

Research Article

The effect of single dose of thymoquinone, the main constituents of *Nigella sativa*, in guinea pig model of asthma

Rana Keyhanmanesh^{1*}, Laleh Pejman², Hasan Omrani², Zahra Mirzamohammadi², Amir A Shahbazfar³

¹ Tuberculosis and Lung Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

² Department of Physiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

³ Department of Pathology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

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Abstract

Introduction: In previous studies, the relaxant and antihistaminic effects of thymoquinone, the main constituents of *Nigella sativa*, have been demonstrated on guinea pig tracheal chains. In the present study, the prophylactic effect of (IP) single dose of thymoquinone on tracheal responsiveness and lung inflammation of guinea pig model of asthma was examined.

Methods: Thirty guinea pigs were randomly divided to 3 groups; control (C), sensitized (S) and pretreated group with (TQ); 3 mg/kg, IP (S+TQ). Tracheal responsiveness to methacholine and ovalbumin (OA), total and differential cell count in bronchoalveolar lavage, lung pathological changes and blood Interleukin 4(IL-4) and Interferon gamma (IFN γ) level in three groups were measured.

Results: Increased tracheal responsiveness to methacholine and OA, lung lavage fluid white blood cell (WBC) and eosinophil count, IL-4 and IFN- γ levels and pathological changes were seen in sensitized group in comparison to control group ($p < 0.001$ to $p < 0.05$). Decreased tracheal responsiveness to methacholine and OA, pathological changes and bronchoalveolar lavage eosinophil were observed in S+TQ group compared to S group ($p < 0.001$ to $p < 0.05$). However, tracheal responsiveness to methacholine and OA, contractility, bronchoalveolar lavage WBC and eosinophil and most of pathological changes in S+TQ group were significantly higher than those in controls ($p < 0.01$ to $p < 0.05$).

Conclusion: These results showed the preventive effect of single dose of thymoquinone on guinea pig model of asthma.

Introduction

Nowadays the prevalence of asthma, a global health problem, is increasing. Asthma is a common chronic disease characterized by various features including reversible airways obstruction, an increased airway responsiveness and airway inflammation¹ which is a key feature of bronchial asthma.² Studies on animals and humans have shown the release of inflammatory mediators from mast cells causing bronchoconstriction. Moreover many other inflammatory cells, including eosinophils, macrophages and neutrophils, are involved in the pathogenesis of airway inflammation in asthma.³ Many chemical drugs were used in treatment of this disease like quick relief and long-term controller medications which control smooth muscle contraction and decrease inflammation. Although these drugs are effective, they have side effects too. So scientists are working with traditional or folk medicines in parallel with modern medicines. Among these herbal medicines, *Nigella sativa*

(*N. sativa*) has been used as traditional treatment of asthma as well as other inflammatory diseases.^{4,5} Thymoquinone (TQ) active ingredient of *N. sativa* has been investigated for its anti-oxidant, anti-inflammatory and anticancer properties in both *in vitro* and *in vivo* models.⁶ The therapeutic effects of *N. sativa* oil on patients with allergic diseases (including allergic rhinitis, bronchial asthma, and atopic eczema) were also demonstrated.⁷ TQ is a potent inhibitor of the inflammatory changes associated with asthma.⁸ TQ inhibits T helper 2 (Th2) cytokines and eosinophil infiltration into the airways and decreases allergic airway inflammation; this evidence demonstrates its potential anti-inflammatory role during the allergic response in the lung. However, little is known about the factors and mechanisms underlying these all effects.⁹ Our earlier studies showed that 33-day oral administration of TQ had preventive effect on tracheal responsiveness and lung inflammation in ovalbumin-induced asthmatic guinea pigs.^{10,11} Following previous studies



*Corresponding author: Rana Keyhanmanesh, Email: keyhanmaneshr@tbzmed.ac.ir



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for determining the effect of TQ in pathophysiology of asthma, in this investigation the effect of single dose intraperitoneal administration of TQ was assessed on tracheal responsiveness to methacholine and ovalbumin (OA), total and differential cell count in bronchoalveolar lavage, lung inflammation and blood cytokines of sensitized guinea pigs.

Materials and methods

Animal sensitization and animal groups

Thirty adult Dunkin-Hartley guinea pigs (400–700 g, male sex) were used. The animals were group-housed in individual cages in climate-controlled animal quarters and were given water and food ad libitum, while a 12-h on/12-h off light cycle was maintained.

Animals were randomly divided into three groups; control group (C), sensitized group with OA (S), and sensitized group pretreated with thymoquinone (S+TQ). TQ with 3 mg/kg dose was injected intraperitoneally on day 10 of induction protocol.

Sensitization of animals to OA was performed using the method of our previous study.¹² Briefly, guinea pigs were sensitized to OA (Grade II Sigma Chemical Ltd., UK) dissolved in saline by injecting 100 mg i.p. and 100 mg s.c. on the first day and a further 10 mg via intraperitoneal on eighth day. From day 14, sensitized animals were exposed to an aerosol of 4% OA for 18 ± 1 days, 4 min daily. The aerosol was administered in a closed chamber, dimensions $30 \times 20 \times 20$ cm. Control animals were treated similarly but saline was used instead of OA solution. The study was approved by the ethical committee of the Tabriz University of Medical Sciences.

Tissue preparation

Guinea pigs were killed by a blow on the neck and the trachea was removed. In each animal, one tracheal chain was prepared as follows: the trachea was cut into 10 rings (each containing 2–3 cartilaginous rings). All the rings were sutured together to form a tracheal chain and then they cut open opposite the trachealis muscle except two terminal rings. Tissue was then suspended in a 20-mL organ bath (Schuler organ bath type 809, Germany) containing Krebs-Henseliet solution of the following composition (mM): NaCl; 120, NaHCO₃; 25, MgSO₄; 0.5, KH₂PO₄; 1.2, KCl; 4.72, CaCl₂; 2.5 and dextrose 11. The Krebs solution was maintained at 37 °C and gassed with 95% O₂ and 5% CO₂. Tissue was suspended under isotonic tension of 1 g and allowed to equilibration for at least 1 h while it was washed with Krebs solution every 15 min.¹³

Responses were measured using an isometric transducer (ADInstruments, Spain) with a sensitivity range of 0–25 g and amplified with an amplifier (ML/118 quadribridge amp; March-Hugstetten, Germany) and recorded on a powerlab (ML-750, 4 channel recorder; March-Hugstetten, Germany).

Assessment of tracheal response to Methacholine

In each experiment, a cumulative concentration-response curve of methacholine hydrochloride (Sigma Chemical Ltd., UK) induced contraction of the tracheal chain was obtained. Consecutive concentrations (including 10^{-7}

to 10^{-2} M, dissolved in saline) were added every 3 min. The contraction due to each concentration was recorded at the end of 3 min and the effect reached a plateau in all experiments. To obtain the curve, the percentage of contraction of the tracheal smooth muscle because of each concentration of methacholine in proportion to the maximum contraction obtained by its final concentration was plotted against log concentration of methacholine. A concentration-response curve of methacholine was performed in the tracheal chain of each studied animal. The effective concentration of methacholine causing 50% of maximum response (EC50) was measured from the methacholine response curve in each experiment using 50% of the maximum response in the Y axis and measuring the dose of methacholine causing this response in the X axis. The contractility response to 10 μM methacholine as the magnitude of contraction was also measured.

Measurement of tracheal response to Ovalbumin (OA)

The tracheal response of all animals to a 0.1% solution of OA was measured in each studied animal as follows: 0.5 mL of 4% OA solution (dissolved in saline) was added to the 20-mL organ bath and the degree of tracheal chain contraction was recorded after 15 min and was expressed as a proportion (in percentage) to the contraction obtained with 10 μM methacholine.

The measurements of tracheal response to methacholine and OA were performed in random order.

Lung lavage and its white blood cell (WBC) count

Coincident with preparing the tracheal chain a cannula was located into the remaining trachea, and the lungs were lavaged with 5 mL of saline 4 times (total: 20 mL). One mL of lung lavage fluid (LLF) was stained with Turk solution; consisted of 1 mL of glacial acetic acid, 1 mL of gentiac violet solution 1% and 100 mL of distilled water and counted in duplicate in a hemocytometer (in a Burkner chamber). The remaining LLF was centrifuged at $2500 \times g$ at 4 °C for 10 min. The supernatant was removed. The smear was prepared from the cells and stained with Wright-Giemsa. According to staining and morphological criteria, differential cell analysis was carried out under a light microscope by counting 100 cells twice and the percentage of each cell type calculated.¹⁴

Lung pathological evaluation

Guinea pigs were sacrificed by a cervical dislocation and their lungs and trachea were removed and placed into 10% buffered formalin (37%, Merck, Germany). Seven days later, the tissues were dried using an Autotechnicon apparatus by passage the tissues through 70% ethanol and xylol to clear the tissues and then paraffin block the tissues. The specimens were cut into 4-μm slices and stained with hematoxylin and eosin (H&E stain). The tissues were then evaluated under a light microscope.

The pathologic changes in the lung of sensitized and treated groups included the vascular and airway membrane hyperplasia, the presence of mucosal plug and emphysema, respiratory epithelial denudation and cellular infiltration. The pathological changes were scored according to previous studies¹¹ as follows: (1) no pathologic changes, 0; (2) patchy changes, 0.5; (3) local

changes, 1; (4) scattered changes, 2; (5) severe changes (in the most parts of the lung), 3.

Measurement of blood interleukin 4 (IL-4) and interferon-gamma (IFN- γ) levels

Five milliliters of peripheral blood was obtained immediately after sacrificing the animals and placed at room temperature for 1 h. The samples were then centrifuged at 2500 rpm at 4 °C for 5 min. The supernatant was collected and immediately stored at -70 °C until analysis. Finally, blood IL-4 and IFN- γ levels were measured using the sandwich enzyme-linked immunosorbent assay (ELISA) method with commercial kits (Glory science co. Ltd, USA).

Statistical analysis

The data of tracheal response to methacholine (EC₅₀), tracheal contractility response, tracheal response to OA, total WBC numbers, differential WBC counts, lung pathology and cytokines were quoted as mean \pm SEM. The data of two sensitized groups were compared with control's using one-way analysis of variance (ANOVA) with Tukey-Kramer post-test. Moreover, the data of the sensitized group were compared with that of control and treated guinea pigs using one-way analysis of variance (ANOVA) with Tukey-Kramer post-test. Significance was accepted at $p < 0.05$.

Results

Tracheal response to methacholine

Concentration response curves to methacholine showed leftward shift of the curve in group S compared to group C. The curve of S+TQ group was shifted to right compared to group S, although there was leftward shift in comparison to control (Fig. 1).

The mean value of EC₅₀ in tracheal chains of group S (1.50 \pm 0.36 μ M) was significantly lower than that in group C (5.31 \pm 0.71 μ M, $p < 0.001$) and group TQ (2.35 \pm 0.14 μ M, $p < 0.01$). However, the mean value of EC₅₀ in tracheal chains of S+TQ group was still significantly lower than that in group C ($p < 0.05$, Fig. 2).

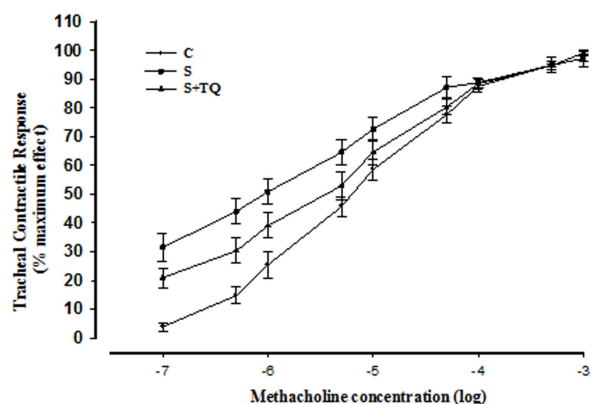


Fig. 1. Cumulative log concentration-response curves of methacholine induced contraction of isolated trachea in control (C), sensitized (S), S treated with thymoquinone (S+TQ) guinea pig tracheal chains in the organ bath (for each group, $n=7$).

Tracheal response to ovalbumin

Tracheal responsiveness to OA in tracheal chains of group S (62.88 \pm 9.12%, range 42.85-100%) was significantly higher than that in group C (4.85 \pm 3.18%, range 0-23%, $p < 0.001$). Pre-treatment with TQ (22.49 \pm 4.42%, range 11.11-40 %) was significantly improved compared to group S ($p < 0.01$). However, tracheal response to OA in S+TQ group was still significantly higher than that in group C ($p < 0.01$, Fig. 3).

Contractility

The contractility response of tracheal chains to methacholine of group S (1.63 \pm 0.08 g $p < 0.001$) and group S+TQ (1.28 \pm 0.19 g, $p < 0.01$) was significantly higher than that of group C (0.58 \pm 0.04 g). The contractility response in treated group with TQ caused no significant decrease

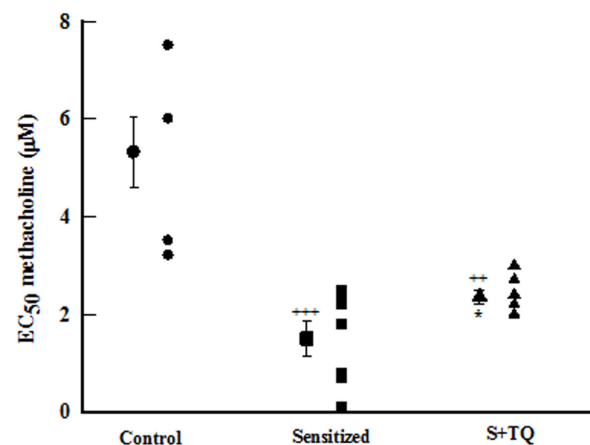


Fig. 2. Individual values and mean \pm SEM (big symbols with bars) of tracheal response to methacholine (EC₅₀) in control (C), sensitized (S), S treated with thymoquinone (S+TQ) guinea pigs (for each group, $n=6$). Statistical differences between control and different groups: ++, $p < 0.01$, +++, $p < 0.001$. Statistical differences between S+TQ vs. sensitized group: *; $p < 0.05$.

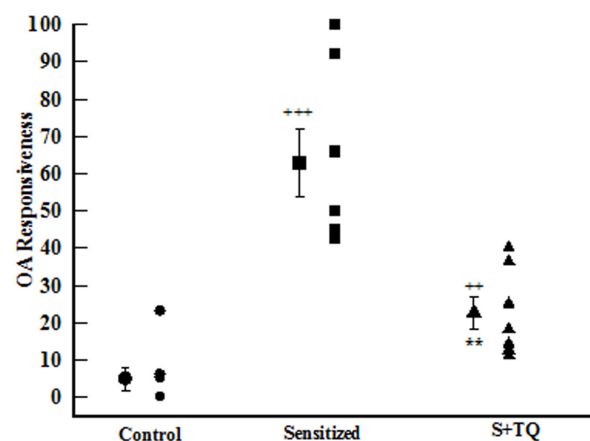


Fig. 3. Individual values and mean \pm SEM (big symbols with bars) of tracheal response to ovalbumin (percent concentration in proportion to contraction obtained by 10 μ M methacholine) in control (C), sensitized (S), S treated with thymoquinone (S+TQ) guinea pigs (for each group, $n=6$). Statistical differences between control and different groups: ++, $p < 0.01$, +++, $p < 0.001$. Statistical differences between S+TQ vs. sensitized group: **; $p < 0.01$.

compared with S group (Fig. 4).

Total white blood cell count in Lung Lavage fluid (LLF)

The mean value of total WBC in LLF of group S (9421.43 ± 169.1) and group S+TQ (6073.14 ± 879.86) was significantly higher than that of group C (2580 ± 180.02 , $p < 0.01$). The WBC in S+TQ group was not significantly lower than that in sensitized animals (Fig. 5).

Differential count of WBC in Lung Lavage fluid

Results showed that in LLF of group S neutrophil, lymphocyte and monocyte were significantly decreased but remarkable increase of eosinophil and non-significant rise of basophil was determined in comparison to group C ($p < 0.001$ for all cases). Administration of TQ caused

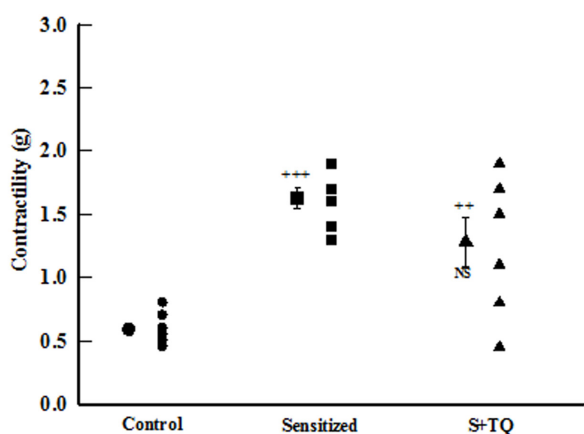


Fig. 4. Individual values and mean \pm SEM (big symbols with bars) of tracheal contractility response to 10 μ M methacholine in control (C), sensitized (S), S treated with thymoquinone (S+TQ) guinea pigs (for each group, $n=6$). Statistical differences between control and different groups: ++, $p < 0.01$, +++, $p < 0.001$. Statistical differences between S+TQ vs. sensitized group: NS: non-significant differences.

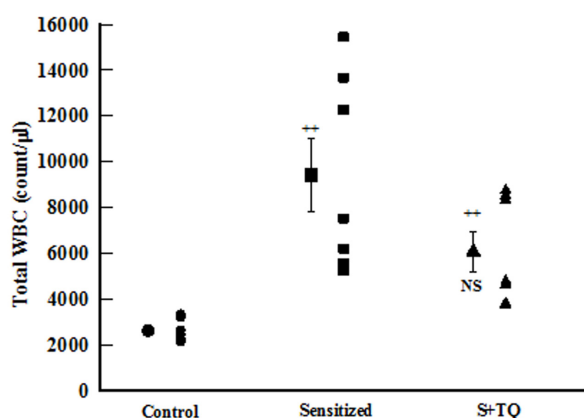


Fig. 5. Individual values and mean \pm SEM (big symbols with bars) of total LLF WBC number in control (C), sensitized (S), S treated with thymoquinone (S+TQ) guinea pigs (for each group, $n=6$). Statistical differences between control and different groups: ++, $p < 0.01$, +++, $p < 0.001$. Statistical differences between S+TQ vs. sensitized group: NS: non-significant differences.

significant decline in eosinophil ($p < 0.001$), basophil ($p < 0.05$) and condensable increment in neutrophil, monocyte and lymphocyte count ($p < 0.001$ for all cases) in comparison with S group. However there is a remarkable difference in eosinophil, lymphocyte and monocyte counts between S+TQ and C groups ($p < 0.01$ for all, Fig 6a-e).

Pathology

With regard to this scoring, all pathological changes in the S group, including the vascular membrane hyperplasia (3 ± 0.30), airway membrane hyperplasia (2.54 ± 0.28), the presence of mucosal plug ($2.720.14$), respiratory epithelial denudation (2.18 ± 0.23), cellular infiltration (1.55 ± 0.20) and emphysema (2 ± 0.27) were significantly higher than those in the group C (0.33 ± 0.21 , 0.17 ± 0.16 , 0 ± 0 , 0.17 ± 0.16 , 0 ± 0 and 0.17 ± 0.16 respectively, $p < 0.05$ for all cases, Fig 7, Fig. 8a-e).

Pretreatment with TQ caused a significant improvement in all pathological changes ($p < 0.05$). However, there were still significant variations in presence of mucosal plug, respiratory epithelial denudation and emphysema between

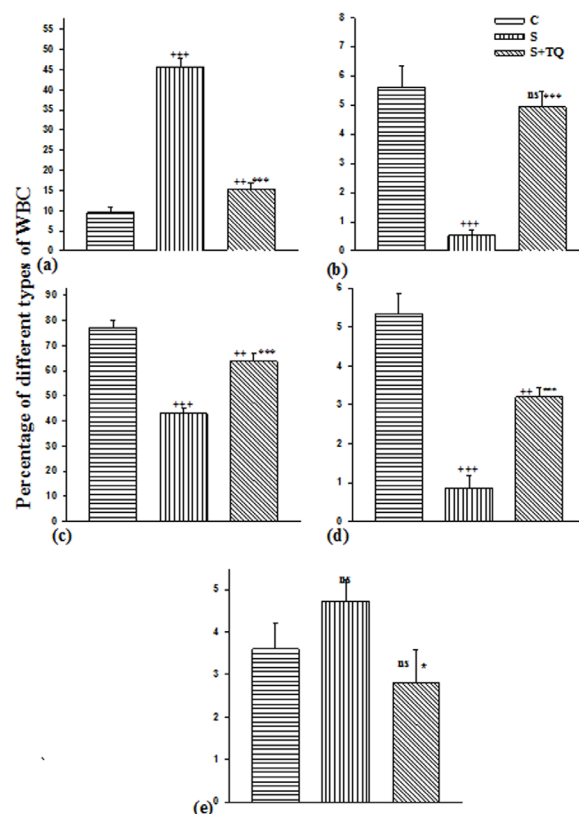


Fig. 6. The percentages of eosinophil (a), neutrophil (b), lymphocyte (c), monocyte (d) and basophil (e) of lung lavage fluid in control, sensitized (S), S treated with thymoquinone (S+TQ) guinea pigs (for each group, $n=15$). Statistical differences between control and different groups: ns; non-significant differences, ++, $p < 0.01$, +++, $p < 0.001$. Statistical differences between S+TQ vs. sensitized group: *: $p < 0.05$, ***: $p < 0.001$.

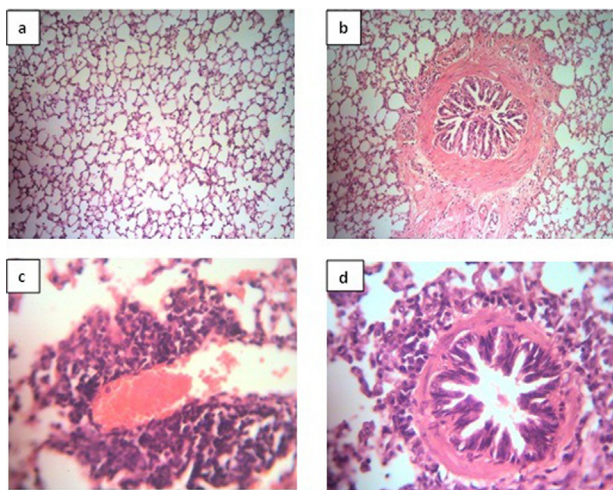


Fig. 7. Lung pathological changes in different groups. Normal lung tissue in control group (a, $\times 200$), airway membrane hyperplasia and the presence of mucosal plug in group S (b, $\times 200$), cellular infiltration in group S (c, $\times 800$) and group S+TQ (d, $\times 800$).

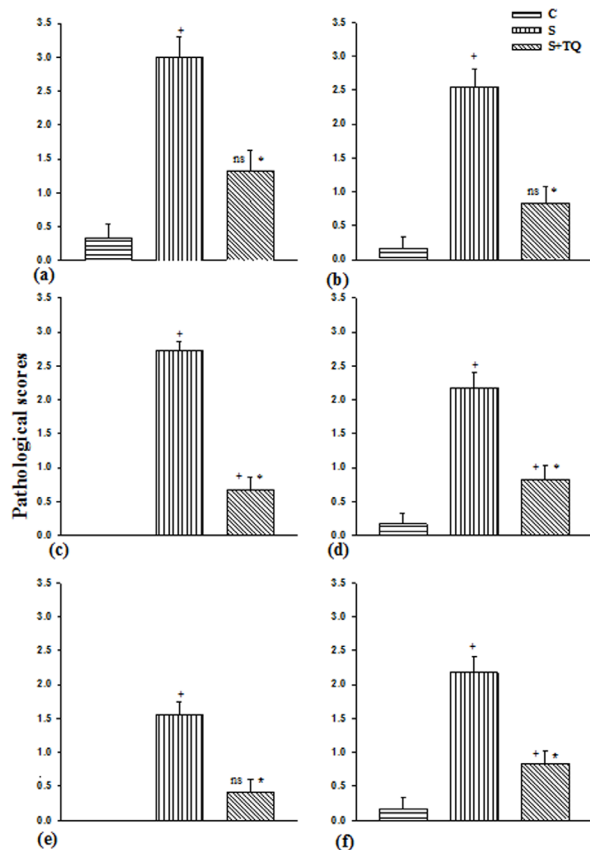


Fig. 8. The vascular (a) and airway membrane hyperplasia (b), mucosal plug (c), local epithelial denudation (d), eosinophil and lymphocyte infiltration (e) and emphysema (f) of lungs in control (C), sensitized (S), S treated with thymoquinone (S+TQ) guinea pigs (for each group, $n = 8$). Statistical differences between the control and the different groups: ns; no-significant difference, +; $p < 0.05$, ++; $p < 0.01$. Statistical differences between S+TQ vs. sensitized group: *; $p < 0.05$.

group C and the pretreated group ($p < 0.05$, Fig 8a-e).

IL-4 and IFN- γ levels

The mean value of the blood IL-4 levels in group S (47.41 ± 1.98) was significantly higher than that in group C (39.78 ± 2.01 , $p < 0.05$). The blood IL-4 level in the treated group, S +TQ (43.88 ± 1.70), showed non-significant decrease compared to that of the S group. However, the mean value of the IL-4 in this group was not significantly higher than that in group C (Fig 9b). The mean value of the blood IFN- γ level of group S (117.37 ± 2.71) was significantly higher than that of group C (104.97 ± 5.94), $p < 0.05$). Treatment with TQ caused a significant increase in blood IFN- γ in the S +TQ group (124.93 ± 2.31) compared to that in the S group ($p < 0.05$). In addition, the mean value of IFN- γ in this group was significantly higher than that in group C ($p < 0.01$, Fig 9b).

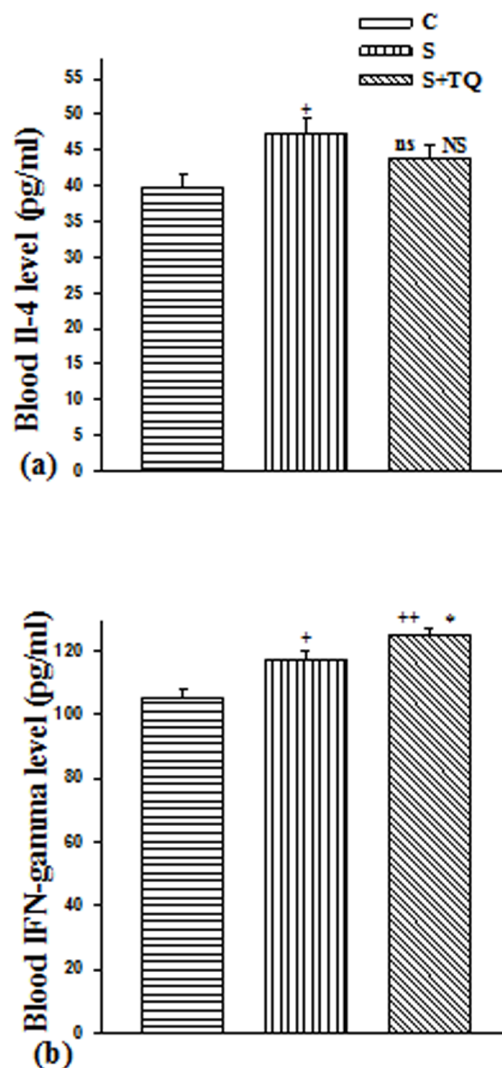


Fig. 9. The blood IL-4 (a) and IFN- γ (b) levels (pg/ml) in control, sensitized (S), S treated with thymoquinone (S+TQ) guinea pigs (for each group, $n = 8$). Statistical differences between the control and the different groups: ns; no significant difference, +; $p < 0.05$, ++; $p < 0.01$. Statistical differences between S+TQ vs. sensitized group: NS; no significant difference, ** $p < 0.01$

Discussion

In the present study, we assessed the preventive effect of single intraperitoneal dose of TQ on the tracheal responsiveness to methacholine and OA, the contractility response to methacholine, the total and differential WBC count of lung lavage fluid, lung pathological changes and blood cytokine (IL-4 and IFN- γ) levels of sensitized guinea pigs. Induction protocol caused a further increase in the contractility response and the tracheal responsiveness to methacholine and OA and a decrease in EC50 value compared to controls. In addition, increased lung lavage fluid WBC and eosinophil count, IL-4 and IFN- γ levels and pathological changes were seen in sensitized group in comparison to control. These results were relevant to the results of previous studies.^{10-12,14-16}

The main pathological feature of asthmatic patients is airway inflammation, which causes the most characteristic feature of the disease, increased airway responsiveness. All drugs used to treat asthma aim to reduce this inflammation. In this study, administration of TQ could decrease all these inflammatory changes. The exact mechanism of anti-inflammatory action for TQ is not known but the inhibitory effect of TQ on Histamine H₁ receptors was seen in previous studies.¹⁷ Moreover, other studies confirmed the therapeutic effect of TQ on 152 patients with allergic rhinitis, bronchial asthma and atopic eczema.⁷ The anti-inflammatory and antitussive effect of TQ has already been demonstrated in our previous studies,^{10,11} although in these studies TQ was orally administrated in 33 days and in present study, single dose of this drug was administrated intraperitoneally. The preventive effect of TQ may be due to its ability to suppress airway inflammation which is indicated by Hajhashemi in 2004.¹⁸ However, Boskabady in 2005 failed to show any relaxant effect for TQ on tracheal smooth muscle in organ bath.¹⁹ It is possible that TQ has an anti-inflammatory effect without having a direct relaxant effect.

One proposed mechanism of action for TQ is the regulation of the Th1 and Th2 balance. It has been shown that the inflammatory condition of an asthmatic lung is regulated by the balance of these two mutually inhibited subsets of CD4⁺ helper T cells. Th₁ cells produce IL-2 and IFN- γ but little or no IL-4, whereas Th₂ cells produce IL-4 and IL-10 but no IFN- γ or IL-2. Th₂ cells promote the activity of macrophages and regulate the pro-inflammatory response, whereas Th₁ cells inhibit the activity of macrophages directly or indirectly by inhibiting Th₂ activity and thus regulating the anti-inflammatory response.²⁰ In fact, asthma is associated with a shift in immune responses away from a Th₁ (IFN- γ) pattern and toward a Th2 (IL-4, IL-5 and IL-13) profile.^{21,22}

In the present study, asthma induction increased IL-4 and IFN- γ and TQ administration decreased IL-4 and increased IFN- γ compared with sensitized group. These results have also been reported in previous studies.^{11,14} Reduced level of IL-4 and increased level of IFN- γ in serum of sensitized animals pretreated with TQ may reveal stimulatory effect of TQ on Th₁ and a suppressive

effect on Th₂ cells.

The inflammatory cells produce more reactive oxygen species than cells got from normal subjects.²³ Reactive oxygen species contract airway smooth muscles and simulate both histamine release from mast cells and mucus secretion from airway epithelial cells.²⁴ TQ has been shown to have strong antioxidant properties²⁵⁻²⁸ and to suppress the expression of inducible nitric oxide (NO) synthesis in rat macrophages.²⁹ It has been shown that TQ has inhibitory effects on both the cyclooxygenase and the 5-lipoxygenase pathways of arachidonic acid metabolism and on membrane lipid peroxidation.³⁰

Conclusion

In conclusion, these results showed the preventive effect of TQ on tracheal responsiveness to methacholine and OA and pathological and cytokine changes in sensitized guinea pigs; therefore, the results may suggest the prophylactic effects of this substance on asthma.

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Ethical issues

The study was approved by the ethical committee of the Tabriz University of Medical Sciences.

Competing interests

There is none to be declared

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