Effects of black seed (Nigella Sativa) on type 2 cytokines gene expression and mucus production in the airways of asthmatic mice

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Abstract

Background: Black Seed (BS) is used in traditional medicine as a therapy for a variety of diseases including allergic asthma.

Materials and Methods: In the present study, anti-inflammatory and immunomodulatory effects of BS on cytokine gene expression, lung airway eosinophilia and goblet cell hyperplasia were examined in a mouse mice model of allergic asthma. Groups of 6-week-old female BALB/c mice were sensitized by intraperitoneal injections of OVA plus alum on days 1 and 14. On days 24, 26, 28 and 30, the mice were exposed to OVA in saline for 30 min with nebulizer. Similar experiments were conducted with mice receiving saline as a negative control.

Results: The mouse allergic asthma model received BS by food on days 23, 25, 27 and 29. Then, the percentage of inflammatory cells as well as mRNA expression levels of interleukin (IL)-4, IL-5, IL-13 and mucin (MUC5a) genes were survived in Broncho-alveolar lavage fluid (BALF). Furthermore, we attempted to examine histopathological examination of the lung. Mice receiving BS showed a significant decrease in the number of eosinophils, and a potential inhibitory effect on mRNA expression levels of Th2-driven immune response cytokines and mucin, resulting in decreased production of interleukin and mucin in allergic asthma.

Conclusion: Our findings suggested that BS has an anti-inflammatory and immunomodulatory effect during the allergic response in the lung, and can be a promising treatment for allergic asthma in humans.

Keywords: Black Seed, allergic asthma, Th2-type cytokines mRNA, mucin.

Introduction

Allergic Asthma is a multifactorial disease of airways characterized by airway spasm, lung eosinophilia, mucus hypersecretion resulting from goblet cells, elevated serum IgE levels and increased airway responsiveness to allergens. Approximately 300 million people from all ages suffer from asthma worldwide (1-6). Th2 cytokines, such as IL-4, IL-5 and IL-13, are believed to play an important role in asthma development and progression. These cytokines are involved in overproduction of IgE, activated and increased eosinophils in the airways as well as development of airway hyper responsiveness (AHR) to allergens (7).

Nigella sativa, an annual flowering plant in the
family Ranunculaceae, is one of the most widely used traditional medicine generally known as black seed (BS) or black cumin (8, 9). Since ancient times, it has been used as a food additive, preservative and herbal remedy for various diseases. A lot of studies have shown that BS is an excellent cure capable to prevent a variety of diseases, including those related to the gastrointestinal, immune and nervous systems, as well as respiratory disorders such as asthma (8, 10, 11). Black seed oil (BSO) is commonly used in traditional medicine because of the presence of highly-active constituents such as conjugated linoleic acid, thymoquinone, nigellone (dithymoquinone), thymol, thymohydroquinone, melanthin, nigilline and trans-anethole (11-13). These constituents allow BS to prevent and control infections, allergies and chronic diseases. Recent studies on the pharmacological effects of BS have demonstrated its anti-inflammatory and immunomodulatory properties (6, 8). In addition, several studies showed that BS has the inhibitory effect on cytokine production and activation (12-15).

The aims of this study were to investigate the immunomodulatory and anti-inflammatory effects of BS on ovalbumin (OVA)-induced allergic asthma in mice. The extent of antigen induced inflammatory infiltration in the bronchial airways and histopathological changes in lung tissues was investigated. In addition, we evaluated the effect of BS on the expression level of IL-4, IL-5, IL-13 and MUC5a genes in OVA-induced asthmatic mice.

Methods

Animals

Forty-two 6-week-old female BALB/c mice were purchased from Pasteur institute of Iran (Tehran, Iran). The mice were kept in an allergen-free environment in plastic cages, and received food and water ad libitum at 18-24°C temperature with a relative humidity of 30-60% and a 12-hour light/dark cycle. Water, food, drinking containers and storage cage supplies were sterilized and changed daily.

Antigen Sensitization, challenge and treatment

The mice were immunized with two methods, intraperitoneal injection and inhalation aerosol of Ovalbumin (OVA). The mice were divided into three groups of the same gender, race and age as follows:

- Group 1) receiving BS by food on days 23, 25, 27 and 28, and 30 for 30 min per day (Positive control);
- Group 2) includes allergic asthma models (similar to group 1) receiving BS by food on days 23, 25, 27 and 29; and group 3 contains healthy mice receiving normal saline (as a negative control). After day 31, exams were done and the groups were compared using statistical methods.

RNA extraction from broncho-alveolar lavage fluid (BALF)

BAL fluid was collected from animals, centrifuged in sterile centrifuge tubes for 5 minutes at 500 × rpm at 4°C, and then washed twice with PBS. Afterwards, the cell pellet was suspended in PBS. Total RNA was extracted from BAL fluid using the TRizol reagent according to the manufacturer’s specifications. RNA concentration was quantified by measuring optical density (OD) at 260 nm (Eppendorf, Homburg, Germany). The extracted RNA was stored at -70°C until cDNA synthesis.

Construction of cDNA from total RNA of BALF

Total RNA samples were converted to single-stranded cDNA by using the Maxima First Strand cDNA Synthesis Kit, according to the manufacturer’s instruction (RevertAid, Fermentas Life Sciences, International INC, Canada). The product of the first strand cDNA synthesis was used directly in qPCR or stored at -20°C for up to one week and -70°C for longer storage.

Real-Time quantitative PCR

In the present study, real-time PCR was carried out using SYBR Green I as a fluorescent dyes. The primer design was based on the target gene nucleotide sequences from the NCBI Nucleotide database (Gene Bank, access: http://www.ncbi.nlm.nih.gov/nuccore). The optimized primers (Table 1) were designed and validated by Roche® Probe Finder 2.5 (access: http://qpcr.probefinder.com/organism.jsp) using Primer3 version 1.1.4. PCR amplification was carried out in the Rotor-Gene SYBR Green PCR kit from Qiagen and using the thermocycler Rotor Gene Q (Qiagen, Hilden, Germany). After an initial activation
step at 95°C for 5 min (hot start DNA polymerase activation). Reactions were run under the following conditions: 40 cycles of denaturation at 95°C for 5 s, combined annealing/extension at 60°C for 10 s at which the fluorescence was acquired. Amplification specificity was checked using the melting curve generated by heating the PCR product slowly at a rate of 1°C s−1 from 60°C to 95°C. Relative levels of gene expression were normalized to GAPDH mRNA levels. To avoid an impressing effect of inter-run variations on reference gene stability, the gene expression of all reference genes was quantified for each sample in triplets within the same qPCR plate. The PCR efficiency of the target genes, IL-4, IL-5, IL-13, MUC5a and endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was calculated from the slopes of the standard curves.

**Histopathology**

The number of eosinophils in bronchoalveolar lavage fluid was quantified in all animals. Histopathological sections were prepared and stained with Hematoxylin and Eosin (H&E) to detect eosinophilic infiltration and mucus-secreting goblet cells, respectively.

**Statistical analysis**

Gene expression changes were shown in the graphs on logarithmic scales. Columns in this graph represent average gene expression (in triplicate) ± standard deviation. The Kolmogorov-Smirnov test was used for normal distribution. One-way analysis of variance (ANOVA) and student's t-test were used to compare results from different groups, and differences in each group before and after treatment, respectively. The Mann-Whitney U test was applied to compare the differences between positive and negative groups. A p Value less than 0.05 were considered to be significant.

**Results**

**Airway inflammation in mouse model of allergic asthma**

To confirm the allergic airway inflammation model, the positive and negative controls were compared in terms of differences between inflammatory cell infiltration around blood vessels and lung airways, the presence of eosinophils in the inflammatory cells and lavage fluid, increased numbers of goblet cells and mucus-secreting cells in the airways. The Mann-Whitney U test was used to compare positive and negative control groups. There were significant differences between the two groups in terms of eosinophilic lavage (P<0.05) (Fig. 1-A), eosinophilic lung inflammation around the airways (P<0.05) (Fig. 1-B) and mucus secretion (P<0.05) (Fig. 1-C). However, no significant difference was found in alveolar wall inflammation between negative and positive controls (P<0.8). This indicates an appropriate experimental model, because pulmonary alveolitis should not be detected in the asthma and allergic inflammation model.

**Real time-PCR for IL-4, IL-5, IL-13 and mucin genes**

Asthmatic mice treated with BS were able to suppress type-2 cytokines expression in bronchoalveolar fluid cells. In this study, mice sensitized with OVA displayed a significant increase in the relative expression of IL-4. Simply stated, IL-4 expression (Fig. 2) led to an increase about 4-8 fold, as compared to the control. However, OVA-stimulated mice treated with SB suppressed IL-4 expression. IL-5 (Fig. 2) and IL-13 (Fig. 2) displayed the same expression pattern as IL-4. However, the expression level was significantly diminished in mice receiving BS. OVA-stimulated BS treated mice showed down regulation of mucin gene expression. OVA-stimulated mice expressed high levels of mucin. BS- received mice had 11-12 fold decrease of mucin gene expression (Fig. 2). Therefore, it can be deduced that BS could reduce mucin expression in mice stimulated with OVA.

**Discussion**

Previous studies have shown that BS has immunomodulatory (16, 17), anti-inflammatory (18), anti-carcinogenic, mutagenic and anti-oxidant (19, 20) activities in a number of diseases, including allergic airway inflammation (21) and bronchial asthma (22).

As compared to the controls, mice sensitized and challenged with OVA showed significant inflammatory changes, including increased Th2 cytokines (such as IL4, IL5 and IL13), the presence of lung tissue eosinophils in Broncho-alveolar lavage (BAL) fluid, and a marked increase in the number of goblet cells. There is evidence that IL-4 is able to
induce the proliferation of Th2-type cells, differentiate B cells and, more importantly, decreases the production of Th1 cells (23, 24). In the airways of OVA-sensitized and challenged mice, IL-5 plays a crucial role in the maturation and differentiation of eosinophils that leads to its numerical increase in the airways subsequent to activation (25). IL-13 is involved in AHR, promoting IgE isotype switching in B cells and importantly mucus secretion in the airway (26). Consistent with the above-mentioned studies, the modulation of local Th1/Th2 cytokine equilibrium could explain airway smooth muscle spasm improvement, decreased airway inflammation, decreased levels of OVA-specific IgE and IgG1, as well as increased levels of IgG2a (27, 28).

In the present study, decreased Th2 cytokine expression in BS-treated mice compared with the sensitized group; the difference was clearly proved with statistical data. Therefore, it can be deduced that BS has a significant effect on IL-4, IL-5, IL-13, and MUC5a in the airway. It suggests that BS has the anti-inflammatory effect on experimental asthma. Histological examination of lung tissue demonstrated that BS has the potential ability to inhibit allergen-induced lung eosinophilic inflammation and mucus production. In the present study, mice treated with BS could potentially suppress IL-4, IL-5, IL-13, and MUC5a gene expression in OVA sensitized and challenged mice. It demonstrates that BS acts mainly by suppressing type 2 cytokines and mucus secretion from the airways.

These results demonstrated that BS has a protective role in allergic asthma in mice. In agreement with our findings, previous studies demonstrated that BS-treated mice not only decrease type 2 cytokines and MUC5a expression, but also exhibit a substantial decrease in eosinophil levels and mucus in asthmatic mice (22, 29).

In addition, Balaha, M.F., et al. previously showed that OVA-sensitized and -challenged mice orally-treated with BS Oil are able to significantly decrease the number of total leukocytes, macrophages

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and eosinophils, as well as IL-4, IL-5 and IL-13

Fig. 1. Effects of BS on airway cellular infiltration 24 h after the final antigen challenge. PBS: PBS-exposed group; OVA: non-treated bronchial asthma group; BS: bronchial asthma group treated with 1 mg/kg/day BS. Chart results of eosinophils in the lavage fluid cell count (A), inflammation around lung bronchi (B), and the mucus secretion in the lung Bronchi (C) in studied groups.

Fig. 2. Effect of BS on Th2 cytokine mRNA expression. Total RNA was extracted and used in Real Time-PCR to assess IL-4, IL-5, IL-13, and MUC5ac gene expression. Results from 14 Balb/c mice per each group are given. Data is given for PBS, OVA and BS group. Cytokines mRNA expression in mic BALF expressed as a percent ratio to GAPDH (internal control) mRNA determined by Real Time-PCR. Data is shown as mean ± SD for 14 BALB/c each group. A significant increase in the mRNA expression for all cytokines was noted in OVA-sensitized mice (OVA-group) as compared to PBS-treated controls. BS significantly reduced mRNA expression of IL-4 (P<0.05), IL-5 (P<0.05), IL-13 (P<0.05) and MUC5ac (P<0.05) in mice treated with BS.
one of the diagnostic features of these diseases, is an increase in the numbers of eosinophils (eosinophilia) in the peripheral blood and sputum (33). In this study, we surveyed mucus gene expression by real time-PCR localization using MUC5a gene in the BS-treated group and non-asthmatic airways animals. In the previous studies, decreased expression of bronchial epithelial cells was recorded by Xiao et al who demonstrated that E-cadherin expression was significantly lower in the bronchial biopsies of asthmatic subjects compared with non-asthmatics ones (34). Additionally, Kulshreshtha et al showed that E-cadherin is a trans-membrane protein in epithelial cells that provides essential architectural structure and immunological function to the airway epithelium (35). Mucus hypersecretion in asthma results from airway inflammation and contributes to clinical symptoms, airway obstruction, and mortality (36). In human asthma and in animal models, mucus overproduction correlates with airway eosinophilia. Th2 cells stimulate airway eosinophilia and a marked increase in mucus production, Mucus could be induced by IL-13 (37). In the present study, we dissect further mechanisms of Th2-induced mucus production (38).

We surveyed the anti-inflammatory and immunomodulatory effects of BS in a mouse model of allergic asthma by cytokines balancing with attention to airway function, antigen-induced inflammatory infiltrates in the airways, local Th2 cytokine production, secretion of mucus in the airways, and histopathological changes of lung tissues. These data suggested that BS had immunomodulatory, anti-inflammatory, and mucus hypersecretion effects on OVA-induced asthma in mice due to inhibition of Th2 cytokine production, eosinophil infiltration, and excess mucus production into the airways. Therefore, BS can be recommended as a therapeutic agent for the treatment of allergic asthma. In addition, it is considerable to elucidate that BS could have such anti-inflammatory and immunomodulatory effects when was operated orally as the clinical operation route of the drug.

Conflicts of Interest

There is no conflict of interest.

Acknowledgment

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