}, H_{02} and H_{04} were 0.0071, 0.0100 and 0.0355, respectively (Table 4). These *P*-values imply that the tissue-CL strongly correlated with *Ce*, *Eo* and *Fi*. However, the *P*-value for H_{03} was 0.5836, which suggests that there is no significant correlation between tissue-CL and *Ed*. The effect estimates $\hat{\beta}_1 = 0.00271$ and $\hat{\beta}_2 = 0.00717$ suggest that the cumulative probabilities at the 'intensive' end of both *Ce* and *Eo* increase as the tissue-CL increases; the effect estimate $\hat{\beta}_4 = -0.00181$ suggests that the cumulative probability at the 'intensive' end of *Fi* increases as tissue-CL decreases. In summary, our results indicate that tissue-CL correlate positively with the abundance of inflammatory cells and, to a greater extent, the percentage of eosinophil infiltration, and negatively with the amount of fibrosis, but not with the severity of edema.

Endoscopy score, tissue-CL, and eosinophil infiltration correlated with each other and were closely associated with the BHR/asthma phenotype in nonallergic NP patients

Again, similar to tissue-CL and endoscopy score, eosinophil infiltration was closely associated with the BHR/asthma phenotype in nonallergic NP patients (Fig. 5A).

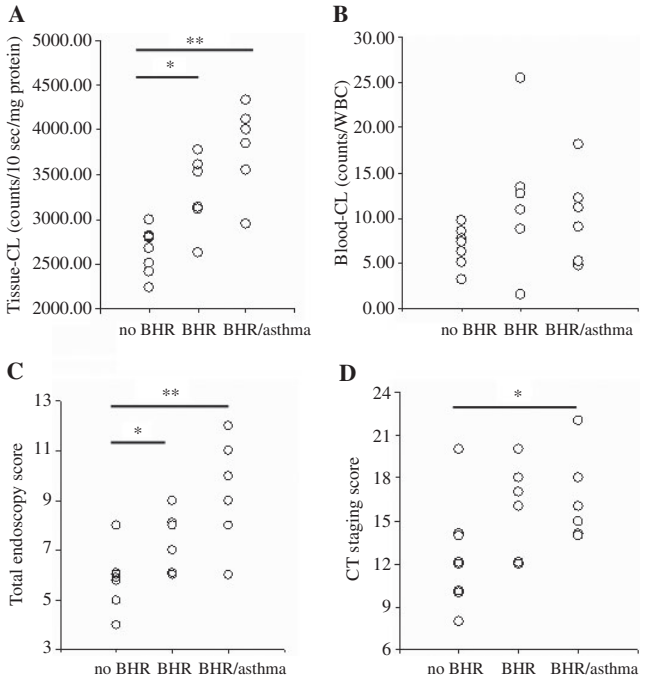

Figure 4. Tissue-CL, blood-CL, endoscopy score and CT score in the three groups of NP patients. (A) The distribution of tissue-CL in the three NP groups, $*P < 0.05$; $**P < 0.01$. (B) The distribution of blood-CL in the three NP groups. (C) The distribution of endoscopy score in the three NP groups, $*P < 0.05$; $**P < 0.01$. (D) The distribution of CT score in the three NP groups, $*P < 0.05$.

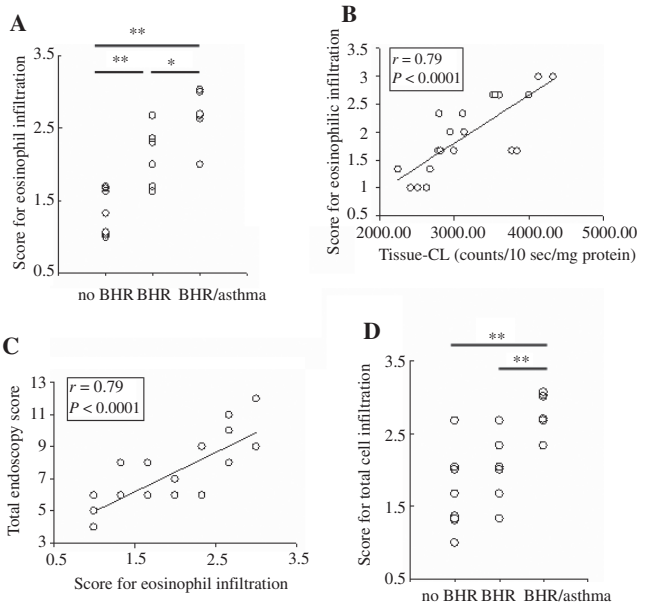

Figure 5. Relation between endoscopy score, tissue-CL and eosinophil infiltration in polyp biopsy specimens from NP patients. (A) Eosinophil infiltration in the three groups of NP, $*P < 0.05$; $**P < 0.01$. (B) Correlation between tissue-CL and eosinophil infiltration ($r = 0.79$; $P < 0.0001$). (C) Correlation between endoscopy score and eosinophil infiltration ($r = 0.79$; $P < 0.0001$). (D) Endoscopy score in the three groups of NP, $*P < 0.05$; $**P < 0.01$.

However, contrary to tissue-CL and endoscopy score, eosinophil score is the only one parameter that can show significant difference between each of the three groups. When eosinophil infiltration was compared with total endoscopy score and with tissue-CL, the three parameters correlated with each other, suggesting a causal relation (Table 2; Fig. 2B, Fig. 5B and C). Scores for the abundance of total inflammatory cells was significantly increased in the BHR/asthma group when compared with the BHR ($P < 0.01$) and the isolated NP groups ($P < 0.01$); however, there was no difference between the BHR and the isolated NP groups (Fig. 5D).

Discussion

Although no single factor can account for the development of NP in all patients, an underlying inflammatory and/or infectious process, such as microbial colonization or infection can lead to the formation and perpetuation of this specific local tissue pathology (1, 3, 4). The high concentration of inflammatory cells found in NP potentially results in the overproduction of FRs (7, 12).

To assess the generation of FRs directly in biological systems is difficult because of the extreme instability of the ROS. Therefore, the measurement of the oxidative stress in tissue is usually performed by an indirect method (8–11, 16, 17). Both luminol- and lucigenin-enhanced CL are used for the measurement of ROS production from different types of cells by employing different activators, such as TBHP and zymosan. Pasto et al. meticulously assessed superoxide production in NP by means of electron spin resonance (ESR) measurement, superoxide dismutase (SOD) inhibitable reduction of cytochrome c, and lucigenin-enhanced CL. Among those methods, CL was proved to be a convenient, reliable and sensitive method and was therefore applied to their subsequent assessment of O_2^- production in nasal polyps (10). Lucigenin-CL reacts more specifically with O_2^-, while luminol-CL levels reflect the extent of myeloperoxide activity which results mainly from hydrogen peroxide, peroxynitrite and hypochlorous acid (16, 17, 27). In this study, we estimated the oxidative stress in blood and in tissues by an ultra-sensitive luminol-CL method. The mean level of tissue-CL in NP was significantly higher than in control specimens; no significant difference in blood-CL between NP and control groups was found. This result is compatible with previous reports and supports that NP is a specific inflammatory pathology of local tissues (4).

Physiologically, FRs are kept in balance with the antioxidant defense system in the body, a condition called redox homeostasis. These ROS are normally controlled by the antioxidant defense mechanisms, including enzymatic antioxidants such as SOD, catalase and glutathione peroxidase (GPX), and nonenzymatic antioxidants, low molecular mass compounds such as glutathione (GSH), α-tocopherol, β-carotene, retinol and ascorbic acid. Oxidative stress ensues when there is an oxidant/antioxidant imbalance due to an excess of ROS production or a depletion of antioxidants (7). For example, MDA is a metabolite of FR-mediated lipid peroxidation, which can be used as an indicator of FR levels (8, 9). Dogru et al. reported that MDA levels in NP from 19 patients were significantly higher than in mucosa from lateral lamella of the middle turbinates from nine control subjects (8). Dagli et al. investigated the role of FRs and antioxidants in NP from 31 patients compared with 19 control subjects (9). According to their results, in polyp tissue, the levels of oxidant (MDA) were increased, and the levels of antioxidant (GSH and α-tocopherol) were decreased compared with control tissues from inferior turbinates.

When evaluating the association between tissue FR levels and clinical parameters, we found endoscopy score, but not nasal score or CT score, was correlated with tissue-CL in NP patients, although there was a deviation of no less than one in the polyp score due to the inclusion criteria. It is possible that nasal score is a relatively subjective parameter to assess the severity of NP leading to a wide variation among individuals. It is also possible that CT score, a standard and reliable staging system for the severity and outcome survey of CRS, could not be so concordant with NP (19).

On the basis of the histological characteristics, NP had been classified into several types. Both eosinophilic edematous type and chronic inflammatory or fibrotic type are most commonly seen in clinical practice (1). Believing that eosinophils, edema, inflammatory cells, and fibrosis are the most prominent histological characteristics, we applied a scoring system to correlate tissue types with the tissue-CL (10). The data indicated that high tissue-CL counts were positively associated with inflammatory cell abundance and, to a greater extent, eosinophil infiltration, and negatively with the abundance of fibrosis; tissue-CL did not correlate with the severity of edema. Our results differed a little from that found by Pasto et al. who noticed that lucigenin-CL significantly correlated with the abundance of eosinophils and fibrosis, but not with inflammatory cell abundance. It has been known that eosinophils produce more superoxide anion than neutrophils (28); however, it is also plausible that inflammatory cells, including neutrophils and eosinophils, attributed to the oxidative stress. Moreover, in noneosinophil-dominated inflammation, such as antrochoanal polyp and nasal polyps based on cystic fibrosis and primary ciliary dyskinesia, a high recurrence rate was noted as well as eosinophil-dominated NP (2). Thus, pathophysiological mechanisms other than eosinophilia may be of importance (1, 2, 29).

Both allergic rhinitis and NP are common chronic inflammations of upper airway and are found in the majority of patients with asthma. The histology of chronic hypertrophic sinusitis (CHS/NP), characterized by epithelial damage, basement membrane thickening, and eosinophilic inflammation, is more similar to that of asthma; therefore, etiologic factors in NP attract increasing interest (6, 30). Unlike asthma, the importance of FR damage and oxidative stress in NP is only beginning to become apparent. Although, tissue damage and remodeling in NP as well as in asthma are closely related to eosinophil infiltration; however, inflammatory cells, especially the eosinophils, play a pivotal role in all these three diseases. To prevent the influence of allergy, we applied a CL method to estimate the footprints of ROS in nonallergic NP patients. The results indicated tissue-CL, but not blood-CL, was significantly increased in polyps tissues than in control specimens, suggesting oxidative stress play a role in this disease with a local, but not systemic, fashion. Increased tissue-CL correlated with endoscopy score and histological eosinophil infiltration. In fact, these three parameters correlated with each other and associated with the BHR/asthma phenotype in NP, implying a causal relation not only in the sinonasal microenvironment, but also in the interaction of upper and lower airways. It is also notable that only the eosinophil score can show significant difference between each of the three groups, indicating a unique position of eosinophils in this linkage. Further studies on the mechanism of oxidative stress in NP are required.

Acknowledgments

We would like to express our gratitude to Dr Chi-Long Chen and Dr Tze-Yi Lin for their expertise in the pathological advice.

This work was supported by a grant (grant no.: NSC 92-2314-B-039-026) from the National Science Council (Republic of China) and was also supported in part by a research grant (grant no. DMR-93-031) from the China Medical University Hospital. All Authors have no financial relationship with a biotechnology and/or pharmaceutical manufacturer that has an interest in the subject matter or materials discussed in the submitted manuscript.

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