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Improvement of Th17/Treg balance and Asthma Control Test score by *Nigella sativa* supplementation in asthmatic children: a new approach to managing asthma

Astmalı Çocuklarda *Nigella sativa* Verilmesi ile Astma Kontrol Test Skoru ve Th17/Treg Dengesinde Düzelme: Astmanın Tedavisinde Yeni Bir Yaklaşım

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Abstract

Introduction: Th17 and Treg cells have important roles in the development and progression of the allergic diseases, such as asthma. Several studies reported that *Nigella sativa* could improve chronic airway inflammation, and inhibit airway remodeling by regulating the immune system. To examine the effect of *Nigella sativa* supplementation on Th17/Treg cells, and asthma control test score in asthmatic children.

Materials and Methods: Twenty-eight children with asthma meeting the inclusion criteria in Saiful Anwar Hospital, Indonesia, participated in this study. All subjects took the standard treatment based on asthma guidelines. *Nigella sativa* oil (NSO) is given as additional treatment at the dose of 15–30 mg/kg/day for eight weeks in a randomized, single-blind-controlled trial. Th17 and Treg in peripheral blood mononuclear cells were analyzed by flow cytometry tool. Improvement of asthma control was accessed by ACT (Asthma Control Test) Score.

Results: There was a significant decrease of Th17, and an increase of Treg percentages in NSO treatment group. Th17/Treg ratio was lower in NSO group compared to the standard treatment group (1.06±0.33 vs. 2.30±1.08, p=0.001). The ACT score improvement was significantly higher in NSO compared to that in the standard treatment group (3.71±1.634 vs. 1.86±0.864, p=0.033). However, there was no significant correlation between Th17/Treg ratio and ACT score in pre- and post-treatment (p=0.552, p=0.344 respectively).

Conclusion: NSO supplementation improves Th17/Treg balance and clinical symptoms in asthmatic children.

Keywords: Th17, Treg, ACT score, asthma, *Nigella sativa*

Öz

Giriş: Th17 ve Treg hücrelerinin, astma gibi allerjik hastaların ortaya çıkması ve ilerlemesinde önemli rolleri bulunur. Bir dizi çalışma, *Nigella sativa*nın kronik hava yolu inflamasyonunu azalttığını ve bağışıklık sistemini düzenleyerek hava yollarının etkilenmesini azalttığını göstermektedir.

Gereçler ve Yöntemler: Çalışmada, Endonezya Saiful Anwar Hastanesinde astma tanısı koyulan 28 olgu irdelendi. Tüm hastalar, astma rehberine uygun olarak standart olarak tedavi aldı. Nigella sativa yağı (NSY), randomize tek kör çalışmada günde 15-30 mg/kg dozda 8 hafta boyunca verildi. Treg ve Th17 hücreleri periferik kan mononükleer hücrelerin içinde akan hücre ölçer ile sayıldı. Astmadaki düzelme, Astma Kontrol Testi (AKT) ile ölçüldü.

Bulgular: NSY verilen grupta Th17 hücrelerinde anlamlı bir azalma, Treg hücrelerinin oranlarında da artış saptandı (1.06±0.33'e karşın. 2.30±1.08, p=0.001). AKT skoru NSY verilen grupta, standart tedavi uygulanan hastalara göre anlamlı derecede daha yüksek idi (3.71±1.634'e karşın 1.86±0.864, p=0.033). Ancak, tedavi öncesi ve sonrasında alınan örneklerde Th17/Treg oranları ve AKT skorlarında bir değişim görülmedi (sırası ile; p=0.552, p=0344).

Sonuç: NSY verilmesi Th17/Treg dengesini ve astması olan çocuklarda klinik bulguları iyileştirir.

Anahtar Kelimeler: Th17, Treg, AKT skoru, astma, *Nigella sativa*

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Introduction

Asthma is a chronic inflammatory disease associated with airway obstruction and hyper-reactivity. Asthma characterizes any history of respiratory symptoms such as wheezing, coughing, shortness of breath, tightness in chest whose intensity varies over time, recurrent, reversible, and is usually initiated by exposure to an allergic substance. According to the Global Initiative for Asthma 2016, asthma is a serious global health problem because it affects all groups of age, and causes high morbidity and mortality rates.[1] Based on Global Burden of Disease Study, it was reported that in 2008–2010 there were 334 million people with asthma, and global asthma death rate was 250.000 people/year. Meanwhile, the prevalence of asthma in children between 5–17 years old was estimated at about 8.7 million in 2011.[2]

Asthma has a complex pathogenesis involving both genetic and environmental factors. One of the mechanisms underlying airway inflammation is the dysregulation of T helper cells. It is known that there is an imbalance between Th1/Th2 cells in asthmatic patients, and this correlates with initiation, progression, and asthma severity. Th2 cells are functionally upregulated, while Th1 cells are inhibited, which enables Th2 cytokines to promote airway inflammation by activating eosinophils as well as IgE secretion.[3] However, a Th1/Th2 imbalance is not only the main mechanism underlying asthma pathogenesis. There are other immunological pathways that regulate airway inflammation. Currently, T-regulatory (Treg) cells have been recognized as immunoregulatory cells, capable of inhibiting immune response and inducing immune tolerance.[4] Moreover, there are also Th17 cells as a specific T functional cell group that play key roles in mediating allergic disease, autoimmunity, inflammation, and mucosal host defense against pathogens. Recent studies have also found coexistence of Th17/Treg imbalance in patients with asthma.[5,6]

By knowing this mechanism, finding new ways to ameliorate the immune response is critical to reduce the burden of asthma. It has been reported that black seed or *Nigella sativa* has anti-inflammatory and immunomodulatory properties.[7] In several preclinical and clinical studies, *Nigella sativa* showed positive effects on clinical and biochemical markers of asthma inflammation.[8] The aim of this study was to investigate the effect of *Nigella sativa* on Th17/Treg balance, and improvement of asthma control in children with asthma. Furthermore, *Nigella sativa* and its potent immunomodulatory effect may be used as therapeutic agents in conjunction with standard treatment in asthmatic patients.

Materials and Methods

Subject and Study Design

This research is an experimental single-blind-randomized clinical trial. Research subjects were patients diagnosed with asthma based on Global Initiative for Asthma criteria between the ages of 4–14 years old. This study was conducted in Allergy, Immunology and Respirology Outpatient Clinic, Department of Child Health, Dr. Saiful Anwar General Hospital, Indonesia. Subjects who met the inclusion criteria were included in the study and then randomized into two groups, namely treatment and control groups. This study has been approved by the Ethical Committee of Medical Faculty Brawijaya University. Written informed consent has also been obtained from all parents/guardians of all subjects.

Nigella sativa treatment

In this study, we used soft gel capsules contained 500 mg *Nigella sativa* oil (NSO) (Minyak Habbatussauda MADINAH, Indonesia). It has been licensed as a herbal medicinal product in Indonesia, and has got a registration number as POM TR.123 329 761. All patients were on asthma medications according to Global Initiative for Asthma guidelines for standard asthma.[1] In the treatment group, we give NSO as an adjunctive therapy at the dose of 15–30 mg/kg/day for eight weeks.[9]

Isolation of PBMCs

A peripheral blood sample of 5 ml was obtained from each of the 28 subjects, kept at room temperature; and the test was carried out on the same day. Isolation of PBMCs (Peripheral Blood Mononuclear Cells) was performed by centrifugation with Ficoll gradient density. The PBMC suspension was incubated overnight at 37°C in RPMI 1640 (Invitrogen) medium with 100 U/ml penicillin and 100 U/ml streptomycin, two mM glutamine and 10% FBS.

Th17 and Treg cell count

The absolute counts of Th17 and Treg cells were assessed by flow cytometry. PBMCs were adjusted to the concentrations of 1×10^6 cells/L and incubated with various antibodies. Anti-human CD4+CD25+Foxp3-

PE antibodies (BioLegend, San Diego, CA.) were used for Treg detection. And anti-human CD4+IL-17A-PE antibodies (BioLegend, San Diego, CA.) were used for Th17 detection. All samples were analyzed by Flow Cytometer software BD Cell Quest Pro.

ACT score

Assessment of asthma control level was done with Asthma Control Test (ACT) which is also commonly used by healthcare providers in Indonesia. The ACT consists of five questions in a total scale of 5–25 with each scale from 1–5. Fully controlled is determined as total ACT score of 25.

Statistical analysis

All statistical analyses were performed using SPSS version 19.0 (SPSS Inc., USA) with a significance level of $p<0.05$. Normally distributed variables were reported as the mean±standard deviation. The normality and homogeneity of data were tested with Shapiro-Wilk test and Levene test, followed by Paired and Unpaired T-Test for comparative analysis. Pearson's correlation was used to measure the association between two variables.

Results

Characteristic of subjects

Clinical characterization of the participants in this study is summarized in Table 1. There were 14 children with NSO treatment, and 14 children with standard treatment. During our research, all patients completed the study, and there were no side effects observed in the treatment. There were no differences in the mean age in both groups. The subjects were mostly females having good nutritional status. Most of the participants had the family history of atopic diseases, such as rhinitis, asthma, eczema, urticaria, and conjunctivitis. The most common clinical manifestations were cough and dyspnea. From the assessment of asthma severity, there were 14 patients with intermittent asthma, and 14 patients with mild persistent asthma.

Table 1. Clinical characteristic of subjects

Characteristic	NSO treatment group	Standart treatment group
a. Age (mean±SD)	8.79±2.940	8.71±3.771
b. Gender (n)		
Male	5/14	6/14
Female	9/14	8/14
c. Nutritrional status (n)		
Good	12/14	13/14
Underweight	2/14	1/14
d. Family history of atopic disease (n)		
Yes	10/14	9/14
No	4/14	5/14
e. Clinical manifestation (n)		
Cough	2/14	2/14
Dyspnea	4/14	5/14
Cough + Dyspnea	8/14	7/14
f. Asthma Classification (n, %)		
Intermittent	7/14	7/14
Mild Persistent	7/14	7/14

Th17 and Treg cell percentages

Flow cytometry was used to evaluate the Th17/Treg dynamics in all subjects before and after treatment. Percentages of Th17 and Treg in NSO and standard treatment group are shown in Figure 1. At the beginning of the study, the percentages of Th17 and Treg were not significantly different in NSO and in standard treatment group (Th17 cell percentages were 32.65±12.06 vs. 25.69±15.09, respectively, p=0.084; Treg cell percentages were 15.83±6.13 vs. 17.81±5.89, respectively, p=0.640). After eight weeks of treatment, there was a significant decrease of Th17 percentages in NSO group compared to the standard treatment group (20.64±7.03 vs. 24.79±12.01, respectively, p=0.038). However, Treg percentage showed no significant difference in NSO group and standard treatment group after treatment (21.24±9.71 vs. 21.35±6.31, respectively, p=0.283). There was a significant decrease of Th17 percentage and increase of Treg percentage in NSO group after treatment compared to before treatment (p=0.007, p=0.015, respectively (Figure 2).

ACT Score

At the end of the study, the mean ACT score of standard treatment group was not different from that of NSO group (p=0.692). However, there was a significant increase of ACT score between pre- and post-treatment both in the standard treatment group (17.57±1.222 vs. 19.36±1.151 respectively, p=<0.001) and NSO treatment group (16.57±2.533 vs. 20.29±1.816, p=0.000). The difference of ACT score improvement was significantly

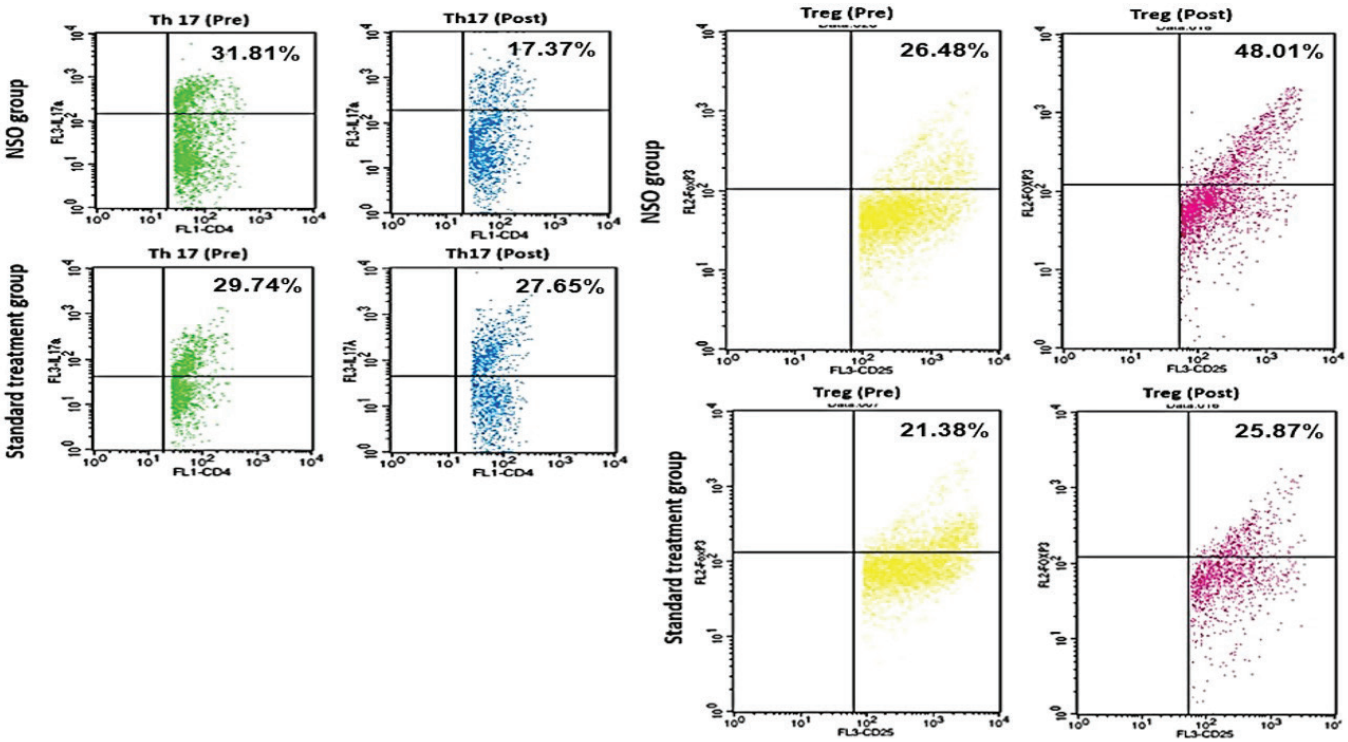

Figure 1. Representative graphics from flow cytometry for Th17 and Treg in NSO and standard treatment group pre and post treatment.

higher in NSO compared to that in the standard treatment group (3.71±1.634 vs 1.86±0.864, respectively, p=0.033) (Figure 3).

Correlation of Th17/Treg ratio and ACT Score

Th17/Treg ratio was significantly lower in NSO group compared to that in the standard treatment group (1.06±0.33 vs 1.31±0.77, respectively, p=0.002). We found a significant decrease of Th17/Treg ratio in NSO group after *Nigella sativa* treatment compared to before treatment (2.30±1.08 vs. 1.06±0.33, respectively, p=0.001). And there was no difference in Th17/Treg ratio in the standard treatment group before and after treatment (1.71±1.55 vs. 1.31±0.77, respectively, p=0.180). However, we found no

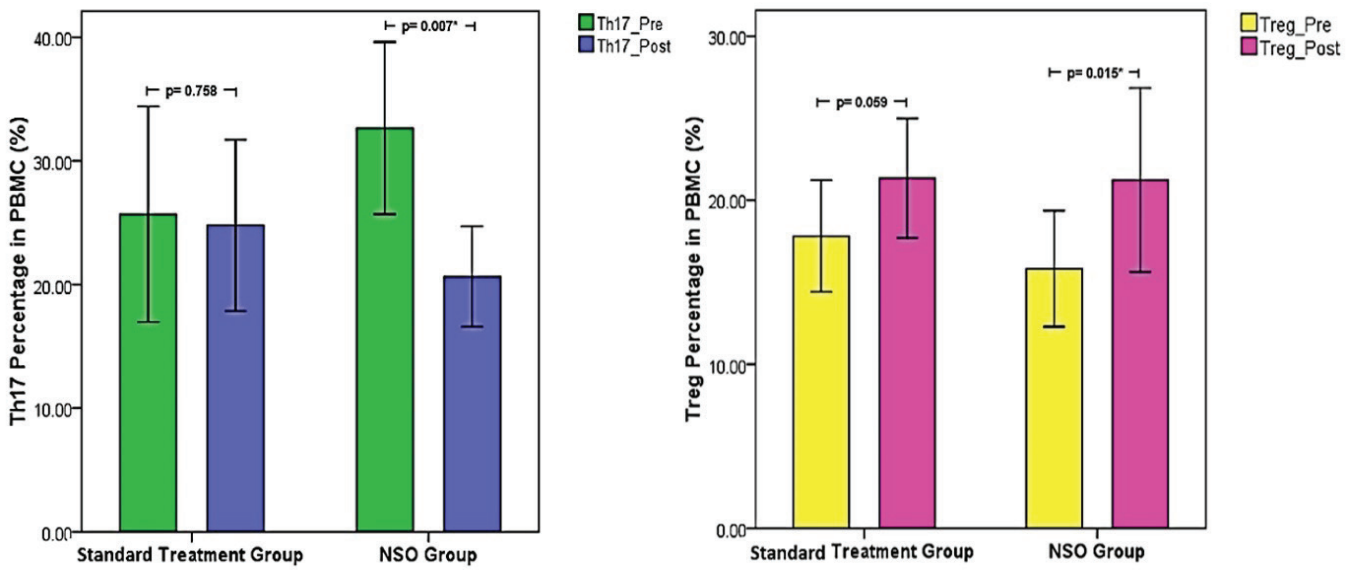

Figure 2. Th17 and Treg percentages in PBMCs by study group (standard treatment and NSO groups) measured by flow cytometry

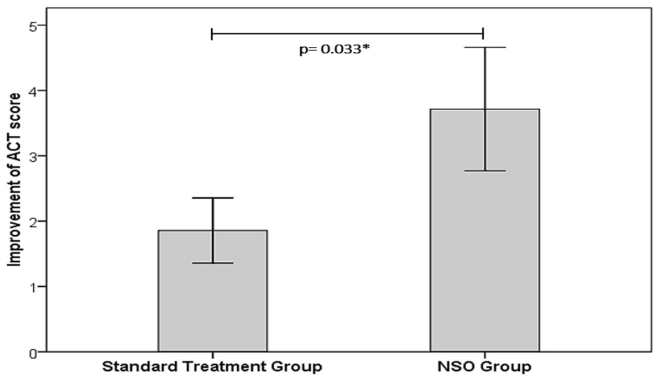

Figure 3. Improvement of ACT score in standard and NSO treatment groups

significant correlation between Th17/Treg and ACT score both in pre- and post-treatment (p=0.552, p=0.344).

Discussion

Nigella sativa (NS) seed, known as the black seed, is a spice and a traditional herbal medicine used in various diseases, such as bronchial asthma.[7] Various medical databases reported the therapeutic effects of NS and its active metabolites in asthma. Several preclinical studies have described multiple effects of NS in animal or cellular models of asthma including bronchodilation, antihistaminic, anti-inflammatory, anti-leukotrienes, and immunomodulatory effects. Furthermore, clinical studies showed improvements in different asthma outcomes including symptoms, pulmonary function, and laboratory parameters.[8]

The use of NS as a herbal medicine, as a complementary, and alternative medicine is common in Indonesia. However, therapies with NS often had insufficient evidence for their effectiveness in asthma patients. In this study, we examined the effect of *Nigella sativa* oil (NSO) supplementation on the improvement of asthma outcomes. We used a single-blind-randomized clinical trial design on pediatric asthma patients in one of tertiary hospitals in Indonesia. Peripheral blood Th17/Treg cells and asthma control test (ACT) score were measured as readouts.

Our study demonstrated that there is an increase in ACT score in both NSO and standard treatment group. However, the difference of ACT improvement was higher in NSO compared to ACT improvement in standard treatment group. Several studies reported that *Nigella sativa* supplementation had beneficial effects on improving asthma control.[8] A randomized, double-blind, placebo-controlled trial conducted by Koshak et al. showed that NSO improves mean ACT score in children with asthma.[9] Another single-blind, placebo-controlled, randomized study conducted by Salem et al. indicated that *Nigella sativa* powder supplementation with inhaled maintenance therapy improves some measurement of pulmonary function and inflammation in partly controlled asthma.[10] Our previous research also reported that the administration of *Nigella sativa* powder among asthmatic children with immunotherapy and probiotic therapy significantly increases the ACT scores.[11,12] Another similar study described that giving NSO as additional treatment in children with asthma shows a significant reduction in PI (Pulmonary Index) and improvement of PEFR (Peak Expiratory Flow Rate).[13] Boskabady et al. also reported that prophylactic therapy of aqueous extract of NS could improve the severity of asthma symptoms.[14]

In the development of asthma, there is a role of Th17 in mediating airway inflammation and hyper-responsiveness. Th17 cell is a particular T functional cell group that secretes IL-17a, IL-17f, and IL-6 and TNF-cytokines; these cytokines play important roles in activating neutrophils and eosinophils, which contribute to the inflammatory process in asthma. Besides, there are Treg cells that have been recognized as important immunoregulatory cells, capable of inhibiting immune response and inducing immune tolerance.[15] As the roles of Treg are explored in the development of asthma, it has been demonstrated that Treg can reduce allergic airway inflammation in mice model.[16] Several studies found that the imbalance of Th17/Treg ratios contributed to asthma progress and rebalancing of Th17/Treg could be a potential therapeutic target.[17,18] In our study, the results suggest that NSO treatment can reduce the Th17 cell percentage and increase the Treg cell percentage in peripheral blood in children with asthma. Th17/Treg ratio is lower in NSO group compared to that in the standard treatment group.

The chemical composition of NS has been studied in considerable detail. The activity of NS appears to be mainly attributed to thymoquinone.[7] One proposed mechanism of action for the *Nigella sativa* as immunomodulator is its regulation of T helper/Treg balance. It has been shown that in inflammatory condition, as in asthma, there is a balance of T helpers and Treg. T helpers, especially Th2

and Th17 cells, promote the activities of macrophages and regulate the pro-inflammatory response, whereas Treg cells inhibit the activity of T helpers.[19] A study conducted by Kheirouri et al. determined that *Nigella sativa* treatment reduced CD8(+) and CD4(+), and increased CD4(+) CD25(+) T cell (Treg) in female patients with rheumatoid arthritis (RA).[20] Another possible mechanism of action for *Nigella sativa* may be due to its effect on dendritic cells (DCs), antigen-presenting cells involved in the initiation of both innate and adaptive immunity, and thus critically important for the regulation of the immune response. Xuan et al. reported that thymoquinone from *Nigella sativa* extract inhibits maturation of LPS-stimulated DCs, and its cytokine secretion. Thymoquinone induces tolerogenic dendritic cells, and increases T regulator proliferation that suppresses inflammation.[21] With these several mechanisms, it is suggested that *Nigella sativa* has a therapeutic effect on asthma improvement.

However, there was no correlation between Th17/Treg ratio and ACT score in this study. Our previous research showed that the combination of immunotherapy with *Nigella sativa* powder and probiotics did not decrease the number of peripheral blood Th17 nor increase the number of peripheral blood Treg cells in asthmatic children, but it can improve the clinical symptoms.[11,12] It can proposed that there are other possible mechanisms of *Nigella sativa* in improving asthma condition. NSO treatment also induces elevation of IFN-γ and reduction of IL-4 cytokines but not in the number of Th1 and Th2 cells.[22] Further investigations are required to study the mechanism of specific cellular and molecular targets of *Nigella sativa* for its clinical application. Besides, the use of small sample size limits the number of inferences we can get from this study. Therefore, studies with larger sample sizes are needed. Finally, in this research, we concluded that NSO supplementation improves Th17/Treg balance and clinical symptoms in asthmatic children.

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Ethics Committee Approval: This study has been approved by the Ethical Committee of Medical Faculty Brawijaya University.

Informed Consent: Written informed consent has also been obtained from all parents/guardians of all subjects.

Peer-review: Externally peer-reviewed.

Conflict of Interest: No conflict of interest was declared by the authors.

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