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Oral *Nigella sativa* oil ameliorates ovalbumin-induced bronchial asthma in mice

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ABSTRACT

Nigella sativa oil (NSO) is used in folk medicine as a therapy for many diseases including bronchial asthma. We investigated the possible modulating effects of NSO on asthma-like phenotypes in a mouse model of bronchial asthma. BALB/c mice were actively sensitized by intraperitoneal injections of 50 μg ovalbumin (OVA) with 1 mg alum on days 0 and 12. Starting on day 22, they were exposed to OVA (1% (w/v), in sterile physiological saline) for 30 min, three times every 4th day. Negative control animals were exposed to saline in a similar manner. NSO was administered orally for 31 day from day 0 to day 30. On the day of sensitization and challenge, NSO was given 30 min before the treatment. Airway function, number of inflammatory cells in bronchoalveolar lavage fluid (BALF), levels of interleukin (IL)-4, IL-5, IL-13 and interferon (IFN)-γ in BALF, serum levels of total IgE, OVA-specific IgE, IgG1 and IgG2a, and histopathological examination of lung tissues were investigated. Oral treatment with NSO showed significant decrease in airway hyperresponsiveness, the number of total leukocytes, macrophages and eosinophils, levels of IL-4, IL-5 and IL-13 in BALF, serum levels of total IgE, OVA-specific IgE and IgG1, and significant increase in BALF level of IFN-γ and serum level of OVA-specific IgG2a, indicating restoration of local Th1/Th2 balance. Furthermore, it significantly abrogated the histopathological changes of the lungs, as the images were nearly normal. These results suggest that the treatment with oral NSO could be a promising treatment for bronchial asthma in humans.

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1. Introduction

Bronchial asthma is an inflammatory disorder of the airways affecting about 300 million persons of all ages worldwide and increasing by 50% per decade [1]. It is characterized mainly by structural and functional abnormalities of the bronchial epithelium, accumulation of inflammatory cells in the bronchial mucosa, remodeling of the airway tissue structure, and airway hyperresponsiveness (AHR). Many kinds of cells play important roles in the development of bronchial asthma, in particular, T lymphocytes, eosinophils, macrophages, neutrophils, mast cells and epithelial cells [2,3]. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and coughing, especially at night and early in the morning. These episodes are usually associated with variable airflow obstruction that is often reversible. Allergic airway inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli [4].

Allergic airway inflammation is thought to be occurred due to the imbalance of Th1/Th2 cytokine production with over-production of Th2 cytokines such as interleukin (IL)-4, IL-5 and IL-13, and at the same time, Th1 response is suppressed with the decrease in interferon (IFN) -γ production. IL-4 is a principal Th2 cytokine for the production of IgE and for the development of Th2 cells from naïve $CD4^+$ T cells. IL-5 increases the number of eosinophils in the airway. IL-13 is involved in AHR and mucus secretion in the airway. In contrast, IFN-γ produced by Th1 cells can inhibit the inflammatory process of asthma [4–6]. However, the exact mechanism of the pathogenesis of asthma is still unclear.

Although currently available treatments for management of asthma, including inhaled corticosteroids and β_2 agonists, target at dampening airway inflammation and relaxing airway smooth muscles, the disease attacks and exacerbations still occur, as they do not alter the underlying pathology [7]. Moreover, inhaled corticosteroids have several local and systemic side effects including, oropharyngeal candidiasis, dysphonia, lingual hypertrophy, pharyngitis, growth failure, bone metabolism disorders, adrenal suppression and glaucoma, especially, if used with large doses and for long time [8]. In addition, patients receive β_2 agonists regularly for treatment of asthma, frequently, experience tremor, palpitations and the most important is tolerance to the bronchodilatory effect of β_2 agonists [9]. Therefore, these facts highlight the need for novel molecules with fewer side effects, preferably of natural origin, for the treatment of allergic asthma.

Nigella sativa is a grassy plant belonging to the Ranunculaceae family with green to blue flowers and small black seeds. The black

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seeds have been used for thousands of years in many of the Middle East and Far East countries as a spice and food preservation. Both the seed and its oil (*N. sativa* oil, NSO) are used as nutritional supplements. NSO is used in folk medicine as a natural therapy for many symptoms and diseases such as headache, fever, dizziness, inflammation, cough, bronchitis, eczema, asthma, hypertension, diabetes and gastrointestinal disorders [10–12]. NSO is so beneficial due to its content of over 100 constituents such as aromatic oils, trace elements, vitamins and enzymes. Moreover, four alkaloids namely thymoquinone (TQ), thymohydroquinone (THQ), dithymquinone (DTQ) and thymol (THY) have been isolated from NSO and are supposed to be its active constituents. These constituents are necessary for balancing and strengthening the immune system, enabling it to prevent infections, allergies, and controlling chronic illnesses [13,14]. Indeed, oral administration of NSO was reported to decrease the severity of disease in patients with allergic rhinitis and bronchial asthma [15]. In addition, recent studies on the pharmacological effects of NSO have shown its anti-inflammatory and immunomodulatory effects [16,17]. Among them, several studies showed the inhibitory effect of NS on systemic cytokines levels [16–18]. Moreover, local immune responses in the induction of allergic inflammation in the lungs are reported to be more important than that of the systemic immune responses in a mouse model of allergic asthma [19] and the effect of NSO on local TH1/TH2 cytokines balance is still unclear. Therefore, we investigated the anti-inflammatory and immunomodulatory effects of oral NSO with regard to airway function, antigen-induced inflammatory infiltrates in the airways, local Th1/Th2 cytokine production, serum immunoglobulin levels, and histopathological changes of lung tissues in a mouse model of allergic asthma.

2. Materials and methods

2.1. Animals

Eight-week-old male BALB/c mice (Japan SLC, Hamamatsu, Japan) were used. The animals were housed in plastic cages in an air-conditioned room at 22±1 °C with a relative humidity of 60±5%. The mice were fed a standard laboratory diet and given water *ad libitum*. The mice were divided into 4 groups of 6 mice each; mice of group 1 (saline) were saline injected and challenged as a control group, group 2 (OVA) was ovalbumin (OVA)-induced bronchial asthma group, group 3 (NS1) was OVA-induced bronchial asthma group treated with 1 ml/kg/day NSO, and group 4 (NS4) was OVA-induced bronchial asthma group treated with 4 ml/kg/day NSO. All experiments were carried out following the guideline for the care and use of experimental animals in Gifu Pharmaceutical University with an approval of Animal Experiment Committee of the University.

2.2. Reagents

The following reagents were commercially obtained and used; Ovalbumin (OVA, Seikagaku Kogyo, Tokyo, Japan); *Nigella sativa* oil (NSO, 100% pure, endotoxin free, obtained by cold processing, Egyptian Ministry of Health Registration No. 2906/2002, Eng. Mohamed Dorra Co., Alexandria, Egypt); thymoquinone (TQ) and thymol (THY) (TCI Co., Ltd., Tokyo, Japan); dithymoquinone (DTQ) and thymohydroquinone (THQ) (were prepared from TQ as described by Smith and Tess (1944) [20] and El-Dakhakhny (1963) [21], respectively and their purity and identity are confirmed by nuclear magnetic resonance spectroscopy and melting point); diphenyl sulfone (DPS, TCI); methanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan); 2-propanol (Nacalai Tesque, Inc., Kyoto, Japan); bovine serum albumin (BSA, Millipore, Kankakee, IL, USA); Block-Ace (Yukijirushi Co., Sapporo, Japan); Türk solution (Wako Pure Chemical Industries); sodium pentobarbitone (Abbott Lab., Chicago, IL, USA); disodium ethylenediaminetetraacetic acid (EDTA-2Na) and acetylcholine chloride (Ach) (Nacalai Tesque); Diff-Quick solution (Sysmex, Kobe, Japan); pancuronium bromide (Sigma, St. Louis, MO, USA); monoclonal anti-dinitrophenol (DNP) IgE antibody (monoclonal anti-DNP IgE, clone SPE-7, Sigma); peroxidase-labeled polyclonal anti-mouse IgE goat IgG antibody (Nordic immunological laboratories, Tilburg, Netherland); monoclonal rat anti-mouse IgE antibody (LO-ME-3, Serotec Co., Ltd., Oxford, UK); polyclonal goat anti-mouse IgG1 antibody (STAR81, Serotec); monoclonal rat anti-mouse IgG2a antibody (LO-MG2a-7, Serotec); peroxidase-conjugated streptavidin (Dakopatts a/s, Glostrup, Denmark).

2.3. High-performance liquid chromatographic (HLPC) analysis of NSO

The four supposed active constituents of NSO, namely, TQ, THQ, DTQ and THY were analyzed as described previously by Ghosheh et al. [22]. Briefly, the extraction of NSO constituents were carried out using SEP-PAK C_{18} cartridges (Waters Co., Milford, MA, USA) followed by quantification of the recovered constituents by HPLC (Shimadzu Co., Kyoto, Japan) on a reversed-phase C_{18} analytical column (5 μm× 4.6 mm×150 mm, Inertsil ODS-3, GL Sciences, Inc., Tokyo, Japan), using an isocratic mobile phase of water:methanol:2-propanol (50:45:5% (v/v)) at a flow rate of 2 ml/min. DPS was used as a calibration standard and was added to SEP-PAK eluate. Ultraviolet detection was performed at 254 nm. Calibration curves were obtained by injecting 20 μl of serial dilution of standard solution (100, 50, 25, 12.5 and 6.25 ng/μl for TQ, THQ, and DTQ, and 200, 100, 50, 25 and 12.5 ng/μl for THY) with a constant amount of DPS 200 ng/μl, then plotting concentration versus area under-curve for absorption for each concentration. Three analyses were done for each point on the calibration curves. From the calibration curves, levels of TQ, THQ, DTQ and THY were calculated.

2.4. Sensitization and challenge

Experiments were performed in accordance with the methodology previously reported by Nagai et al. (1997) [23]. Mice were actively sensitized by intraperitoneal injections of 50 μg OVA with 1 mg alum on days 0 and 12. Starting on day 22, they were exposed to OVA (1% (w/v), in sterile physiological saline) for 30 min, three times every 4th day. The negative control animals were exposed to saline in a similar manner. The aerosol (particle size 2.0–6.0 μm) was generated by a nebulizer (Ultrasonic nebulizer UN-701, Azwell Co. Ltd., Osaka, Japan) driven by filling a perspex cylinder chamber (diameter 5.5 cm, height 12 cm) with a nebulized solution. Twenty-four hours after the final antigen challenge, measurement of bronchial responsiveness to Ach, collecting bronchoalveolar lavage fluid (BALF), and collecting lungs for histopathology were performed. The NSO was administered orally for 31 day from day 0 to day 30. On the day of sensitization and challenge, NSO was given 30 min before the treatment.

2.5. Measurement of airway function

Bronchoconstriction was measured according to the overflow method described by Tanaka et al. (2001) [24]. In short, mice were anesthetized with sodium pentobarbitone (60 mg/kg, i.p.) and the jugular vein was cannulated for intravenous injection of Ach. Mice were injected with pancronium bromide (0.1 mg/kg, i.v.) to suppress spontaneous respiration, and ventilated with a rodent ventilator (New England Medical Instruments Inc., Medway, MA, USA) with air supplemented with oxygen at 120 strokes/min and a stroke volume of 0.3 ml/animal. Bronchoconstriction was measured using a bronchospasm transducer (Ugo Basil 7020, Milano, Italy) connected to the tracheal cannula. To evaluate bronchial responsiveness to Ach, changes in respiratory overflow volume were measured using increasing doses of Ach. The increase in respiratory overflow volume provoked by Ach was represented as a percentage of the maximal overflow volume (100%) obtained by clamping the tracheal cannula. The area under the curve (AUC) was calculated from the dose–response curve for Ach (range:

31.25 to 2,000 μg/kg). Briefly, each dose was converted logarithmically then AUC was calculated and represented as arbitrary units.

2.6. BAL study

The accumulation of inflammatory cells in BALF was examined to evaluate airway inflammation. Experiments were carried out according to the previously described method by Tanaka et al. (2001) [24]. Briefly, 24 h after the final inhalation of antigen (day 31), animals were killed by intraperitoneal injection of sodium pentobarbitone (100 mg/kg). The left bronchus was tied for histological examination. Then, the right air lumen was washed four times with 0.5 ml of calcium-magnesium-free phosphate buffered saline (PBS) containing 0.1% BSA and 0.05 mM EDTA-2Na and the procedure was repeated three times (total volumes 1.5 ml, recovery > 85%). BALF from each animal was pooled in a plastic tube, cooled in ice, and centrifuged (150 × *g*) at 4 °C for 10 min. Cell pellets were resuspended in the same buffer (1 ml). A portion of the cell suspension was mixed with Türk solution and nucleated cells were counted in a Bürker chamber. A differential count was made on a smear prepared with a cytocentrifuge (Cytospin II, Shandon, Cheshire, UK) and stained with Diff-Quick solution. Based on standard morphologic criteria, 300 cells were counted (magnification × 500).

2.7. Cytokine levels in BALF

The amount of cytokines in the supernatant of BALF was measured using enzyme-linked immunosorbent assay (ELISA) Kits (Kits for IL-4 and IFN-γ from Pierce Biotechnology, Inc., Rockford, IL, USA; kits for IL-5 and IL-13 from R&D Systems, Inc., Minneapolis, MN, USA). The detection limit of each kit was 5 pg/ml for IL-4, 10 pg/ml for IFN-γ, 7 pg/ml for IL-5 and 1.5 pg/ml for IL-13, respectively.

2.8. Measurement of immunoglobulin (Ig) levels in serum

After the final inhalation at day 30, blood was collected and sera were obtained by centrifugation and stored at −30° C. Total IgE, OVA-specific IgE, OVA-specific IgG1 and OVA-specific IgG2a in the serum were measured using ELISA as formerly described by Komai et al. and Nagai et al. [25,26]. In short, total serum IgE was measured by coating flat-bottomed 96-well microtiter plates (Immuno-Plate I 96-F, Nunc, Roskilde, Denmark) with monoclonal rat anti-mouse IgE antibody at a concentration of 5 μg/ml. After blocking with 1% Block-Ace, a standard and serum dilutions were incubated for 1-h followed by peroxidase-labeled polyclonal anti-mouse IgE goat IgG antibody. Sequentially diluted monoclonal anti-DNP IgE (SPE-7) was used as a standard.

Serum OVA-specific IgE was measured by coating the microtiter plates with monoclonal rat anti-mouse IgE antibody (LO-ME-3) at a concentration of 5 μg/ml. After blocking with 1% BSA, serum dilutions were incubated for 1-h followed by biotinylated-OVA and peroxidase-conjugated streptavidin. Serum OVA-specific IgG1 was measured by coating the microtiter plates with polyclonal goat anti-mouse IgG1 antibody (STAR81) at a concentration of 2 μg/ml. After blocking with 1% BSA, serum dilutions were incubated for 1 h followed by biotinylated-OVA and peroxidase-conjugated streptavidin. Serum OVA-specific IgG2a was measured by coating the microtiter plates with OVA solution at a concentration of 20 μg/ml. After blocking with 1% BSA, serum dilutions were incubated for 1 h followed by peroxidase-conjugated monoclonal rat anti-mouse IgG2a antibody (LO-MG2a-7). Sequentially diluted monoclonal anti-OVA IgE, IgG1, and IgG2a (donated by Dr. Kiniwa, Taiho Pharmaceutical Co. Ltd., Tsukuba, Japan) were used as standards.

Optical densities after the enzymatic reactions were read using an automatic ELISA plate reader (Multiscan MS, ver 8, Lab systems Oy, Helsinki, Finland) at 492 nm (reference 690 nm), and analyzed using Deltasoft 3 (Biometallics Inc., Princeton, NJ, USA). Each detection limit was 1 ng/ml for total IgE, anti-OVA IgE, and anti-OVA IgG1, and 3 ng/ml for anti-OVA IgG2a respectively.

2.9. Histopathological study

The left lungs were excised and immersed in fresh 10% buffered formalin for 24 h. Tissues were embedded in paraffin, and 5 μm sections were prepared and stained with hematoxylin and eosin for histopathological examination.

2.10. Statistical analysis

Values are presented as the mean ± S.E.M. Statistical significance between saline-injected animals (saline) and OVA-sensitized and challenged animals (OVA) was estimated using the two-tailed Student's *t*-test or the Mann–Whitney's *U*-test after the variances of data were evaluated using the F-test. To define significant differences among OVA-sensitized and challenged animals (OVA) and NSO treated animals (NS1 and NS4), the data were subjected to Bartlett's analysis, followed by parametric or non-parametric Dunnett's multiple range test. A *P* value less than 0.05 was considered to be significant.

3. Results

3.1. NSO constituents' quantification

HPLC analysis of NSO used in the present work revealed that it contained TQ, THQ and DTQ at levels of 1258.61 ± 43.92, 290.80 ± 10.63 and 5.85 ± 0.08 μg/ml, respectively. In contrast, THY was under the detection limit (16.1 ± 0.18 μg/ml) (Fig. 1).

3.2. Effect of NSO on antigen-induced AHR

Repeated OVA inhalation induced AHR to Ach compared with that in saline-exposed mice. Treatment with NSO during OVA sensitization and inhalation significantly inhibited OVA-induced AHR in a dose-dependent manner (Fig. 2 A and B).

3.3. Effect of NSO on antigen-induced airway eosinophilic inflammation

Repeated OVA inhalation significantly increased the number of inflammatory cells, including macrophages and eosinophils in the BALF. The accumulation of inflammatory cells in the BALF was markedly inhibited by 4 ml/kg/day NSO (Fig. 2 C–F). Paralleled with inflammatory infiltrates, the levels of Th2 cytokines, IL-4, IL-5 and IL-13 in the BALF, were significantly increased, however, the levels of Th1 cytokine, IFN-γ in the BALF, was significantly decreased by repeated antigen challenge (Fig. 3). In parallel with the effect on airway eosinophilic inflammation, NSO dose-dependently suppressed significantly the increased levels of Th2 cytokines, IL-4, IL-5 and IL-13, and restored effectively the decreased level of Th1 cytokine, IFN-γ in the BALF (Fig. 3).

3.4. Effect of NSO on serum Igs

Fig. 4 shows the effect of NSO on the serum Ig levels on day 30, immediately after the final OVA challenge. Systemic sensitization and local OVA challenge significantly increased serum levels of total IgE, OVA-specific IgE, IgG1 and IgG2a. The treatment with NSO significantly inhibited the increases in the levels of total IgE, OVA-specific IgE and IgG1 dose-dependently, whereas NSO significantly augmented the increase in the level of OVA-specific IgG2a at a dose of 4 ml/kg/day (Fig. 4).

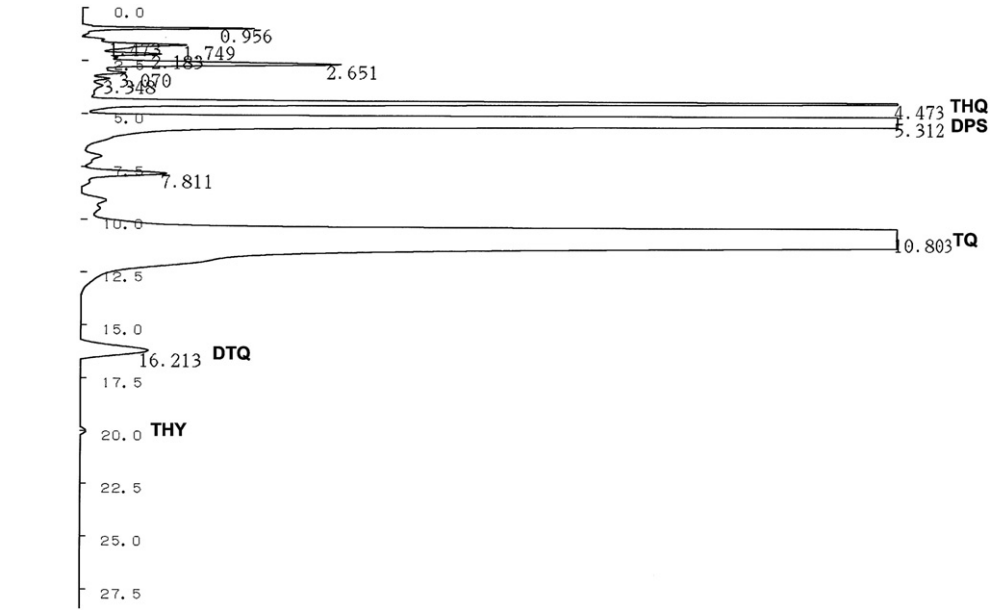

Fig. 1. HPLC analysis of *Nigella sativa* oil supposed active constituents. TQ, thymoquinone; THQ, thymohydroquinone; DTQ, dithymoquinone; THY, thymol and DPS, diphenyl sulfone.

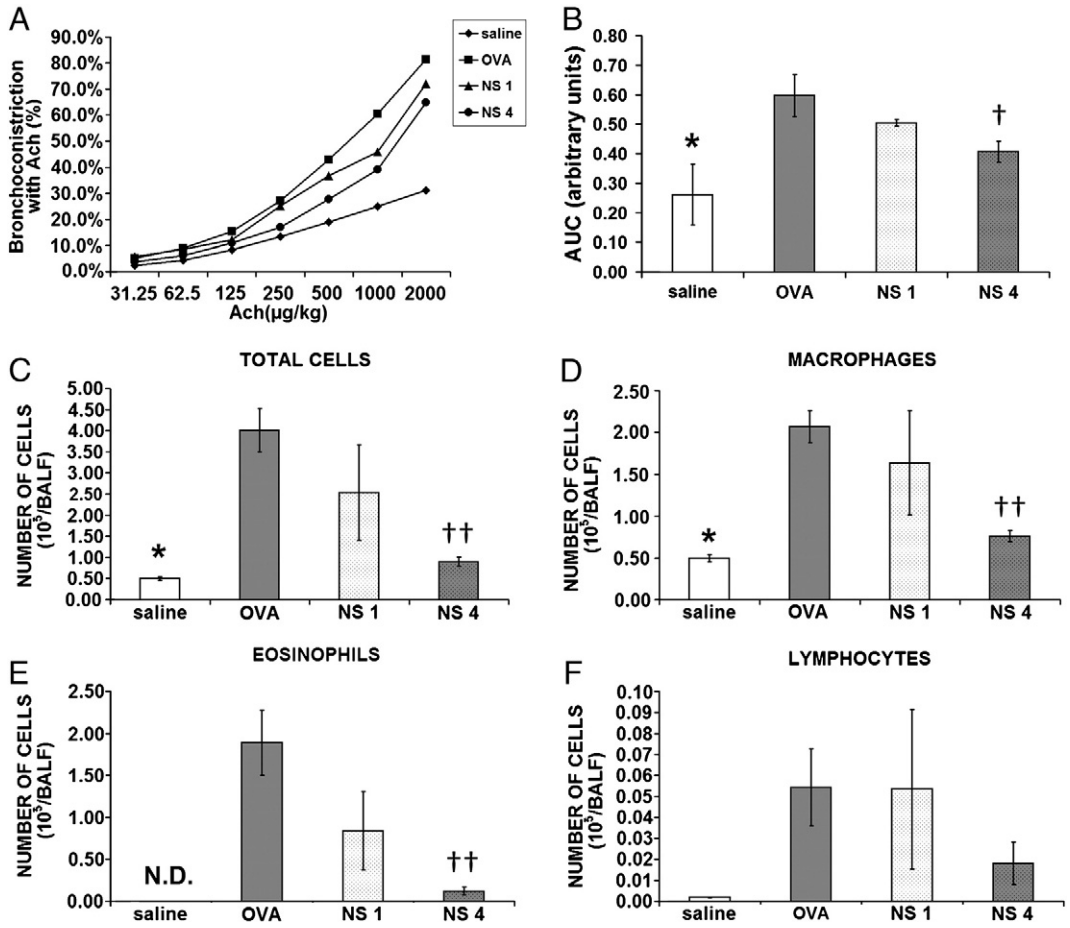

Fig. 2. Effect of *Nigella sativa* oil on increased airway responsiveness to acetylcholine and inflammatory infiltrates in the bronchoalveolar lavage fluid of ovalbumin-induced bronchial asthma in BALB/c mice. A: Airway responsiveness to acetylcholine 24-h after the final antigen challenge. B: Area under the curve (AUC) calculated from the dose–response curves of bronchoconstriction to acetylcholine (range: 31.25–2000 µg/kg). C–F: number of leukocytes in the bronchoalveolar lavage fluid 24-h after the final antigen challenge. Values represent the mean ± S.E.M. of six mice in each group. saline, saline exposed group; OVA, non-treated bronchial asthma group; NS1, bronchial asthma group treated with 1 ml/kg/day *Nigella sativa* oil; NS4, bronchial asthma group treated with 4 ml/kg/day *Nigella sativa* oil; N.D., not detected; * $P<0.05$ (vs. OVA group); † $P<0.05$, †† $P<0.01$ (vs. OVA group).

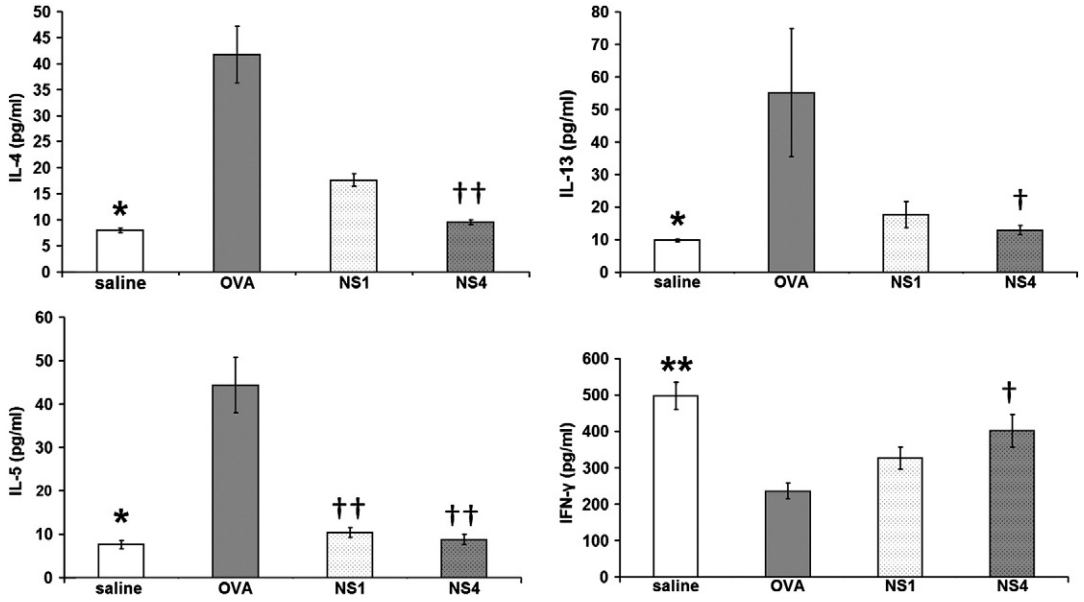

Fig. 3. Effect of *Nigella sativa* oil on increased cytokine levels in the bronchoalveolar lavage fluid of ovalbumin-induced bronchial asthma in BALB/c mice 24-h after the final antigen challenge. Values represent the mean ± S.E.M. of six mice in each group. saline, saline exposed group; OVA, non-treated bronchial asthma group; NS1, bronchial asthma group treated with 1 ml/kg/day *Nigella sativa* oil; NS4, bronchial asthma group treated with 4 ml/kg/day *Nigella sativa* oil; * $P<0.05$, ** $P<0.01$ (vs. OVA group); † $P<0.05$, †† $P<0.01$ (vs. OVA group).

3.5. Effect of NSO on histopathological changes

Fig. 5 shows the representative microscopic images of tissue sections of each group stained with hematoxylin and eosin. Repeated OVA challenge caused perivascular and peribronchial cellular infiltration, increased bronchial muscle thickening, epithelial hyperplasia, and detached epithelial lining. Oral administration of NSO at a dose of 4 ml/kg/day significantly prevented these histopathological changes.

4. Discussion and conclusion

In the present study, we investigated the anti-inflammatory and immunomodulatory effects of oral NSO in a mouse model of allergic asthma with regard to airway function, antigen-induced inflammatory infiltrates in the airways, local Th1/Th2 cytokine production, serum immunoglobulin levels, and histopathological changes of lung tissues. Different animal models have been developed in order to investigate

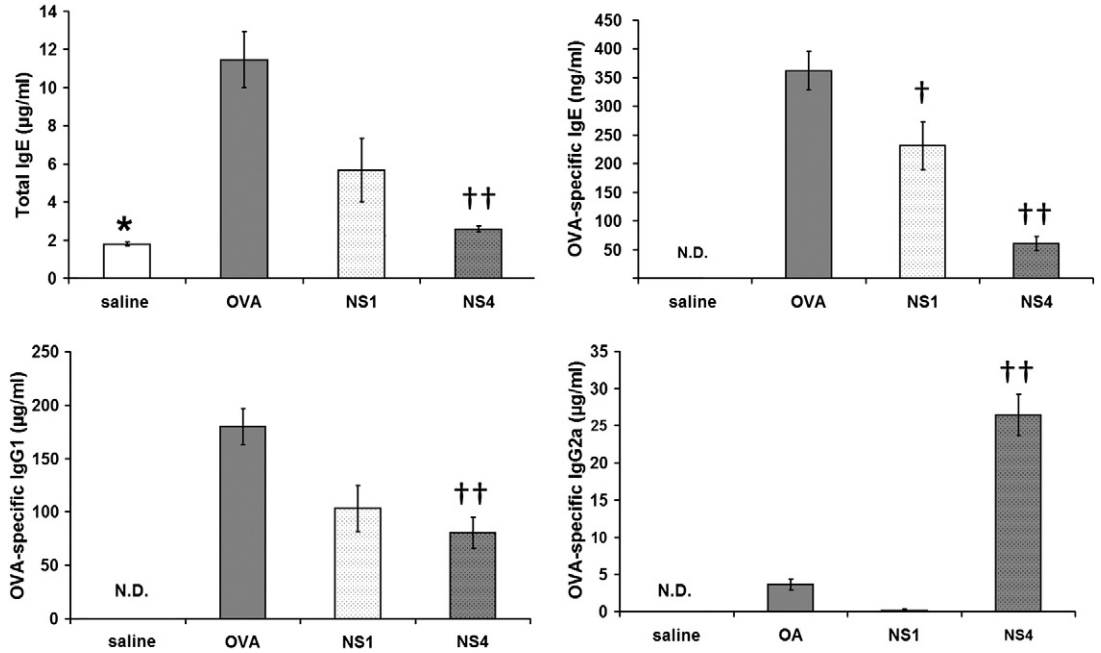

Fig. 4. Effect of *Nigella sativa* oil on increased serum immunoglobulin levels of ovalbumin-induced bronchial asthma in BALB/c mice after the final allergen challenge. Values represent the mean ± S.E.M. of six mice in each group. saline, saline exposed group; OVA, non-treated bronchial asthma group ; NS1, bronchial asthma group treated with 1 ml/kg/day *Nigella sativa* oil; NS4, bronchial asthma group treated with 4 ml/kg/day *Nigella sativa* oil; N.D., not detected; * $P<0.05$ (vs. OVA group); † $P<0.05$, †† $P<0.01$ (vs. OVA group).

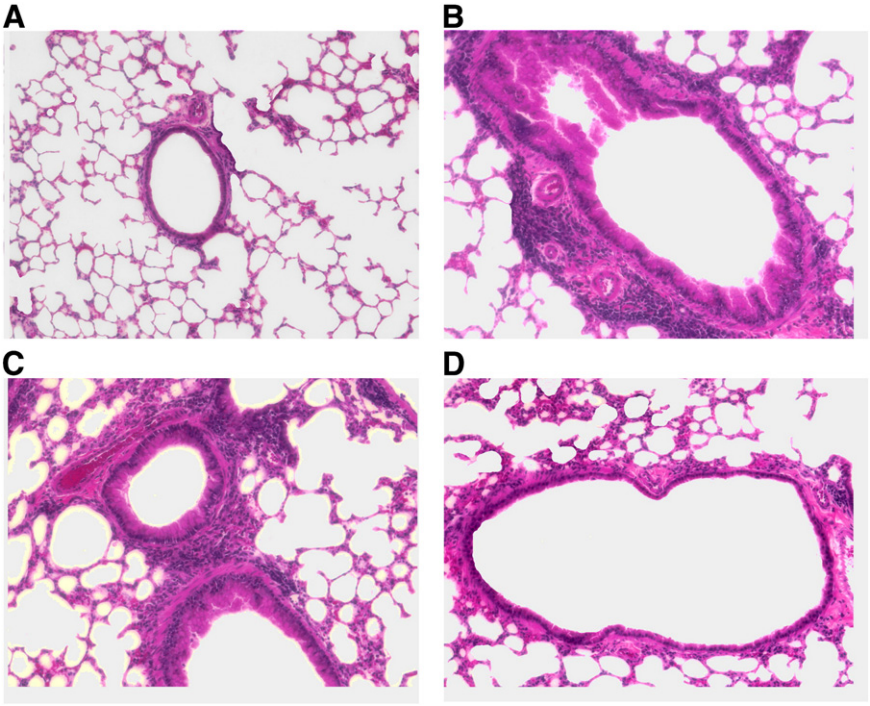

Fig. 5. Effect of *Nigella sativa* oil on histopathological changes of lung tissues of ovalbumin-induced bronchial asthma in BALB/c mice. A: Saline exposed group, B: Non-treated bronchial asthma group, C: Bronchial asthma group treated with 1 ml/kg/day *Nigella sativa* oil, D: Bronchial asthma group treated with 4 ml/kg/day *Nigella sativa* oil, hematoxylin and eosin staining, ×200.

the therapeutic effect of NSO on allergic asthma thus far. However, we selected our model as it shows all the salient features of allergic airway inflammation in humans including increased serum antigen-specific IgE, increased Th2 cytokine levels and decreased Th1 cytokine levels in BALF, enhanced airway responsiveness to Ach, airway eosinophilic inflammation, and goblet cell hypertrophy and hyperplasia [27,28]. The results of our study showed that oral treatment with NSO in mice sensitized and challenged with OVA significantly improves AHR, decreases the number of total leukocytes, macrophages and eosinophils, levels of IL-4, IL-5 and IL-13 in BALF, serum levels of total IgE, OVA-specific IgE and IgG1, and significantly increases the level of IFN-γ in BALF and serum level of OVA-specific IgG2a with abrogation of histological changes in lung tissues in a dose-dependent manner.

HPLC analysis of NSO used in the present work revealed that it contained TQ, THQ and DTQ. However, its THY content was under the detection limit. The NSO and its active ingredients have shown potent anti-inflammatory and immunomodulatory effects, which are, depend on NSO nature and dose of constituents [14]. TQ has been reported to have anti-inflammatory, tracheal smooth muscle relaxant and anti-asthmatic effects [29–31]. THQ has been shown to have anti-inflammatory effect [32]. DTQ has been demonstrated to have anti-histaminic and anti-inflammatory effects [31,33]. Moreover, DTQ attenuated symptoms of patients suffering from bronchial asthma when given orally to them [34]. Therefore, we examined the effect of NSO, but not each active ingredient, on allergic airway inflammation in the present study. Although, some of NSO constituents have immunomodulatory effect, we believed that the crude NSO is more effective as immunomodulatory and auto-regulator to the immune system due to complementary effects of its ingredients. In addition, NSO contain other constituents such as, antioxidants, trace elements, vitamins and enzymes that are essential co-factors for balancing and strengthening the immune system [13,14].

IL-4, IL-5, and IL-13 are key Th2-type cytokines that play important roles in the development of allergic asthmatic responses in humans [35]. IL-4 promotes the differentiation and proliferation of Th2-type T cells, switching of B cells to produce IgG1 and IgE, and the blocking of IL-4 by monoclonal antibodies decreases IgE level and airway eosinophilia in allergic mice [5,36,37]. IL-5 plays an important role in the differentiation, maturation and survival of eosinophils that leads to its numerical increase in the airways subsequent to activation. Moreover, previous studies have shown that eosinophilic inflammation could not be developed in the absence of IL-5 or its signaling in the airways of OVA-sensitized and challenged mice [38,39]. IL-13 is involved in AHR, promoting IgE isotype switching in B cells, mucus secretion in the airway mucosa, and IL-13 knockout mice fail to develop AHR after allergen sensitization and challenge even in the presence of severe airway inflammation [40,41]. In contrast to these Th2 cytokines, IFN-γ produced by Th1 cells can enhances production of IgG2a and it also reported to inhibit asthma-like phenotypes as it inhibits antigen-induced AHR, IgE production, and antigen-induced eosinophil recruitment [42,43]. Therefore, restoration of local Th1/Th2 cytokine balances is a rational strategy for developing new treatment approaches for allergic asthma.

In the present study, we clearly demonstrated that the oral treatment with NSO dose dependently inhibited Th2 cytokine production (IL-4, IL-5 and IL-13) and recovered the decreased Th1 cytokine production (IFN-γ) in the BALF after antigen challenge in sensitized mice. This restoration of local Th1/Th2 cytokine balance could explain the improvement of AHR, reduction of airway inflammation and cellular infiltration, and reduction of the level of total IgE, OVA-specific IgE and IgG1 as well as increased level of IgG2a, as our previous results show, using IL-4 gene-disrupted mice [25]. To our best knowledge, this is the first time to show its strong immunomodulatory effects on local Th1/Th2 imbalance by measuring cytokine production in the BALF with airway function, systemic IgE/IgG1/IgG2a production and histopathological changes.

Certainly, Boskabady et al. [16] recently showed that the hydroethanolic extract of *N. sativa* seeds suppress IL-4 production and enhance IFN-γ production in the blood of OVA sensitized guinea pigs. However, they did not measure cytokine production in the BALF, and the level of IFN-γ in the blood of sensitized and challenged guinea pigs was higher than that of control guinea pigs, and IL-4 level in the blood was very low and near detection limit. Using a rat model, Shahzad et al. [44] have demonstrated that the treatment with NSO in sensitized

rats showed attenuation of eosinophilic inflammation associated with significant inhibition of elevated mRNA expression of IL-4 and IL-5, and reduction of delayed AHR. Furthermore, Abbas et al. [18] have reported that *N. sativa* significantly reduced the number of peripheral blood eosinophils, the elevated serum IgG1 and IgG2a levels, Th2 cytokine profiles, and inflammatory cells in the lung tissue, but that it did not affect IFN-γ level.

Using TQ, a supposed active constituent of NSO, El Gazzar et al. [30] demonstrated that intraperitoneal treatment with TQ significantly attenuated airway eosinophilic infiltration due to its inhibitory effects on IL-4, IL-5 and IL-13 production. However, they did not show its significant effect on IFN-γ production in the BALF of OVA-sensitized mice. In their model, they have not demonstrated the improvement of Th1/Th2 imbalance, because both the level of IFN-γ in the BALF and serum IgG2a level of sensitized and challenged mice were still higher than those of naïve mice. In contrast, the efficacy of TQ has been reported by Keyhanmanesh et al. [17] using an asthma model of guinea pigs. TQ suppress IL-4 production and enhance IFN-γ production in the blood of OVA sensitized guinea pigs. However, they measured cytokine production in the blood and not in the BALF, the level of IFN-γ in the blood of sensitized and challenged guinea pigs was higher than that of control guinea pigs, and IL-4 level in the blood was very low and near detection limit [17].

These controversial results regarding IFN-γ production may be due to the animal model used. Animal asthma models are quite different from each other in immunization protocol, antigen, and frequency and concentration of challenging antigen. These differences could influence asthma-like phenotype [45,46]. Therefore, we experienced such a problem using animal models as described above. However, as asthma is a heterogeneous disease in humans, we should pay more attention to whether each parameter is associated with the other parameters in each animal model.

In contrast to our result, Büyüköztürk et al. [47] showed that NSO has no effect on the Th1 and Th2 cytokine productions from splenocytes in OVA-sensitized mice. This controversial is due to different regime of treatment, method of sensitization and source of cytokines. In this study, mice were treated with oral NSO before sensitization only, but in our study, the treatment was during the sensitization and challenge. In addition, they sensitized the mice only by i.p. injection of OVA without challenge with OVA inhalation. In contrast to their method, mice were sensitized with OVA plus alum and inhaled OVA repeatedly. Moreover, in their study, they investigated only the systemic cytokines from the cultured splenocytes but not BALF cytokines, and the histopathological picture of lung tissues was not evaluated. In the present study, we investigated the local effect of NSO on BALF cytokines and histopathology of lung tissues [47].

Although our model used in the present study shows all the salient features of allergic airway inflammation in humans, this model still has limitation to detect airway remodeling, especially subepithelial fibrosis and bronchial smooth muscle hyperplasia and hypertrophy. In addition, OVA used as an antigen is not a relevant antigen. Therefore, further investigation is still needed to detect the possible protective effect of NSO using a chronic model [25,48]. Moreover, further investigations are currently in progress to determine whether NSO affects antigen-induced cytokine production from the splenocytes in sensitized mice.

In conclusion, oral administration of NSO in a dose of 4 ml/kg/day was able to ameliorate OVA-induced bronchial asthma in mice. This amelioration is thought to be mainly due to the attenuation of Th1/Th2 cytokine imbalance. We believe that the treatment with oral NSO could be a promising treatment for bronchial asthma in humans.

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