

# Overexpression of Nitric Oxide and Inducible Nitric Oxide Synthase in Mucous Membrane of the Maxillary Sinus Fungus Ball

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## Research

**Keywords:** nitric oxide, inducible nitric oxide synthase, fungal ball, rhinosinusitis

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# Abstract

**Objective:** We aimed to explore the expression and interplay of NO and iNOS to elucidate their roles in human maxillary sinus mucosa with maxillary sinus fungus ball (MSFB) and chronic rhinosinusitis (CRS).

**Methods:** Fifty-one patients with MSFB, CRS and simple maxillary sinus cyst were included in this study. The NO content of the mucosa of each group was detected by Nitric acid reductase method. The expression of iNOS was tested by immunohistochemistry and Western blott. Spearman correlation analysis was used to analyze the correlation between the expression of iNOS and the occurrence of MSFB and CRS.

**Results:** The results showed that NO and iNOS were highly expressed in the maxillary sinus mucosa of MSFB group, which were significantly different from CRS and control group ( $P < 0.01$ ). The expressions of NO and iNOS in the samples of CRS group were higher than that of the control group ( $P < 0.05$ ). Moreover, iNOS was widely presented in the cytoplasm of sinus mucosa epithelium and inflammatory cells in the maxillary sinus mucosa of MSFB patients. Furthermore, Spearman correlation coefficient indicated that the expression of iNOS was positively correlated with the incidence of MSFB ( $r = 0.6395$ ,  $P < 0.05$ ).

**Conclusion:** NO and iNOS are overexpressed in the maxillary sinus mucosa of MSFB and CRS patients, and the increase in the MSFB patients was better than the CRS. The expressions of NO, iNOS were also correlated with the incidence of MSFB. We thus provide a new indicator to assist the diagnosis of MSFB and its distinction from CRS.

## Introduction

Nitric oxide (NO), synthesized by nitric oxide synthase (NOS), profoundly modulates diverse physiological processes, but also encompasses pathological implications [1–4]. Recently, NO has been shown to exert regulatory, protective, and defensive effects against tissue-damaging processes during inflammation [5]. NOS is divided into three isoforms: endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). iNOS is a key mediator of inflammation and a host of the body defense systems. It is induced by various pathological conditions, particularly inflammation. NO synthesis is clearly enhanced locally, at the sites of inflammation, by iNOS activity [6]. The impact of NO on the respiratory system, especially airway function, has drawn much scientific interest. Studies have found that elevated levels of NO and its metabolites in sinusitis might damage healthy sinus epithelium [7]. NO is also closely associated with the occurrence and development of sinusitis. Changes (increase and decrease) in NO release in the nasal exhalation of patients with sinusitis occur in an ununiform manner [7]. Thus, detection of NO and iNOS expression in the sinuses could help us further understand the role of NO in mucosal lesion and explain its involvement in fungal rhinosinusitis as a new molecular mechanism.

Fungus ball is the most common form of non-invasive fungal rhinosinusitis [8]. *Aspergillus fumigatus* (between 44.8% and 75%) and *Aspergillus flavus* (14%) are the two most common species recovered

from fungal rhinosinusitis patients. Fungal ball most commonly occurs in the maxillary sinus [9], and thus it is named maxillary sinus fungus ball (MSFB). The next most common site is the sphenoid sinus [10]. Naraghi [11] showed significantly higher levels of NO metabolites in patients' chronically infected sinuses. The study of Taruya [12] reported that patients suffering from chronic rhinosinusitis with nasal polyps, showed significant expression of iNOS messenger RNA. It is believed that fungal rhinosinusitis is a unique phenotype of chronic rhinosinusitis, with unique clinical and histological characteristics [13]. However, the specific expression of iNOS, especially in MSFB, has not been evaluated, neither in humans nor in animal studies.

In this study, we first measured NO metabolites and iNOS expression in mucosal biopsies from patients with fungal rhinosinusitis or CRS. Differences under the same experimental conditions, so we believe, would be more convincing. Our data demonstrated an obvious increase in iNOS expression and content of NO metabolites in MSFB compared to those in the CRS and control groups. iNOS was also found to be extensively expressed in the sinus mucous epithelium of MSFB patients, primarily in the cytoplasm of inflammatory cells, such as lymphocytes and plasma cells. Studying NO metabolite content and iNOS expression is not only useful for understanding their contribution to MSFB and for comparing the differences between MSFB and CRS, but might also help in diagnosis and for the development of possible therapeutic targets.

## Materials And Methods

### Patients and samples

All patients underwent unilateral endoscopic sinus surgery between July 2017 and October 2019 at the Second Affiliated Hospital of Dalian Medical University, Liaoning, China. This study was performed on 57 cases that were equally split between three groups: normal control group (n = 19), including 7 males and 12 females with an average age of 42 y; CRS patients, without nasal polyps (n = 19), including 10 males and 9 females with an average age of 45 y; and MSFB patients (n = 19), including 8 males and 11 females with an average age of 52 y. Biopsies were obtained from the maxillary sinus ostium mucosa of all patients during endoscopic surgery. All procedures used in this study complied with the ethical standards of Dalian Medical University. The study protocol was approved by the Institutional Review Board, Dalian Medical University (2019-052). Written informed consent was obtained from all patients prior to participation.

### Inclusion and exclusion criteria

Diagnosis was based on histopathological examination of surgically-collected samples from the maxillary sinus. Diagnosis was reconfirmed by CT scans and operation record. Patients who underwent functional endoscopic sinus surgery (FESS) due to simple maxillary sinus cyst but without CRS were included as control. The experimental groups were patients, diagnosed with MSFB and CRS, who underwent FESS.

Diagnostic criteria for MSFB: 1) Medical history: nasal congestion, runny nose, foul odor of nasal secretions, facial pain, and other similar symptoms associated with rhinosinusitis; 2) Onset of unilateral maxillary sinusitis; 3) Ununiform density of all or most lesions as seen in sinus CT scans. In some lesions, the CT value was higher than the soft tissue but lower than that of a calcification spot or bone. In these instances, the CT value was about 100–150 HU; 4) During surgery, sediment and caseous, brown or yellowish green, fungal masses are observed in the diseased sinuses; 5) Pathology: the mass consists of aggregates of mutually entwined fungal filaments. Diagnosis of CRS without nasal polyps was done according to the Chinese Guidelines for the Diagnosis and Treatment of Chronic Sinusitis (2018).

Patients were excluded if the lesions were located in places other than the maxillary sinus, or when lesions were concurrent with bronchial asthma, allergic rhinitis, acute rhinosinusitis within one month prior to FESS, aspirin triad, autoimmune disease, primary ciliary motor dysfunction, or cystic fibrosis.

### Tissue handling

All specimens were quickly rinsed with normal saline and then dried with filter paper. Each specimen was cut into three 4-mm parts. One part was immediately soaked in formalin for 24 h, routinely dehydrated, and embedded with paraffin. The other two parts were placed separately in cryovials and submerged quickly into liquid nitrogen.

### Detection of NO

Quantity of nitrite, a stable metabolite of NO, was measured using the nitrate reductase method, using NO kit (Griess reagent; Beyotime Biotechnology Co., Ltd., Hangzhou, Zhejiang, China). Briefly, the same volume of supernatant was extracted from all samples after centrifugal. Then, 50  $\mu$ L of tissue culture medium were mixed with 100  $\mu$ L Griess reagent. Fresh culture medium was used as a blank control in all experiments. Subsequently, the mixture was incubated at room temperature for 10 min and absorbance at 540 nm was measured by an enzyme label microplate reader. The quantity of nitrite was determined based on a sodium nitrite standard curve.

### Immunohistochemical analysis

Tissues embedded in paraffin were sliced in a cryostat into 5  $\mu$ m slices. Immunostaining was carried out on these sections, using primary antibodies (mouse monoclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA). For antigen retrieval, sections were rinsed with citric acid solution (pH = 6.0) and then exposed to microwave repair at 100 °C (medium-high temperature) for 25 min. Slides were then incubated with primary antibody overnight at 4 °C, according to the manufacturer's recommendations. Color development was achieved using streptavidin–biotin amplification (Zhongshan Jinqiao kit; Solely Biotechnology, Beijing, China). Peroxidase activity was visualized using a diaminobenzidine solution. Sections were counterstained with hematoxylin. Control specimens were assayed without the primary antibody and used to verify the absence of non-specific binding. Consecutive sections were stained with hematoxylin to observe mucosal pathology and assess the degree of iNOS expression.

Immunohistochemical results: iNOS immunohistochemical staining could be observed under the optical microscope, and the positive expression was yellow or brownish yellow particles under the microscope. The number of positive cells in each field of view was counted at high magnification (10 to 40 times) for each section. Perform statistical analysis.

### Western blot analysis

Sinus tissues were minced and homogenized in radio immunoprecipitation assay buffer (RIPA; P0013C, Beyotime Institute of Biotechnology). The homogenate was centrifuged at 12,000 rpm and 4 °C for 15 min, and the supernatant was collected for cytosolic protein analysis. Protein concentration was determined by a BCA protein assay kit (Beyotime Institute of Biotechnology). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on equal amounts of protein samples, and proteins were transferred to polyvinylidene difluoride membrane. After blocking with 5% non-fat milk (Guangming, Shanghai, China), membranes were incubated overnight at 4 °C with anti-iNOS (ab178945; 1:1000, Abcam Biotechnology, Cambridge, United Kingdom ) or anti- $\beta$ -actin (abm52614; 1:4000, Abcam Biotechnology) antibodies. On the next day, the membranes were incubated with anti-rabbit IgG (H + L) (#35568; 1:6000, Thermo Fisher Scientific, Invitrogen) antibody. The signals were subsequently imaged by Odyssey Clx. Intensity values were expressed as relative protein expression, normalized to  $\beta$ -actin.

## Statistical analysis.

Data were analyzed using GraphPad Prism7. Data were presented as means  $\pm$  standard deviation. Student's t-test was used to assess the differences between variables in any two groups. Differences between groups were considered statistically significant when  $p < 0.05$ . \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ . Spearman correlation analysis was used to analyze the correlation between the expression of iNOS and the occurrence of MSFB and CRS. The "p" value should be less than 0.05, and the value range of correlation coefficient "r" is between - 1 and 1.

## Results

To investigate the amount of NO in the sinus mucosa samples, nitrate reductase assay was carried out. Results showed increased level of NO in the MSFB group compared to that in the CRS and control groups ( $p < 0.001$ ) (Fig. 1). The CRS group showed a higher amount of NO compared to the control ( $p < 0.01$ ) (Fig. 1).

To find out whether the observed changes in NO content were due to differences in iNOS expression, western blot was performed. To avoid any possible cross-reaction of the iNOS antibody with eNOS, we performed the western blot, using specific monoclonal antibodies. Consistent with data provided by the antibody maker, two iNOS bands were obtained. Their sizes were approximately 127 kDa and 131 kDa (Fig. 2a). Similar to NO content, the level of iNOS proteins was higher in the MFSB group than that in the CRS and control groups ( $p < 0.01$ ). Moreover, compared with the control group, the level of iNOS protein in

the CRS group was slightly higher ( $p < 0.05$ ). Thus, iNOS protein is differentially expressed in the MSFB and CRS groups (Fig. 2b).

To understand whether the observed changes in protein levels is due to altered iNOS immunoreactivity in the maxillary sinus mucosa, an immunostaining study was performed. Expression of iNOS protein was located to the lymphocytes and plasma cells. Its distribution pattern was similar in all samples. However, staining intensity differed between groups. Strong iNOS staining intensity was observed in the MSFB tissues. Staining intensity was significantly higher than in the CRS and control groups. Staining was weak or absent in the control tissues ( $p < 0.001$ ) (Fig. 3).

The iNOS relative protein gray ratio of each group was obtained by western blot. Spearman correlation coefficient indicated that the expression of iNOS was positively correlated with the incidence of MSFB ( $r = 0.6395$ ,  $P < 0.05$ ), but weakly correlated with the incidence of CRS ( $r = 0.3396$ ,  $P \geq 0.05$ ) (Table 1).

Table 1  
Spearman correlation analysis  
of the correlation between the  
expression of iNOS and the  
occurrence of MSFB and CRS

Status	MSFB	CRS
r value	0.6395	0.3396
p value	$\geq 0.0001$	0.0494

## Discussion

Fungal infection and obstruction of the natural ostium might lead to fungal sinusitis. However, the molecular mechanism involved in MSFB pathogenesis, the most common type of fungal sinusitis, is still not clear. NO, an important cellular signaling mediator, and iNOS are two important agents involved in sinus mucosa infection. The present study examined, for the first time, the level of NO, as well as the expression and localization of iNOS in maxillary sinus mucosa of MSFB patients. These patients showed dramatic increase in iNOS protein expression and NO metabolite content in plasma cells and lymphocytes. Values were lower in the CRS group and lowest in the control. These findings are consistent with findings in previous studies [14]. Our study is the first to explore NO metabolites level and iNOS expression in human MSFB and analyze their correlation with MSFB and CRS. Thus, our study reveals the roles of NO and iNOS levels in MSFB and provide a novel study direction of the diagnosis and treatment of MSFB.

Although a growing body of evidences indicated that NO fulfills critical roles in various processes related to nasal diseases, it remains confusing whether elevated NO and iNOS play an injurious or protective role in fungal rhinosinusitis. Little has been reported regarding the mechanism of fungal infection of the paranasal sinus, and the etiology of fungal sinusitis is unclear. Normally, in healthy individuals, high levels of NO, originating mainly from the maxillary sinus [13], help maintain the bacteriostatic state in the

paranasal sinuses [15]. Elevated or decreased NO levels might cause nasal cavity and sinus diseases. In recent years, nasal NO (nNO) was shown to act as a marker aiding in the assessment of sinusitis severity. However, accuracy of the nasal NO measurements in the upper airway and paranasal sinuses is controversial. Fu et al. [16] reported that preoperative nNO levels were lower in fungal rhinosinusitis patients than in patients with other unilateral sinus-related diseases, and therefore might be useful in diagnosis. However, Hu et al [17] using immunohistochemistry, showed significantly higher NO and iNOS content in noninvasive allergic fungal sinusitis. There are two reasons for this difference. First, the type of fungal sinusitis was not classified. Hu studied NO and iNOS content in noninvasive allergic fungal sinusitis, while Fu did not take into account differences between subtypes of fungal sinusitis. Secondly, there were differences in the process of producing NO from the sinus mucosa, and exhaling it. The anatomical variation of the middle nasal passage and nasal cavity have a great influence on the results. It is uncertain whether the nNO levels measured are the consequence of nasal NO production in the paranasal sinuses and/or are the consequence of mid-nasal meatus obstruction by a nasal polyp or morphological abnormality [18–20]. Since there is no noninvasive method to directly measure NO content in the maxillary sinus, we measured the content of NO metabolites in the sinus mucosa. Expression of iNOS, we found, reflects the involvement of NO in the occurrence and development of unilateral MSFB. In our study, increased NO level and enhanced expression of iNOS protein were detected in maxillary sinus mucosa of the MSFB group, which agrees with Hu's findings.

Moreover, for fungal balls in the maxillary sinus, it is currently believed that fungal spores exist in the nasal cavity and nasal sinuses for a long time, and that under specific conditions they start reproducing and causing the formation of a growing mycelium. We hypothesize that inhaled air might form a vortex in the maxillary sinus as it flows through the middle nasal canal, creating anoxic environment [21]. Local anoxia can serve as an initiating stimulus to trigger local inflammatory response. At the same time, hypoxia-induced deterioration of the microenvironment can increase the expression of some cytokines, which can induce the increase in iNOS expression.

Related studies have shown that fungal rhinosinusitis is a type of immune disease, caused by fungal antigens and mediated by IgG and IgE [22, 23]. During the immune response, a large number of inflammatory cells are produced, and iNOS and NO content increase accordingly. In addition, bacteria and fungi can be detected simultaneously in fungal ball. The bacterial superantigen theory, proposed by Schubert [24], suggests that superantigen or exotoxin produced by bacteria can be presented through a special route, activate T-lymphocytes through non-specific pathways, and thus amplify the immune response. As a biological messenger active in a variety of cells, NO mediates the interaction of various cytokines, leading to inflammation. Inflammatory stimuli, such as cytokines, cause iNOS to be highly expressed [25]. Our study indicates that expression of iNOS isoforms and NO metabolite level in the MSFB group were much higher than those in the CRS group. These results agree with a previous viewpoint. It is suggested that the MSFB and CRS groups belong to two different routes of inflammatory disease mechanisms [15]. Moreover, high level of NO and iNOS might affect and contribute to the pathophysiology and molecular mechanism of sinus fungal ball inflammation. In future studies, the

effects of NOS inhibitors should be considered in order to evaluate their possible role in preventing recurrence.

## Conclusion

Our study shows that iNOS expression and NO level were considerably higher in MSFB mucosa than that in tissue samples from CRS or normal mucosa. The expression was localized to plasma cells and lymphocytes. Therefore, our results indicate that iNOS monitoring could be a useful marker helping distinguish between CRS and MSFB. This quantitative pattern shows that iNOS and NO play a role in the pathophysiology of fungal rhinosinusitis, especially in patients with fungal ball. Future studies should focus on the role of iNOS and NO in the diagnosis of fungal ball. The small sample size in this study might have affected the power of the statistical analysis. A large-scale study that includes a larger number of patients should be conducted to confirm the utility of evaluating levels of NO and iNOS expression to differentiate between fungal and CRS cases. Despite these limitations, this is the first study to confirm that NO and iNOS in sinus are associated with the development of human MSFB. We believe that our results afford a better understanding of the pathogenic mechanism involved and provide a new indicator to assist the diagnosis of MSFB and its distinction from CRS..

## Declarations

- **Ethical Approval and Consent to participate**

All procedures used in this study complied with the ethical standards of Dalian Medical University. The study protocol was approved by the Institutional Review Board, Dalian Medical University (2019-052).

- **Consent for publication**

Not applicable

- **Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

- **Competing interests**

There are no conflicts of interest to declare.

- **Funding**

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- **Authors' contributions**

Hangjin Li, Bo Yu and Lingyan Zhang analyzed and interpreted the patient data. Hangjin Li was a major contributor in writing the manuscript. Hangjin Li and Wei Zhang acquired data, and analyzed and interpreted it. Yan Guo made substantial contributions to the conception. Jizhe Wang and Hui Qu designed of the work and ensured that all listed authors had approved the manuscript before submission, including the names and order of authors. All authors read and approved the final manuscript.

## Acknowledgements

Not applicable

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## Figures

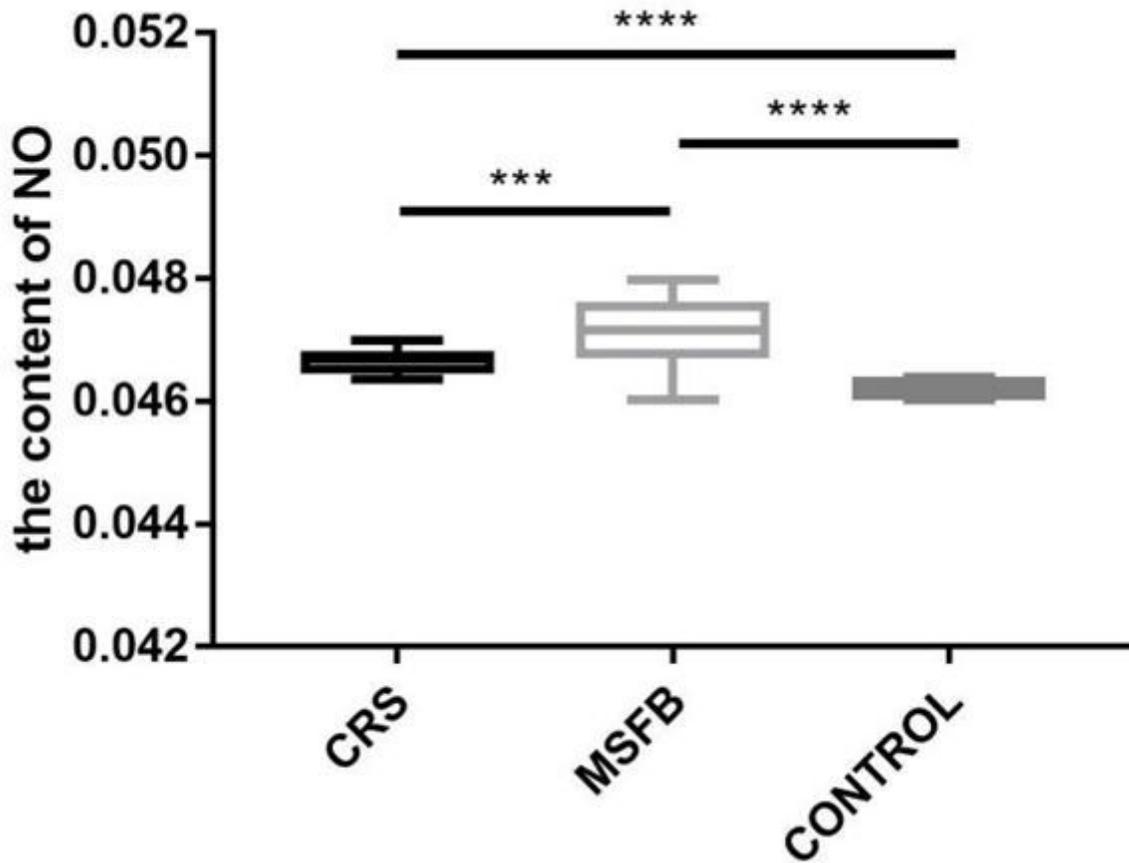
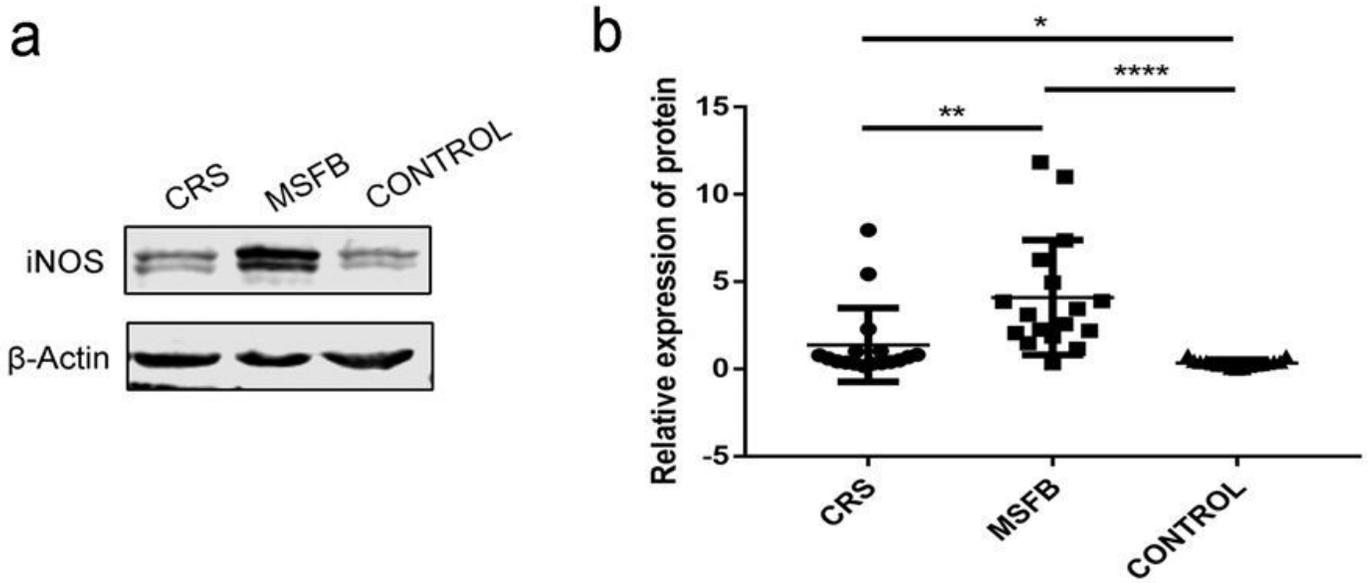


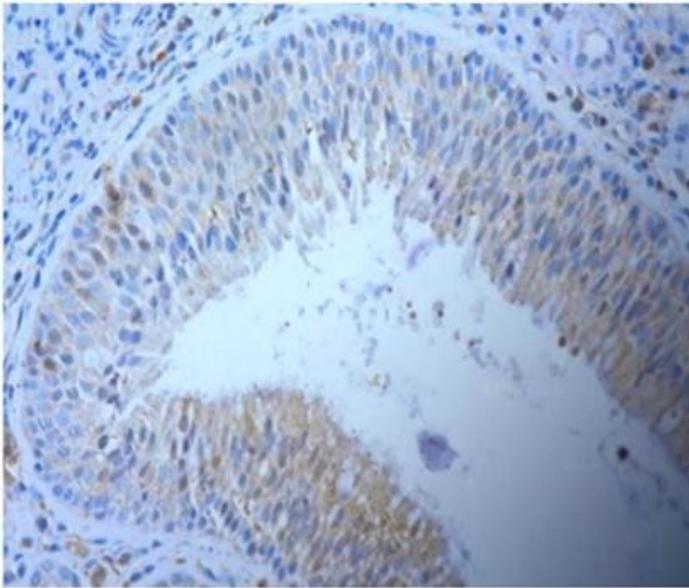
Figure 1

NO content in the three groups of samples by nitrate reductase method. MSFB and CRS had a higher amount of NO compared to the control group ( $p < 0.001$ ). Meanwhile, MSFB had a significantly increased amount of NO compared to CRS ( $p < 0.01$ )

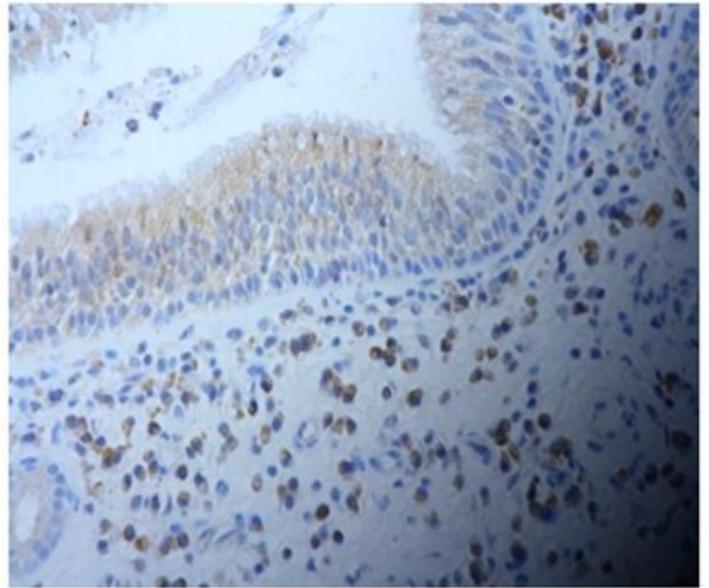


**Figure 2**

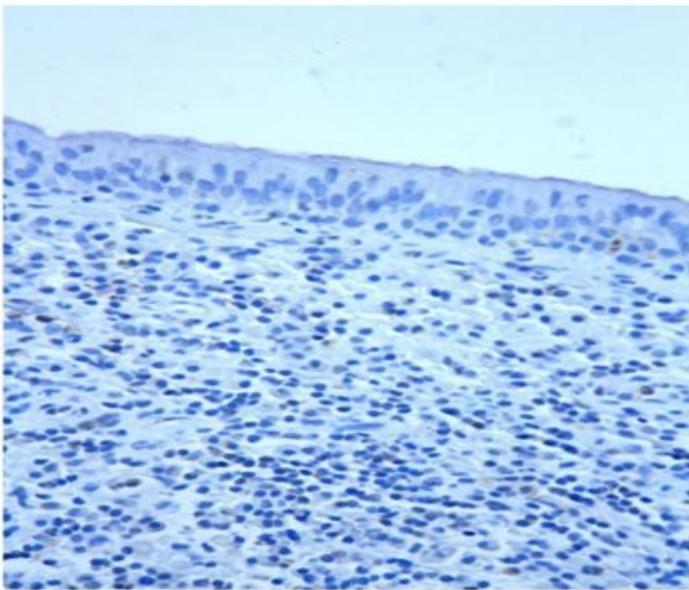
Western blotting determination of iNOS protein in different groups. (a) iNOS protein was expressed more prominently in MSFB group compared to those of CRS and the control. The double iNOS bands size were approximately 127kDa and 131kDa. (b) Quantitative analysis of iNOS expression in each group



CRS



FRS



CONTROL

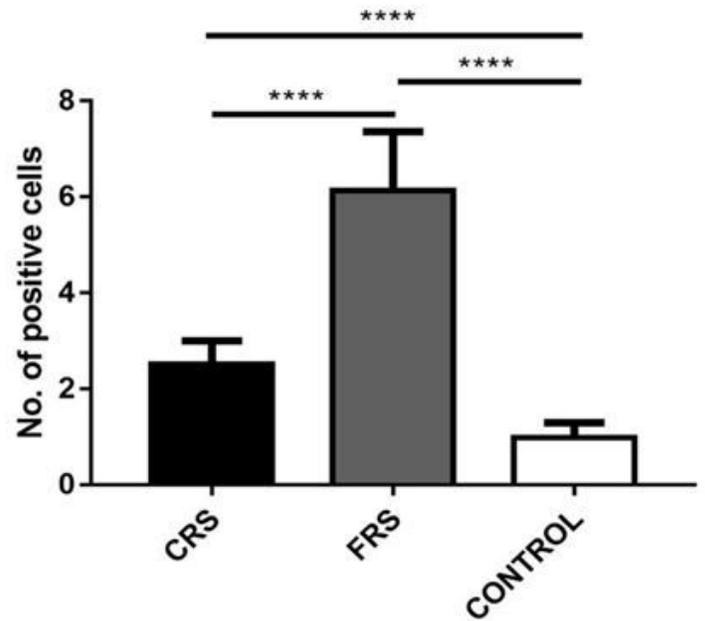


Figure 3

Representative iNOS expression in each group by Immunohistochemistry. The iNOS-positive cells were stained brown. The number of iNOS-positive cells were counted in each group, and the differences were statistically significant