ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
October 2014; 13(5):324-334.

Effect of Dexamethasone and *Nigella sativa* on Inducible Nitric Oxide Synthase in the Lungs of a Murine Model of Allergic Asthma

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Received: 4 May 2013; Received in revised form: 28 July 2013; Accepted: 29 September 2013

ABSTRACT

The aim of this study was to investigate the effects of *Nigella sativa* (NS) fixed oil in comparison to dexamethasone (Dex) on inducible nitric oxide synthase (iNOS), peripheral blood eosinophils (PBE), allergen specific serum IgG1 and interleukins and airway inflammation in a murine model of allergic asthma.

Thirty-one mice were divided into four groups. Group I (n = 6) served as the control group. Group II (n = 10) mice were sensitized intraperitoneally and challenged intratracheally with cone albumin with no treatment. Group III(n = 6) mice were sensitized, challenged, and treated with Dex for 17 days starting at 24 hours after the first challenge. Group IV (n = 9) mice were sensitized, challenged, and treated with NS fixed oil for 17 days as well. For all groups, the following procedures were carried out: immunohistochemical study of iNOS in lung tissues, detection of PBE percentage, and histopathological examination of lung tissues for inflammatory cells.

Lung tissue iNOS expression increased in sensitized, non-treated mice compared with controls, but this increase was not significant. NS fixed oil treatment significantly reduced PBE and lung inflammation but did not significantly reduce lung tissue iNOS expression compared with the control group. These effects were comparable to the effects of Dex.

These results suggest that Nigella sativa exhibits immunomodulatory and anti-inflammatory effect which may be useful for treatment of allergic asthma.

Keywords: Asthma; Dexamethasone; Mice; *Nigella sativa;* Nitric Oxide Synthase

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Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

INTRODUCTION

Asthma is an inflammatory disease of the airways characterized by airway obstruction and increased airway responsiveness that affects about 300 million people of all ages worldwide and is increasing by 50% per decade.[1]

Asthmatic inflammation develops when the sequential interaction of inflammatory cells with resident cells generates a cascade of events that contributes to the chronic inflammation and clinical manifestations associated with the disease, including further inflammation, airway smooth muscle spasm, airway mucus secretion, airway edema and narrowing, and bronchial epithelial damage.[2]

Animal models of allergic asthma exhibit many of the features of human asthma, including airway hyper-responsiveness, airway inflammation, and increased serum IgE levels.[3,4] Nitric oxide is a short-lived molecule that has been shown to have a number of important biological functions in various diseases, including asthma.[5,6] Nitric oxide is generated from L-arginine by the enzyme nitric oxide synthase (NOS), of which there are three known isoforms. Types I and III of NOS are found predominantly in neurons and the endothelium, respectively, and they are constitutively expressed and are dependent upon calcium for activity. The third isoform, NOS II, can be expressed by a wide range of cells primarily after it has been induced by certain cytokines, microbes, or microbial products.[7]

Several studies have demonstrated increased levels of NO in the airways in animal models of asthma and in human patients with asthma.[8,9] Measurement of exhaled NO has been suggested as a method to monitor airway inflammation in asthma, especially in cases of exacerbated asthma[10] and after oral steroid therapy.[11]

Corticosteroids are the most potent nonspecific anti-inflammatory agents. Because of the many undesirable side effects of systemic corticosteroids, inhaled corticosteroids are used as the first line of treatment for asthma and are an effective means of reducing inflammation and bronchial constriction in the majority of patients.[12] Although the most frequently reported side effects of inhaled corticosteroids are local, systemic side effects also have been reported. Adrenal suppression, decreased bone metabolism, and decreased growth are of particular concern in children, a group in which asthma is increasing in frequency.[13] Thus there is a need for new or alternative approaches to control this disease.

Nigella sativa (NS) is a grassy plant related to the Ranunculaceae family that has been used as a herb in traditional medicine for the treatment of a variety of diseases, including diarrhea and asthma, for a long time by different populations. The crude extract of NS seeds exhibits spasmolytic and bronchodilator activities mediated possibly through calcium channel blockade, and this activity is concentrated in the organic fraction. Recent results from the literature have indicated beneficial effects of *N. sativa* in respiratory allergies. Therefore, its usefulness for diarrhea and asthma in traditional medicine appears to be based on a sound mechanistic background.[14,15] Although NS is used to treat a variety of diseases, including asthma, few studies on the efficacy and mechanisms of action of NS in the treatment of asthma exist.

This study aimed to investigate the effects of NS fixed oil (derived from NS) in comparison to dexamethasone (Dex) on inducible nitric oxide synthase (iNOS), peripheral blood eosinophils (PBE), serum IgG1, and airway inflammation in a murine model of allergic asthma.

MATERIALS AND METHODS

Experimental Protocol

Thirty-one approximately 6-week-old male (CD1) albino mice weighing 18-20 g were purchased from the Experimental Research Center of the Theodor Bilharz Institute (Cairo, Egypt). They were maintained in animal facilities of the Biotechnology Research Laboratory at the Gastroenterology Surgery Center at Mansoura University and were provided with food and water ad libitum. All animal experiments were conducted according to the guide for the care and use of laboratory animals prepared by the National Academy of Sciences and published by the National Institutes of Health.

The animals were divided into four groups. Group (n=6) were neither sensitized nor treated and kept as controls. Group II (n=10) mice were sensitized and challenged with conalbumin (Sigma Chemical Co., USA). Group III (n=6) and Group IV (n=9) mice were treated with dexamethasone or *Nigella sativa* fixed oil (purchased from a local herb store, Mansoura, Egypt) after respiratory challenge, respectively.

Antigen Sensitization, Challenge, and Treatment

Mice were sensitized by two intraperitoneal injections of 200 µg of cone albumin and 2 mg of alum in 0.3 ml of PBS one week apart. Seven days after the last sensitization, the mice were anesthetized and challenged intratracheally by aspiration.[16] Intratracheal challenges with conalbumin were repeated on days 20 and 30. Twenty-four hours after the first antigen challenge, the third group of mice (Group 3) received 0.5 mg/kg of dexamethasone intraperitoneally.[17] Group 4 was shamven *N. sativa* fixed oil at a dose of 5 ml/kg intragastrically using a 25-gauge stainless steel blunt feeding needle.[18] All mice in Groups III and IV were treated with dexamethasone and *N. sativa*, respectively, once per day for the next 17 consecutive days.[17] Twenty-four hours after the last treatment, animals of all groups were sacrificed by decapitation, and blood samples were collected. Blood films were produced directly, and then sera were separated by centrifugation at 3000 g for 10 min and stored at -20°C. The lungs were removed and fixed in neutral buffered formaldehyde then sectioned for histopathological studies.

Peripheral Blood Eosinophils

Peripheral blood eosinophils of all mice were examined using Leishman stain film and light microscopy.

Measurement of Serum IgG1

Sera were examined for IgG1 in mice by enzyme-linked immunosorbent assay (ELISA).[19] A polystyrene plate was coated with 50 g/ml of conalbumin in a coating buffer (pH 9.6) and incubated overnight at room temperature. After washing, the free binding sites were blocked by 200 µ/well of 0.2% non-fat milk in a coating buffer for 1 hour at room temperature. After washing, 50µl of 1:100 dilution of mouse serum were added per well; the plate was incubated at 37°C for 3 hours then washed. Alkaline phosphatase-labeled anti-mouse IgG1 at a dilution of 1:500 in a conjugate buffer was also added. The plate was incubated for 1 hour at 37°C. For development of the color reaction, the plate was incubated with 50µl/well of freshly prepared paranitrophenyl phosphate in a substrate buffer at 37°C for 30 minutes. Optical densities were read at 405 nm using a micro-ELISA plate.

Histopathological Examination

Paraffin sections (5-6 µm thick) were stained with hematoxylin and eosin and examined for the presence of peribronchial inflammation. The peribronchial inflammation was staged according to the severity of inflammation from 1+ to 3+ (1+: inflammation around the bronchiole which occurs only in a focal place; 2+: inflammation surrounds the bronchiole in five or less cell lines; and 3+: inflammation surrounds the bronchiole in more than five lines of cells).[20]

Detection of iNOS by an Immunohistochemical Technique

Slides were deparaffinized and blocked for endogenous peroxidase with 1.75% hydrogen peroxide in methanol for 20 minutes. Antigen retrieval was performed using a citrate solution in a 90°C water bath for 30 minutes. The slides were blocked by normal horse serum for five minutes at 37°C. The polyclonal antibody (rabbit IgG polyclonal antibody NOS2) (M-19), which was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), in 1:10 dilution was applied overnight in a humid medium at room temperature followed by a biotinylated goat anti-polyvalent secondary antibody for 15 minutes at 37°C and then the streptavidin peroxidase complex for 15 minutes at 37°C. For the chromogen, 3,3 Diaminobenzidine (DAB) was used for 20 minutes at room temperature. Slides were counterstained with Meyer's hematoxylin, dehydrated, and coverslipped. Staining results were interpreted according to the scheme reported by previous authors.[21,22]

Statistical Analysis

We used the Statistical Package for the Social Sciences (SPSS) version 10.0.1, 1999 (SPSS Inc., Chicago, IL, USA). Our data showed a nonparametric distribution by the Kolmogorov-Smirnov test. For comparison of continuous variables, we used the Mann-Whitney-U test, and for comparison of categorical variables, we chose the Chi-Square test. Significance was considered when the p-value was <0.05; all p-values reported are of the double-sided type.

RESULTS

We found a non-significant increase in iNOS expression for both epithelial and inflammatory cells in sensitized mice compared with controls (Table & Figure 1). Treatment inhibited iNOS expression in epithelial cells; however, the level of inhibition did not reach

statistical significance. We also noted a decrease in inflammatory cell iNOS expression in the NS-treated mice compared with the sensitized group. Moreover, epithelial iNOS expression showed no significant differences between the sensitized group and each of the Dex and NS-treated groups (Table 1 & Figure 1).

Table 1. Effect of Nigella sativa and dexamethasone on iNOS score on conealbumin synthesized mice

Variables		GI (n=6)n (%)	GII (n=10)n (%)	GIII (n=6)n (%)	GIV (n=9)n (%)
iNOS-epithelial score					
	Zero iNOS score	1 (16.7)	0 (0)	1 (16.7)	0 (0)
	Low iNOS score	4 (66.7)	4 (40)	3 (50)	4 (44.4)
	High iNOS score	1 (16.7)	6 (60)	2 (33.3)	5 (55.6)
iNOS-inflammatory score					
	Zero iNOS score	1 (16.7)	0 (0)	1 (16.7)	0 (0)
	Low iNOS score	4 (66.7)	5 (50)	5 (83.3)	8 (88.9)
	High iNOS score	1 (16.7)	5 (50)	0 (0)	1 (11.1)

GI. sham control group, GII. Sensitized group, GIII. Sensitized and Dex treated group GIV sensitized and *Nigella sativa* treated group.

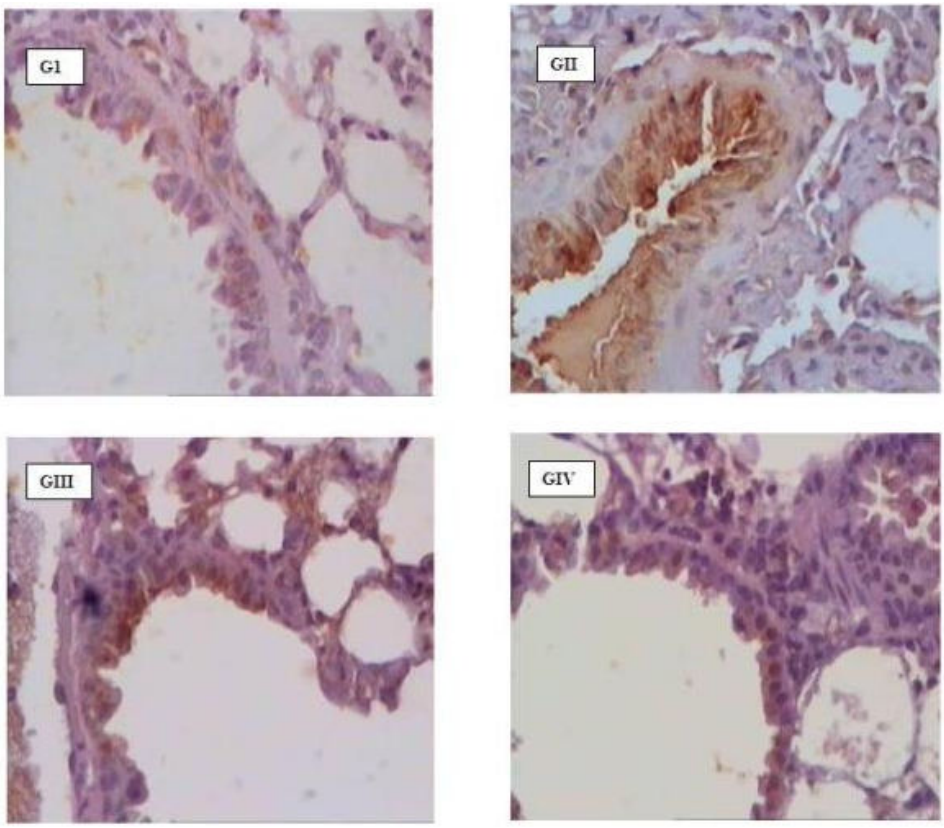

Figure 1. Photomicrographs of immunohistochemical staining for inducible nitric oxide synthase in 4 µm sections of formalin-fixed, paraffin-embedded lung samples (1): G1.Very Low iNOS expression of bronchial epithelium with occasional peribronchial inflammatory cells. GII. Evident iNOS expression of bronchial epithelium with sparing of peribronchial inflammatory cells. GIII. Low iNOS expression of most of bronchial epithelial cells with cytoplasmic reaction. GIV. Most of bronchial epithelial cells as well as peribronchial inflammatory cells expressing iNOS in low intensity (Peroxidase x 400).

Table 2. Effect of Nigella sativa and dexamethasone on Serum IgG_1 and peripheral blood eosinophils on conealbumin synthesized mice

Variables	GP I (n=6)	GP II (n=10)	GP III (n=6)	GP IV (n=9)
IgG1 (mg/dL)				
Mean ± SD	0.16 ± 0.075	2.22 ± 0.086*	1.82 ± 0.28#	1.98 ± 0.23#
Eosinophils (%)				
Mean ± SD	0.67 ± 0.82	4.30 ± 1.70*	1.67±2.07#	2.44 ± 1.59#

GI. sham control group, GII. Sensitized group, GIII. Sensitized and Dex treated group GIV sensitized and *Nigella sativa* treated group.

* Significant difference between sensitized and control group; $p<0.05$ by Mann Whitney U-test

#Significant difference between treated groups and sensitized group; $p<0.05$ by Mann Whitney U-test

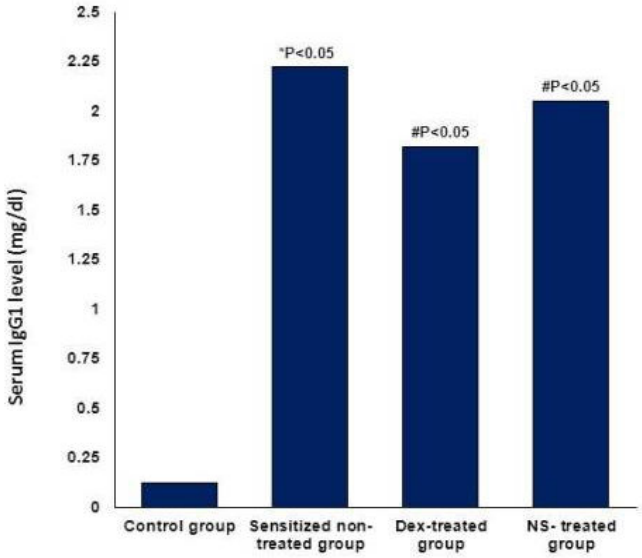

Figure 2. This Figure shows serum IgG_1 level in sham control group, cone albumin sensitized group, sensitized group and Dex treated and sensitized group and *Nigella sativa* treated.

*** Significant difference between sensitized and control group; $p<0.05$ by Mann Whitney U-test**

#Significant difference between treated groups and sensitized non treated group; $p<0.05$ by Mann Whitney U-test.

Table 3. Histopathological findings of the effect of Nigella sativa and dexamethasone on lung tissues of conealbumin synthesized mice

Histopathological score	G I (n=6) n (%)	G II (n=10) n (%)	G III (n=6) n (%)	G IV (n=9) n (%)
No inflammation	3 (50)	0 (0)	3 (50)	3 (33.3)
Focal inflammation	3 (50)	5 (50)*	3 (50) #	5 (55.6)
All around inflammation (but less than 5 cell lines)	0 (0)	5 (50)	0 (0)	1 (11.1)

GI. sham control group, GII. Sensitized group, GIII. Sensitized and Dex treated group

GIV sensitized and *Nigella sativa* treated group.

* Significant difference between sensitized and control group; $p<0.05$ by Mann Whitney U-test

#Significant difference between treated groups and sensitized group; $p<0.05$ by Mann Whitney U-test

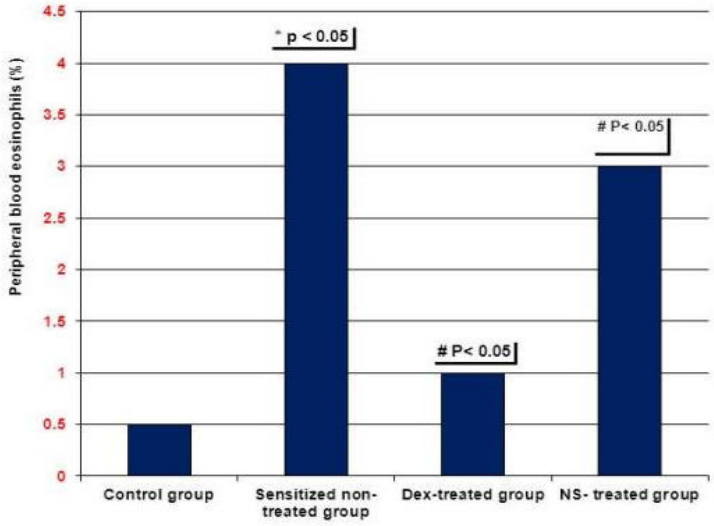

Figure 3. Serum peripheral blood eosinophil measurement in sham control group, conalbumin sensitized group, sensitized group and Dex treated and sensitized group and *Nigella sativa* treated.

*** Significant difference between sensitized and control group; $p<0.05$ by Mann Whitney U-test**

[#]Significant difference between treated groups and sensitized non treated group; $p<0.05$ by Mann Whitney U-test.

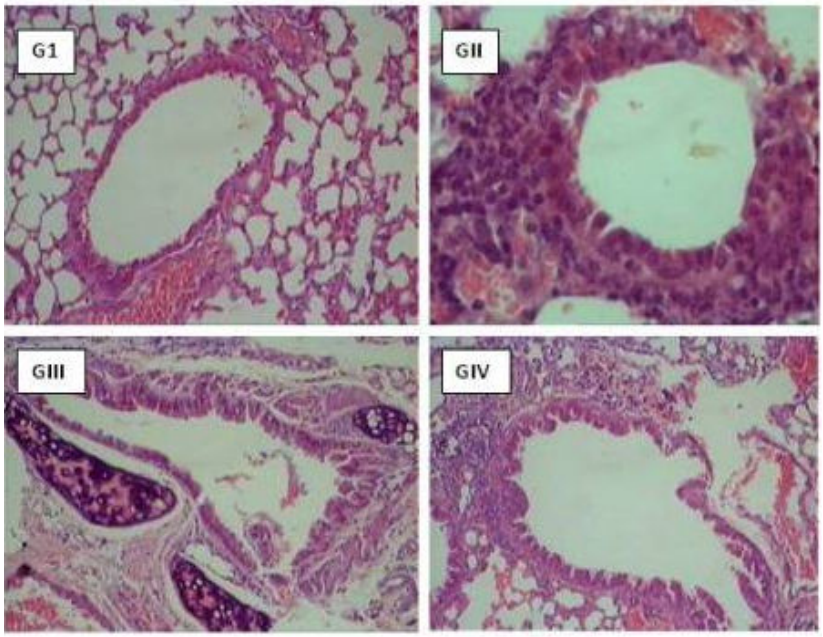

Figure 4. These Photomicrographs showed normal small bronchus without detectable inflammatory reaction. GII, Shows all around peribronchial inflammation, forming less than 5 cell layers. GIII, Main bronchus without detectable inflammatory reaction. GIV, Small focal peribronchial inflammatory reaction around a small bronchus (H&E., X100)

We identified a significant increase in serum IgG1 levels in the sensitized non-treated group when compared with the control group ($p<0.05$). We considered this result to be evidence for successful sensitization. After treatment with Dex, there was a significant decrease in the serum IgG1 level in the Dex-

treated group when compared to the sensitized, non-treated group ($p<0.05$). After treatment with NS, the serum IgG1 level significantly decreased in comparison with the sensitized, non-treated group ($p<0.05$) (Table 2 & Figure 2).

The sensitized, non-treated group of mice in this study showed a significantly increased peripheral blood eosinophilic percentage compared with the control group ($p<0.05$). The group of mice treated with Dex during the airway allergen challenge exhibited a significantly reduced percentage of circulating eosinophils compared with the sensitized, non-treated group ($p<0.05$). In addition, the percentage of circulating eosinophils decreased significantly in the NS-treated group compared to the sensitized, non-treated group ($p<0.05$), as shown in Table 2, and Figure 3.

We observed a significant increase in the number of inflammatory cells invading the lungs in the sensitized, non-treated group when compared with the control group ($p<0.05$) (Figures 3, 4). We also found a significant decrease in the number of inflammatory cells when we compared the Dex-treated group with the sensitized, non-treated group ($p<0.05$). The group of mice that was treated with NS showed a decrease in the number of inflammatory cells invading the lungs compared with the sensitized, non-treated group; however, the difference did not reach statistical significanceusing the Pearson Chi-Square test ($p=0.06$, Table 3, and Figure 4).

DISCUSSION

In this study, we identified a non-significant increase in iNOS expression for both epithelial and inflammatory cells in sensitized mice compared with controls. This result suggests that iNOS may have a limited, if any, role in allergic asthma in mice. Indeed, the relationship between iNOS and asthma is controversial. Previous studies have demonstrated that constitutive Nitric Oxide Synthase (cNOS), not iNOS, is important in allergic inflammation in a rat model[23] and that cNOS was markedly elevated in the airway tissue of mice exposed to ozone, while iNOS did not increase after the same exposure.[24] In guinea pig airways, neural NO-induced relaxation is impaired in allergic inflammation of the airways, even though no change in Neuronal Nitric Oxide Synthase(nNOS) expression has been reported, which indicates altered neural NOS activity in the presence of allergic inflammation that leads to the exacerbation of asthma.[25]

The arginase enzyme controls the transformation of arginine into ornithine, which in turn gives rise to proline and polyamines. Arginine also serves as a substrate for NOS, which generates NO, a critical regulator of airway physiology. The NOS and arginase pathways interfere with each other through substrate competition. During allergic inflammation, increased IL-4 and/or IL-13 (Th2 cytokines) expression results in increased expression of arginase and amplification of the arginase-dependent pathway, with concomitant suppression of NO generation. This process leads to airway hyperresponsiveness and increased generation of mucus and collagen, all of which may contribute to the pathogenesis of asthma.[26] In this case, it appeared that NO inhibition resulted of substrate competition because the expression of arginase (but not NO synthase) was altered in the lungs of the allergen-challenged mice.[27]

Moreover, some studies have suggested that iNOS may be an important mediator in combating infections or in other neutrophilic inflammation excluding allergic inflammation. One study demonstrated that iNOS-deficient mice were as susceptible as steroid-suppressed wild mice to tuberculosis infection in three examined sites (liver, lung, and spleen).[28] In a similar study from another lab, dependence on iNOS in combating infection was manifested in the liver and spleen but much less so in the lung as assessed by colony counts.[29] This last observation was consistent with our finding that iNOS expression in the lungs was increased (but not significantly) following allergic inflammation. In lung transplant recipients, expression of epithelial iNOS is increased and reflects the degree of neutrophilic airway inflammation.[30] Additionally, iNOS in patients with nasal polyps did not correlate with the occurrence of asthma.[31]

In contrast to our results, iNOS was found to be up-regulated in asthma.[10] Another study demonstrated that the expression of iNOS was much higher in ovalbumin (OVA)-induced mice compared with the negative controls.[32]

In this study, we demonstrated that dexamethasone inhibited iNOS expression, both in epithelial and inflammatory cells; however, the inhibition did not reach statistical significance. Based upon this result, we suggest that iNOS inhibition was not a pivotal mechanism in the anti-inflammatory role of steroids in

asthma. In agreement with this finding, another study[33] demonstrated that neither dexamethasone nor budesonide inhibited iNOS mRNA induction in human airway epithelial cells derived from participants with asthma, indicating that these cells do not appear to be directly regulated by glucocorticoids. In contrast to our results, steroid treatment has been previously shown to diminish iNOS expression in cultured cells and to cause a significant reduction in the level of NO in exhaled air of asthmatic patients.[34]

We found a decrease in inflammatory cell iNOS expression in NS-treated mice compared with the sensitized group; however, the difference was not statistically significant. Moreover, epithelial iNOS expression showed no significant difference between the sensitized group and each of the Dex and NS-treated groups. Therefore, we can suggest that NS has an effect on inflammatory and epithelial cell iNOS expression in a similar manner as that of Dex.

In the mouse, two classes of immunoglobulins can sensitize mast cells and trigger anaphylaxis, namely the IgE and IgG isotypes.[35,36] In one study, it was demonstrated that pollen-specific IgE and IgG1 were increased in the serum of rats immunized with pollen grains; the IgG1 antibody response was much higher compared to the IgE response.[37] Mast cells sensitized by IgG could play a role in IgE-deficient mice by secreting mediators and cytokines.[38] Both IgE and IgG1 increased in a murine model of allergic asthma and decreased following Dex treatment.[30] Another murine model of allergic asthma showed a decrease in IgG1 and IgE ovalbumin-specific antibody production after oral NS oil administration in mice.[39] In human studies, several indications that atopic individuals have a reduced capacity to suppress certain immune responses, namely the formation of IgE and IgG antibodies to ingested and inhaled common antigens.[40,41] Another study showed that children produced IgG1 antibodies (but not IgG4) against inhaled allergens between 3 and 12 months of age.[42]

Our study demonstrated increased serum IgG1 levels in the allergic model of asthma with a significant reduction of these levels in both Dex- and NS-treated groups. From these results, we can also suggest that NS has an immunoregulatory effect similar to that of Dex and this effect may be favorable for the treatment of allergic asthma.

In our study, we observed a significant increase in the number of inflammatory cells invading the lungs in the sensitized, non-treated group when compared with the control group. These observations are consistent with some previous studies that have revealed eosinophil, lymphocyte, neutrophil, and monocyte invasion of the lung tissues after sensitization in murine models of atopic asthma.[43,44] We considered this finding a sign of successful sensitization.

We found a significant decrease in the number of inflammatory cells when comparing the Dex-treated group with the sensitized, non-treated group. Dex significantly reduced the accumulation of eosinophils and chronic inflammatory cells in murine chronic asthma.[44,45] Other studies used budesonide and obtained the same results.[46,47]

The group of mice that was treated with NS showed a decrease in the number of inflammatory cells invading the lungs compared with the sensitized, non-treated group, but the difference was not statistically significant using the Pearson Chi-Square test.[39] These data suggest that NS has an anti-inflammatory role in bronchial asthma supporting its use as an alternative (in mild cases) or an adjunct (in moderate and severe cases) to corticosteroids for the long-term control of bronchial asthma, although the effect is less pronounced than that of Dex.

Peripheral blood eosinophilia was documented in bronchial asthma both in humans[48] and in murine models.[49] The development of airway eosinophilia involves eosinopoiesis in the bone marrow and release into the circulating blood, as well as recruitment of eosinophils from the blood into the airways.[50,51] In human studies, eosinophilia was defined either as an absolute count of more than 250-400 cells/cmm[52] or as 5% or more of the total leukocyte count.[53] In this study, we found a significantly increased peripheral blood eosinophilic percentage in the sensitized, non-treated group of mice compared with the control group. Previous studies have documented bone marrow and peripheral blood eosinophilia following airway allergen challenges in mice[54] and in guinea pigs.[55]

The group of mice treated with Dex during an airway allergen challenge exhibited a significantly reduced percentage of circulating eosinophils compared with the sensitized, non-treated group. The reduction of eosinophils in the circulation and tissues, including the airways and bone marrow, by systemically administrated corticosteroids has been reported previously in normal human participants,[56] rats,[57] mice,[58] and patients with asthma.[59]

In addition, the percentage of circulating eosinophils decreased significantly in the NS-treated group compared with sensitized, non-treated group. Our observation that there was a reduced number of airway inflammatory cells (including eosinophils) after the respective Dex and NS treatments may be mediated through the decreased availability of inflammatory cells (including eosinophils) in the peripheral circulation. This finding supports our hypothesis that NS may have an anti-inflammatory role in allergic asthma.

It could be concluded that NS exhibited airway anti-inflammatory and immunomodulatory effects on PBE and serum IgG1V supporting its use as an alternative (in mild cases) or an adjunct (in moderate and severe cases) to corticosteroids in the treatment of asthma.Further investigations are needed to study the effect of different doses of NS on iNOS.

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