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Research Article

Thymoquinone, the Main Constituent of *Nigella sativa*, Could Impact on Adenosine A_2 Receptors in Ovalbumin-sensitized Guinea Pigs

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Abstract

Thymoquinone has demonstrated anti-asthmatic effects in many studies but its exact mechanism is not yet fully known. This investigation aims to demonstrate its prophylactic effect in the presence of selective A_{2A} and A_{2B} adenosine receptors (AR) antagonist; MRS1706 and ZM241365, in sensitized guinea pigs. The gene expression of A_2 AR in blood lymphocytes and lung tissue, lung pathological changes and blood cytokines were evaluated in seven groups. The experiments in blood lymphocytes and lung tissue showed that thymoquinone could increase A_{2A}AR mRNA expression and decrease A_{2B} AR mRNA expression significantly ($P<0.001$ to $P<0.05$); however sensitization had opposite effects. Administration of A_{2A} receptor antagonist attenuated inflammation and A_{2B} receptor antagonist could prevent asthma-induced inflammatory changes. Moreover, the administration of thymoquinone and A_{2A}receptor antagonist together relieved inflammation. Gene expression of A_{2B} receptor showed that thymoquinone administration has more influence on blood lymphocytes while administration of the selective A_{2B} receptor antagonist was more effective in lung tissue. The results showed some of the therapeutic effects of thymoquinone in reducing asthma symptoms might be partially mediated through A_2 adenosine receptors.

Keywords: *Asthma, Adenosine, MRS1706, ZM241365, Gene expression*

Nigella Sativa'nın Biyoaktif Komponenti Olan Timokinon Ovalbuminle Uyarılmış Ginedomuzlarında Adenozin A_2 Reseptörlerini Etkileyebilir

Özet

Timokinonun birçok çalışmada anti-astmatik etkileri olduğu gösterilmesine rağmen tam mekanizması tamamıyla anlaşılamamıştır. Bu çalışma A_{2A} ve A_{2B} adenozin reseptör (AR) antagonistlerinin (MRS1706 ve ZM241365) mevcudiyetinde timokinonun uyarılmış ginedomuzlarındaki proflaktik etkisini araştırmak amacıyla yapılmıştır. Çalışmada toplam yedi grupta kan lenfositleri ve akciğer dokusunda A_2 AR gen ekspresyonları, akciğerdeki patolojik değişiklikler ve kan sitokinleri değerlendirildi. Kan lenfositleri ve akciğer dokusunda yapılan incelemeler timokinonun A_2AAR mRNA ekspresyonunu arttırdığını ve A_{2B} AR mRNA ekspresyonunu ise anlamlı oranda azalttığını, ancak uyarılmanın ters etki yaptığını ortaya koydu ($P<0.001$ to $P<0.05$). A_{2A} reseptör antagonisti yangıyı azaltırken ve A_{2B} reseptör antagonisti astma tarafından oluşturulan yangısal değişikliklere karşı koruyucu etki gösterebilir. Ayrıca, timokinon ve A_{2A} reseptör antagonistinin birlikte uygulanması yangıyı hafifletti. A_{2B} reseptör gen ekspresyonu timokinon uygulamasının kan lenfositleri üzerinde daha etkili olduğunu A_{2B} reseptör antagonistinin ise akciğer dokusunda daha etkili olduğunu gösterdi. Elde edilen sonuçlar astma semptomlarının azaltılmasında timokinon uygulamasının terapötik etkilerinin kısmen A_2 adenozin reseptörleri yoluyla oluştuğunu göstermiştir.

Anahtar sözcükler: *Astma, Adenozin, MRS1706, ZM241365, Gen ekspresyonu*

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INTRODUCTION

Asthma is a chronic and acute respiratory disease of the airways [1] which has been identified by various properties such as the increased responsiveness of airways to physical and chemical stimuli [2]. In asthma, the responses of the immune system and related cells including T lymphocytes are influenced. Asthma is accompanied by the increase in the immune system of Th_2 cells and a decrease in the immune response of Th_1 cells. Th_2 cells are the regulators of pre-inflammatory responses and Th_1 cells directly or indirectly inhibit Th_2 cells [3,4]. Therefore, the increased level of IL-4 (cell markers of Th_2) and decreased level of IFN-γ (cell markers of Th_1) can be seen in this disease [5,6].

Other regulating factors which are propounded in the inflammation are adenosine and adenosine receptors. Adenosine is a purine nucleoside of adenine that mediates the various physiological functions through four transmembrane receptors; A_1, A_{2A}, A_{2B} and A_3 [7]. In asthmatic patients, the level of adenosine in liquid bronchoalveolar lavage is noticeably more than the healthy people [8]. Moreover, inhaled adenosine causes bronchial contraction in asthmatic patients but in healthy people it does not cause any contraction [9]. In fact, adenosine receptors act as sensor and extracellular adenosine acts as a reporter [10]. The stimulation of adenosine A_{2A} receptor inhibits the release of inflammatory mediators [11] and also prevents the activation of T cells [12] and inhibits the mobilization of inflammatory cells in the airways. The stimulation of A_{2B} receptor in human beings activates the mast cells [13], and as a result, increases the release of IL-8 [14]. The inhibition of A_{2B} receptors can have helpful therapeutic effects on asthma [15] and lead to the decrease of bronchospasm in response to such stimuli as AMP [16].

Nowadays, a lot of medicines are used for treating asthma, but the long-term use of them results in therapeutic resistance and will have some undesirable side effects. Therefore, in recent years, the researchers have tried to find new medicines with high effectiveness and fewer side effects. One of these approaches is using herbs. Many studies showed that *Nigella sativa* had anti-inflammatory and therapeutic effects [17-20]. Moreover, our previous studies demonstrated that the extract of *Nigella sativa* and its main constituent, thymoquinone, had preventive effects on asthma and precluded its inflammatory and pathological changes [5,6,21,22].

The exact mechanism of anti-asthmatic effect of thymoquinone is not known yet and the adjustment of Th_1/Th_2 balance has been suggested as one of the possible mechanisms for thymoquinone. In light of this, and in order to find out the intracellular mechanisms of thymoquinone, this investigation was proposed to demonstrate its prophylactic effect on gene expression of A_2 adenosine receptors in blood lymphocytes and lung tissue in the presence of selective A_{2A} and A_{2B} adenosine receptor antagonists; ZM241365 and MRS1706, in asthmatic guinea pigs. In addition, blood IL-4 and IFN-γ level and lung pathological changes were assessed in different groups.

MATERIAL and METHODS

Animal Sensitization and Animal Groups: Seventy male adult Dunkin-Hartley guinea pigs (400-700 g) were used throughout the study. After transportation, they were allowed to acclimatize to the new situation for ten days. They were kept in individual cages at 22±2°C on a 12-h light/dark cycle and allowed free access to standard chow and water. Then the animals were randomly divided into seven groups; control group (C), sensitized group with ovalbumin (S), sensitized group pretreated with thymoquinone (3 mg/kg i.p.; S+TQ) [23], sensitized group pretreated with selective A_{2A} adenosine receptor antagonist (ZM241385, 3 mg/kg i.p.; S+Anta A_{2A}) [24], sensitized group pretreated with selective A_{2B} adenosine receptor antagonist (MRS1706, 3 mg/kg i.p.; S+Anta A_{2B}) [9], sensitized group pretreated with thymoquinone and A_{2A} adenosine receptor antagonist (S+TQ+Anta A_{2A}) and sensitized group pretreated with thymoquinone and A_{2B} adenosine receptor antagonist (S+TQ+Anta A_{2B}). Thymoquinone, ZM241385 and MRS1706 with 3 mg/kg dose were injected intraperitoneally on day 10 of induction protocol. All groups were housed in climate-controlled animal quarters and were given water and food *ad libitum,* while a12-h on/12-h off light cycle was maintained.

Sensitization of animals to ovalbumin (OA) was performed using the method used in our previous study [25]. Briefly, guinea pigs were sensitized to ovalbumin (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 i.p. on the 8th day. From day 14, sensitized animals were exposed to an aerosol of 4% ovalbumin for 18±1 days, 4 min daily. The aerosol was administered in a closed chamber, dimensions 30 × 20 × 20 cm. Control animals were treated similarly but saline was used instead of ovalbumin solution. The study was approved by the ethical committee of the Tabriz University of Medical Sciences (No: TBZMED.REC.1394.862).

Evaluation of the Gene Expression of A_2 Receptors in Blood Lymphocytes and Lung Tissue: One day after the induction period, the animals were killed by cervical dislocation and 5 ml blood sample and approximately, 100 µg of left lung tissue were obtained immediately. At first, blood RBCs were eliminated by RBC lysis buffer containing 9 g NH_4Cl, 1g $KHCO_3$ and 0.2 ml of 0.5M EDTA per liter. The pH was adjusted to 7.3. Then TRIzol reagent was used for RNA extraction. Lung specimens were also grinded and subjected to total RNA extraction by 1 ml of TRIzol reagent (life technologies Co) according to the manufacturer's recommendations. After that, cDNA was

synthesized by Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific Co) using 5 µg of total RNA. The effect of thymoquinone on the gene expression of A_2 adenosine receptors was investigated with RT-PCR. Specific primers were designed for control and receptor genes. A_{2B} primers were designed by OLIGO (version 5.0). The sequences were A_{2A} F: 5′-GCA GAA CGT CAC CAA CTA CTT-3′, A_{2A} R: 5′-CAG GTC ACC AAG CCA TTG TA-3′, A_{2B} F: 5′-CTT TGG CAT TGG ATT GAC TC-3′ and A_{2B} R: 5′-CCA GCA TGA TGA GCA GTG G-3′. β-actin was employed as a housekeeping gene to normalize expression levels of target genes. Primer sequences for β-actin were: β-actin F: 5′- TCC CTG GAG AAG AGC TAC G-3′ and β-actin R: 5′- GTA GTT TCG TGG ATG CCA CA-3′.

The cycling included denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 58°C for 40 s and 72°C for 30 s and a final extension at 72°C for 10 min. PCR products were electrophorized utilizing 1.5% agarose gel and appropriate amount of safe stain (Cinnagen Co,Islamic republic of Iran) and were visualized by gel documentation apparatus. 100 bp DNA ladder (SM0311, Fermentas Co.) was used as size marker. Finally the electrophoresed gene was photographed by document gene device and the intensity of bands was investigated through Image J software (National Institutes of Health, Bethesda, Maryland, USA) and normalized to its actin loading control.

Evaluations of Blood IL-4 and IFN-γ Levels: A total of 5 ml peripheral blood was obtained immediately after sacrificing the animals and placed at room temperature for 1 h. The samples were then centrifuged at 3500×*g* at 4°C for 10 min. The supernatant was collected and immediately put at -70°C until analyzed. Finally, blood IL-4 and IFN-γ were measured using the enzyme-linked immunosorbent assay (ELISA) Sandwich method [6].

Pathological Evaluation: Lungs and trachea were kept in 10% (v/v) buffered formalin. A week later tissues were dried by a range of ethanol concentrations (70%-100%) through Passage method. The samples were saturated in paraffin and put into blocks after being cleared by xylol. Then 4 micron-width slices were prepared using microtome. Finally, the specimens were stained by hematoxylin-eosin (H&E) and evaluated under a light microscope. The pathological changes in the lungs of all groups included vascular and airways smooth muscle hypertrophy and hyperplasia, the presence of mucosal plug, respiratory epithelial denudation, inflammatory infiltration and emphysema [5,21]. The pathological changes were scored according to previous studies as follows: 0: no pathologic changes, 1: patchy changes, 2: local changes, 3: scattered changes and 4: severe changes.

Statistical Analysis: All the results were considered as mean ± SEM. The data of six sensitized groups were compared with controls using one-way analysis of variance (ANOVA) with Tukey-Kramer post-test. Furthermore, the data of five pretreated groups were compared with sensitized guinea pigs using one-way analysis of variance (ANOVA) with Tukey-Kramer. The data of four pretreated groups were compared with S+TQ group using one-way analysis of variance (ANOVA) with Tukey-Kramer.

RESULTS

Analysis of A_{2A} Adenosine Receptor Gene Expression in Blood Lymphocytes: Compared to the control group, the gene expression of A_{2A} adenosine receptor in S and S+Anta A_{2A} groups decreased significantly ($P<0.05$ and percentage changes = -43% for S, $P<0.01$ and percentage changes = -62% for S+Anta A_{2A}) and in S+TQ group increased significantly ($P<0.001$ and percentage changes = 120%, *Fig. 1*). There was a significant increase in A_{2A} adenosine receptor gene expression in S+TQ and S+TQ+Anta A_{2A} groups compared to the sensitized group ($P<0.001$ to $P<0.01$). Percentage changes in these groups are as follows: S+TQ=288% and S+TQ+AntaA_{2A}=91% *(Fig. 2)*.

The gene expression of A_{2A} adenosine receptor in S+

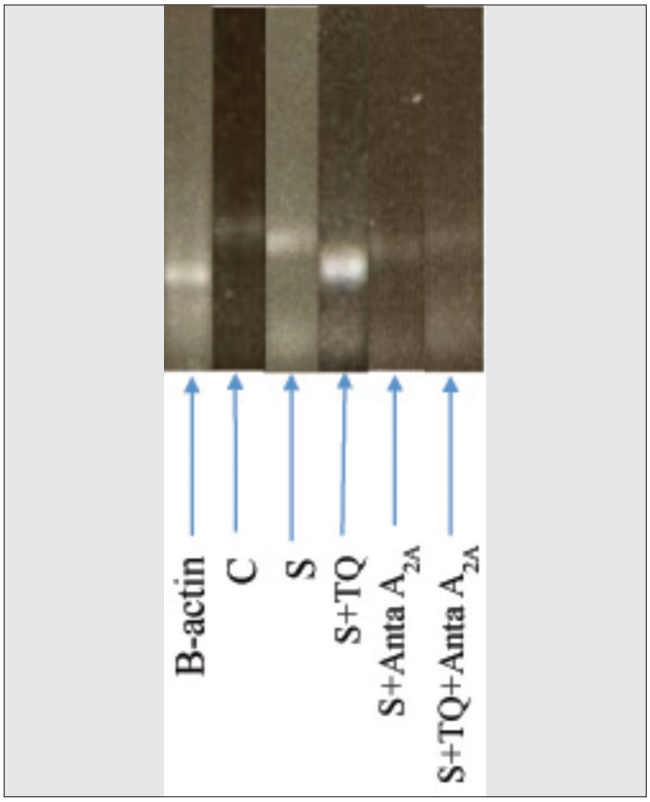

Fig 1. A_{2A} adenosine receptor gene expression analysis using RT-PCR in blood lymphocytes in the control (C), sensitized (S), S pretreated with thymoquinone (S+TQ), S pretreated with A_{2A} adenosine receptor antagonist (S+Anta A_{2A}) and S pretreated with thymoquinone and A_{2A} adenosine receptor antagonist (S+TQ+Anta A_{2A}) guinea pigs

Şekil 1. RT-PCR ile kan lenfositlerinde A_{2A} adenozin reseptör gen ekspresyonu analizi; Kontrol (C), uyarılmış (S), timokinon verilmiş S (S+TQ), A_{2A} adenozin reseptör antagonisti verilmiş S (S+Anta A_{2A}) ve timokinon ile birlikte A_{2A} adenozin reseptör antagonisti verilmiş (S+TQ+Anta A_{2A}) ginedonumuzu

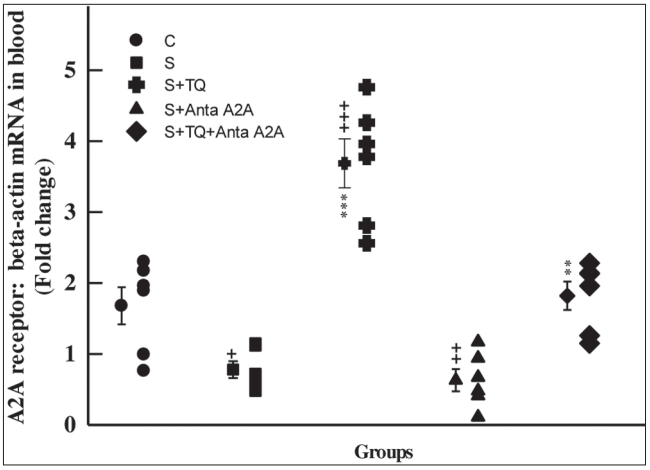

Fig 2. Individual values and mean±SEM (big symbols with bars) of the gene expression of A_{2A} adenosine receptor in blood lymphocytes of control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2A} antagonist (S+Anta A_{2A}), sensitized pretreated with selective A_{2B} antagonist (S+Anta A_{2B}), sensitized pretreated with selective A_{2A} antagonist and thymoquinone (S+Anta A_{2A}+TQ) and sensitized pretreated with selective A_{2B} antagonist and thymoquinone (S+Anta A_{2B}+TQ) groups (for each group, n=6). Statistical differences between control and different groups: + P<0.05, ++ P<0.01, +++ P<0.001. Statistical differences between pretreated groups vs sensitized group: * P<0.05, ** P<0.01, *** P<0.001

Şekil 2. Kan lenfositlerinde A_{2A} adenozin reseptör gen ekspresyonun bireysel değerleri ve ortalama ± SEM (Barlı büyük semboller). Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), timokinon ile birlikte A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}+TQ), timokinon ile birlikte A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) ginedonumuzu (Her grupta n=6). Kontrol ve diğer gruplar arasındaki istatistiksel farklar: + P<0.05, ++ P<0.01, +++ P<0.001. Madde uygulanmış ve uyarılmış gruplar arasındaki istatistiksel farklar: * P<0.05, ** P<0.01, *** P<0.001

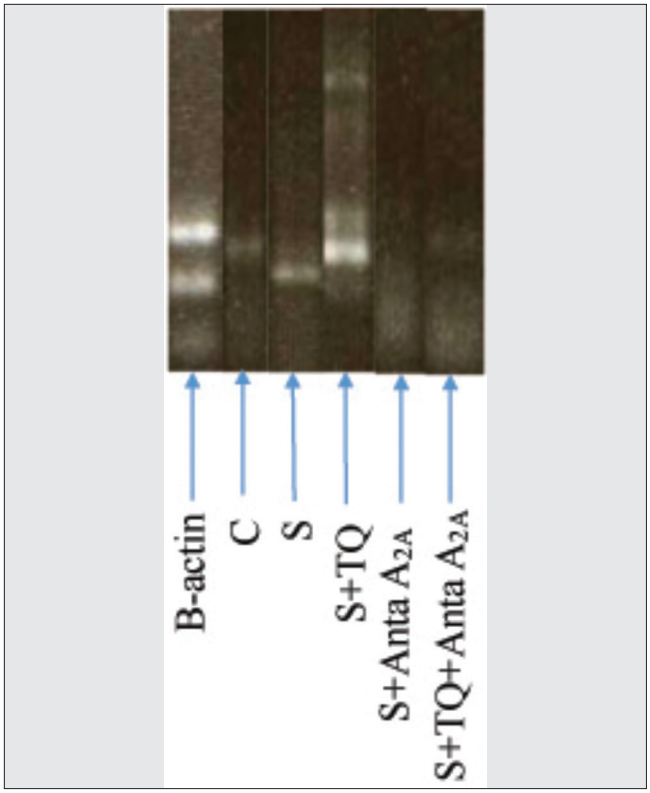

Fig 3. A_{2A} adenosine receptor gene expression analysis using RT-PCR in lung tissue in the control (C), sensitized (S), S pretreated with thymoquinone (S+TQ), S pretreated with A_{2A} adenosine receptor antagonist (S+Anta A_{2A}) and S pretreated with thymoquinone and A_{2A} adenosine receptor antagonist (S+TQ+Anta A_{2A}) guinea pigs

Şekil 3. RT-PCR ile akciğer dokusunda A_{2A} adenozin reseptör gen ekspresyonu analizi; Kontrol (C), uyarılmış (S), timokinon verilmiş S (S+TQ), A_{2A} adenozin reseptör antagonisti verilmiş S (S+Anta A_{2A}) ve timokinon ile birlikte A_{2A} adenozin reseptör antagonisti verilmiş (S+TQ+Anta A_{2A}) ginedonumuzu

Anta A_{2A} and S+TQ+Anta A_{2A} groups decreased significantly compared to thymoquinone pretreated group (P<0.001 and percentage changes = -82% for S+Anta A_{2A}, P<0.001, percentage changes = -50% for S+TQ+Anta A_{2A}). The gene expression of A_{2A} adenosine receptor in S+TQ+Anta A_{2A} group was significantly higher than in S+Anta A_{2A} group (P<0.01, percentage changes= 188%).

Analysis of A_{2A} Adenosine Receptor Gene Expression in Lung Tissue: In comparison to the control group, the gene expression of A_{2A} adenosine receptor in S and S+Anta A_{2A} groups decreased significantly (P<0.05 and percentage changes = -36% for S, P<0.01 and percentage changes = -61%, *Fig. 3*). There was significant increase in A_{2A} adenosine receptor gene expression in S+TQ and S+TQ+Anta A_{2A} groups compared to group S (P<0.01 and percentage changes = 129% for S+TQ, P<0.05 and percentage changes = 76% for S+TQ+Anta A_{2A}, *Fig. 4)*.

A_{2A} adenosine receptor gene expression in S+Anta A_{2A} group was significantly lower than the S group (P<0.05, percentage changes= -39%). The gene expression of A_{2A} adenosine receptor in lung tissue of S+Anta A_{2A} group decreased compared to thymoquinone pretreated group (P<0.001). The gene expression of A_{2A} adenosine receptor in S+TQ+Anta A_{2A} group was significantly more than S+Anta A_{2A} group (P<0.01, percentage changes = 188%).

Analysis of A_{2B} Adenosine Receptor Gene Expression in Blood Lymphocytes: Compared to the control group, the gene expression of A_{2B} adenosine receptor in S and S+TQ groups increased significantly (P<0.001 and percentage changes = 632% for S, P<0.01 and percentage changes = 256% for S+TQ, *Fig. 5*). All pretreated groups showed a significant decrease in A_{2B} adenosine receptor gene expression compared with sensitized group (P<0.001 to P<0.01). Percentage changes in these groups are as follows: S+TQ = -51%, S+Anta A_{2B} = -63% and S+TQ+Anta A_{2B} = -191%, *Fig. 6*).

The gene expression of A_{2B} adenosine receptor in blood of S+TQ+Anta A_{2B} group decreased significantly compared to thymoquinone pretreated group and S+Anta A_{2B} group (P<0.001, *Table 1*).

Analysis of A_{2B} Adenosine Receptor Gene Expression in Lung Tissue: As compared with control group, the

gene expression of A_{2B} adenosine receptor in S and S+TQ groups increased significantly (P<0.001 and percentage changes = 291% for S, P<0.01 and percentage changes = 171% for S+TQ, *Fig. 7*). With regard to the sensitized group, the gene expression of A_{2B} adenosine receptor in lung tissue of all pretreated groups decreased significantly (P<0.001 to P<0.05, *Fig. 8*).

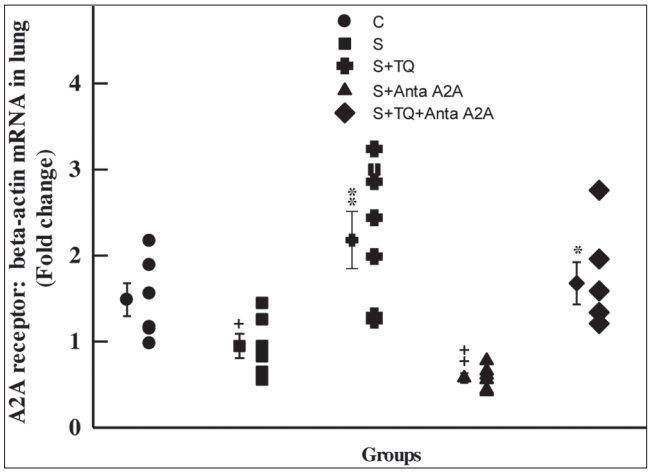

Fig 4. Individual values and mean±SEM (big symbols with bars) of the gene expression of A_{2A} adenosine receptor in lung tissue of control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2A} antagonist (S+Anta A_{2A}) and sensitized pretreated with selective A_{2A} antagonist and thymoquinone (S+Anta A_{2A}+TQ) groups (for each group, n=6). Statistical differences between control and different groups: + P<0.05, ++ P<0.01. Statistical differences between pretreated groups vs sensitized group: * P<0.05, ** P<0.01

Şekil 4. Akciğer dokusunda A_{2A} adenozin reseptör gen ekspresyonun bireysel değerleri ve ortalama ± SEM (Barlı büyük semboller). Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), timokinon ile birlikte A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}+TQ), timokinon ile birlikte A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) ginedonumuzu (Her grupta n=6). Kontrol ve diğer gruplar arasındaki istatistiksel farklar: + P<0.05, ++ P<0.01. Madde uygulanmış ve uyarılmış gruplar arasındaki istatistiksel farklar: * P<0.05, ** P<0.01

The gene expression of A_{2B} adenosine receptor in the lung tissue of S+Anta A_{2B} and S+TQ+Anta A_{2B} groups decreased compared to thymoquinone pretreated group (P<0.001, percentage changes = -68% for S+Anta A_{2B} and percentage changes = -70% for S+TQ+Anta A_{2B}). In the lung tissue of S+TQ+Anta A_{2B} group, the gene expression of A_{2B} adenosine receptor was significantly lower than S+Anta A_{2B} group (P<0.01, percentage changes = -5.50%, *Table 1*).

Pathology: With regard to this scoring, all pathological changes in the S, S+Anta A_{2A} and S+TQ+Anta A_{2A} groups, including the vascular membrane hyperplasia, airway membrane hyperplasia, the presence of mucosal plug, respiratory epithelial denudation, cellular infiltration and emphysema were significantly higher than those in the C group (P<0.05 for all cases). Moreover, the presence of mucosal plug, respiratory epithelial denudation and emphysema in S+TQ and S+Anta A_{2B} groups were significantly higher than those in the C group (P<0.05, *Fig. 9a-g*).

All pathological changes in S+TQ, S+Anta A_{2B} and S+TQ+ Anta A_{2B} groups (except inflammatory infiltration in S+Anta A_{2B}), and the presence of mucosal plug in S+TQ+Anta A_{2A} group were significantly decreased in comparison with the sensitized group (P<0.05). However, there were still significant variations in presence of mucosal plug, respiratory epithelial denudation and emphysema between the C group and S+TQ group (P< 0.05, *Table 2*).

All criteria in S+Anta A_{2A} group, and airway membrane hyperplasia, the presence of mucosal plug and cellular infiltration in S+Anta A_{2B} group were significantly higher than S+TQ group (P<0.001 to P<0.05). There were no differences in the pathological changes between S+TQ+ Anta A_{2A} and S+TQ+Anta A_{2B} groups and the S+TQ group. There was not any significant differences between S+Anta A_{2A} and S+TQ+Anta A_{2A} groups and between S+Anta A_{2B} and the S+TQ+Anta A_{2B} group *(Table 2)*.

Table 1. *The mean value of the gene expression of A_{2B} adenosine receptor in blood lymphocyte and lung tissue and blood cytokines (IL-4 and IFN-g) in control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2A} antagonist (S+Anta A_{2A}), sensitized pretreated with selective A_{2B} antagonist (S+Anta A_{2B}), sensitized pretreated with selective A_{2A} antagonist and thymoquinone (S+Anta A_{2A}+TQ) and sensitized pretreated with selective A_{2B} antagonist and thymoquinone (S+Anta A_{2B}+TQ) groups(for each group, n = 8)*

Tablo 1. *Kan lenfosit ve akciğer dokusu A_{2B} adenozin reseptör gen ekspresyonu ile kan sitokinlerinin ortalama değerleri. Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), A_{2A} antagonisti ve timokinon verilmiş ve uyarılmış (S+Anta A_{2A}+TQ) ve A_{2B} antagonisti ve timokinon verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) (Her grupta n=8)*

Parameter	C	S	S+TQ	S+Anta A_{2A}	S+Anta A_{2B}	S+TQ+Anta A_{2A}	S+TQ+Anta A_{2B}
The gene expression of A_{2B} adenosine receptor in blood lymphocyte	0.54±0.23	3.93±0.51	1.91±0.16	0.63±0.15 +++	1.41±0.34	1.82±0.20 **	0.34±0.13 +++ ###
The gene expression of A_{2B} adenosine receptor in lung tissue	0.59±0.18	2.32±0.27	1.61±0.13	0.58±0.05 +++	0.51±0.08 +++	1.68±0.24 **	0.48±0.14 +++ ##
Blood IL-4 level	39.78±2.01	47.41±1.98	43.88±1.70	49.48±2.74	45.20±2.15	46.24±1.38	39.98±0.82 #
Blood IFN-gamma level	104.97±5.93	117.37±2.71	124.93±2.31	101.14±3.24 +++	120.03±2.95	107.87±2.04 +++	126.04±2.81

*Statistical differences between pretreated groups vs S+TQ group: +++; P<0.001; Statistical differences between S+TQ+Anta A_{2A} group vsS+Anta A_{2A} group: ** P<0.01; Statistical differences between S+TQ+Anta A_{2B} group vsS+Anta A_{2B} group: #; P<0.05; ##; P<0.01, ###; P<0.001*

Table 2. *The mean value of different pathological changes in control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2A} antagonist (S+Anta A_{2A}), sensitized pretreated with selective A_{2B} antagonist (S+Anta A_{2B}), sensitized pretreated with selective A_{2A} antagonist and thymoquinone (S+Anta A_{2A}+TQ) and sensitized pretreated with selective A_{2B} antagonist and thymoquinone (S+Anta A_{2B}+TQ) groups (for each group, n = 8*

Tablo 2. *Değişik patolojik değişimlerim ortalama değeri. Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), A_{2A} antagonisti ve timokinon verilmiş ve uyarılmış (S+Anta A_{2A}+TQ) ve A_{2B} antagonisti ve timokinon verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) (Her grupta n=8)*

Parameter	C	S	S+TQ	S+Anta A_{2A}	S+Anta A_{2B}	S+TQ+Anta A_{2A}	S+TQ+Anta A_{2B}
Vascular smooth muscle hypertrophy and hyperplasia	0.33±0.21	3±0.3 +++	1.33±0.30 **	2.90±0.28 +++ ##	1.42±0.48 *	2.29±0.42 +	0.86±0.34 **
Airways smooth muscle hypertrophy and hyperplasia	0.17±0.16	2.54±0.28 +++	0.83±0.24 ***	2.54±0.31 +++ ###	0.86±0.34 **	2.29±0.42 ++ #	0.71±0.29 **
Presence of mucosal plug	0±0	2.72±0.14 +++	0.67±0.19 ***	2.27±0.23 +++ ###	1.14±0.34 + ***	1.71±0.29 +++ * #	0.29±0.18 ***
Respiratory epithelial denudation	0.17±0.16	2.18±0.23 +++	0.83±0.20 ***	2.45±0.21 +++ ###	0.86±0.26 **	1.71±0.29 ++	0.29±0.18 ***
Inflammatory infiltration	0±0	1.55±0.20 ++	0.41±0.19 **	2.18±0.22 +++ ###	0.71±0.35	1.85±0.34 +++ ##	0.57±0.30
Emphysema	0.17±0.16	2±0.27 +++	0.92±0.19 *	2.18±0.23 +++ ##	1.43±0.30	1.77±0.29 ++	0.71±0.29 *

*Statistical differences between groups vs C group: ++; P<0.01, +++; P<0.001; Statistical differences between pretreated groups vs S group: * P<0.05, ** P<0.01, *** P<0.001; Statistical differences between pretreated groups vs S+TQ group: #; P<0.05, ##; P<0.01, ###; P<0.001*

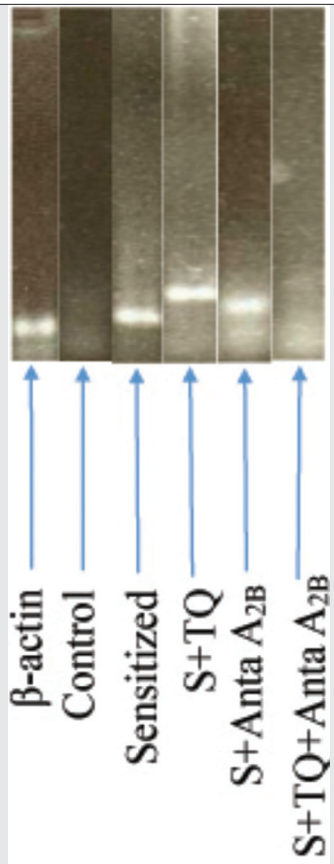

Fig 5. A_{2B} adenosine receptor gene expression analysis using RT-PCR in blood lymphocytes in the control (C), sensitized (S), S pretreated with thymoquinone (S+TQ), S pretreated with A_{2B} adenosine receptor antagonist (S+Anta A_{2B}) and S pretreated with thymoquinone and A_{2B} adenosine receptor antagonist (S+TQ+Anta A_{2B}) guinea pigs

Şekil 5. RT-PCR ile kan lenfositlerinde A_{2B} adenozin reseptör gen ekspresyonu analizi; Kontrol (C), uyarılmış (S), timokinon verilmiş S (S+TQ), A_{2B} adenozin reseptör antagonisti verilmiş S (S+Anta A_{2B}) ve timokinon ile birlikte A_{2B} adenozin reseptör antagonisti verilmiş (S+TQ+Anta A_{2B}) ginedonumuzu

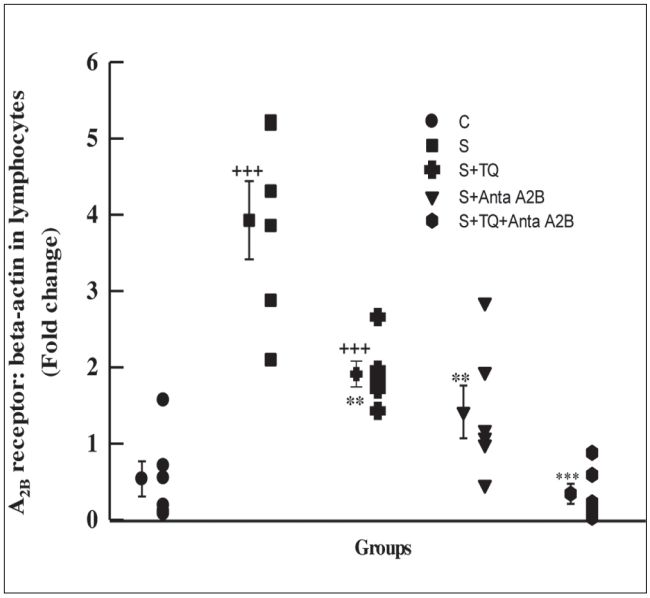

Fig 6. Individual values and mean±SEM (big symbols with bars) of the gene expression of A_{2B} adenosine receptor in blood lymphocytes of control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2B} antagonist (S+Anta A_{2B}) and sensitized pretreated with selective A_{2B} antagonist and thymoquinone (S+Anta A_{2B}+TQ) groups (for each group, n=6). Statistical differences between control and different groups: ++ P<0.01, +++ P<0.001, Statistical differences between pretreated groups vs sensitized group: * P<0.05, ** P<0.01, *** P<0.001

Şekil 6. Kan lenfositlerinde A_{2B} adenozin reseptör gen ekspresyonun bireysel değerleri ve ortalama ± SEM (Barlı büyük semboller). Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), timokinon ile birlikte A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) ginedonumuzu (Her grupta n=6). Kontrol ve diğer gruplar arasındaki istatistiksel farklar: + P<0.05, ++ P<0.01, +++ P<0.001. Madde uygulanmış ve uyarılmış gruplar arasındaki istatistiksel farklar: * P<0.05, ** P<0.01, *** P<0.001

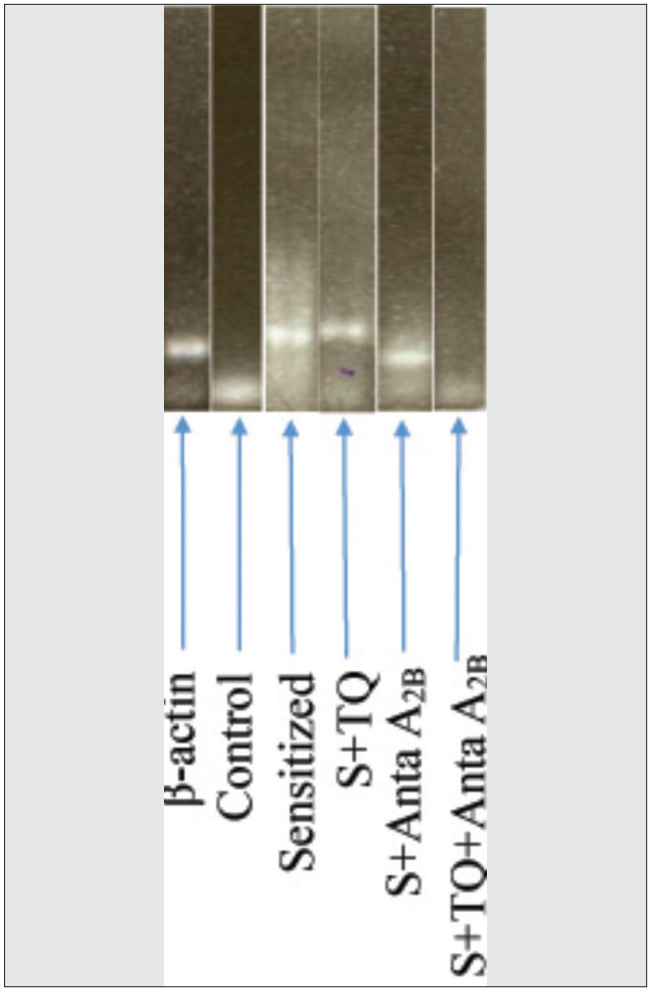

Fig 7. A_{2B} adenosine receptor gene expression analysis using RT-PCR in lung tissue in the control (C), sensitized (S), S pretreated with thymoquinone (S+TQ), S pretreated with A_{2B} adenosine receptor antagonist (S+Anta A_{2B}) and S pretreated with thymoquinone and A_{2B} adenosine receptor antagonist (S+TQ+Anta A_{2B}) guinea pigs

Şekil 7. RT-PCR ile akciğer dokusunda A_{2B} adenozin reseptör gen ekspresyonu analizi; Kontrol (C), uyarılmış (S), timokinon verilmiş S (S+TQ), A_{2B} adenozin reseptör antagonisti verilmiş S (S+Anta A_{2B}) ve timokinon ile birlikte A_{2B} adenozin reseptör antagonisti verilmiş (S+TQ+Anta A_{2B}) ginedonumuzu

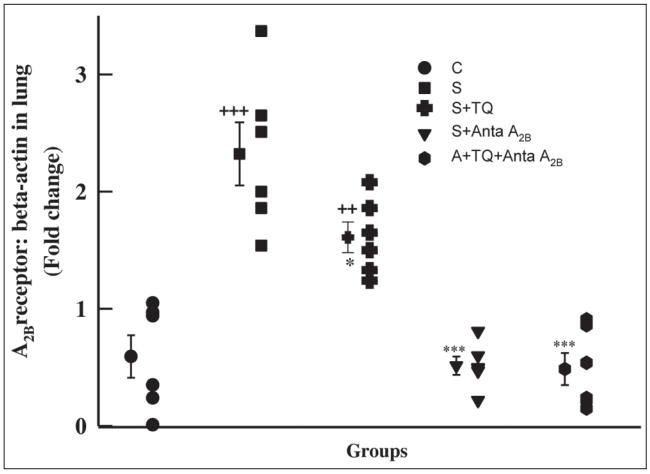

Fig 8. Individual values and mean±SEM (big symbols with bars) of the gene expression of A_{2B} adenosine receptor in lung tissue of control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2B} antagonist (S+Anta A_{2B}) and sensitized pretreated with selective A_{2B} antagonist and thymoquinone (S+Anta A_{2B}+TQ) groups (for each group, n=7). Statistical differences between control and different groups: ++ P<0.01, +++ P<0.001, Statistical differences between pretreated groups vs sensitized group: * P<0.05, *** P<0.001

Şekil 8. Akciğer dokusunda A_{2B} adenozin reseptör gen ekspresyonun bireysel değerleri ve ortalama ± SEM (Barlı büyük semboller). Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), timokinon ile birlikte A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}+TQ), timokinon ile birlikte A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) ginedonumuzu (Her grupta n=6). Kontrol ve diğer gruplar arasındaki istatistiksel farklar: + P<0.05, ++ P<0.01. Madde uygulanmış ve uyarılmış gruplar arasındaki istatistiksel farklar: * P<0.05, ** P<0.01

Blood IL-4 and IFN-γ Levels: The mean value of blood IL-4 level in S, S+Anta A_{2A}, S+Anta A_{2B} and S+TQ+Anta A_{2A} groups was significantly higher than that in the C group (P<0.05). In S+TQ+Anta A_{2B} group, this cytokine is significantly lower than S group (P<0.01). The blood IL-4 level in S+TQ group 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 the C group. There were not significant differences between pretreated groups *(Fig. 10a)*.

The mean value of the blood IFN-γ level of S, S+TQ, S+Anta A_{2B} and S+TQ+Anta A_{2B} groups was significantly higher than that of the C group (P<0.01 to P<0.05). There was significant increase in blood IFN-γ of S+TQ and S+TQ+Anta A_{2B} groups compared to that in the S group (P<0.05); however the mean value of IFN-γ in S+Anta A_{2A} and S+TQ+Anta A_{2A} groups was significantly lower than that in the S group (P<0.01 to P<0.05). The mean value of IFN-γ in S+Anta A_{2A} and S+TQ+Anta A_{2A} groups was significantly lower than that in S+TQ group (P<0.001, *Fig. 10b*).

DISCUSSION

The present study attempted to assess the effect of thymoquinone, alone and in the presence of A_{2A} adenosine receptor antagonist, on the A_{2A} and A_{2B} adenosine receptor mRNA gene expression in blood lymphocytes and lung tissue of ovalbumin-sensitized guinea pigs by the use of Real Time PCR method. In addition, blood IL-4 and IFN-γ level and lung pathological changes were analyzed.

The Effect of Thymoquinone on A_{2A} Adenosine Receptor: Data from these experiments in lymphocytes and lung tissue showed that thymoquinone could increase A_{2A} adenosine receptor mRNA expression significantly; however, administration of selective antagonist of A_{2A} adenosine receptor decreased mRNA expression of A_{2A} receptor. The concurrent administration of these two exogenous factors has a slight effect on the expression of A_{2A} adenosine receptors.

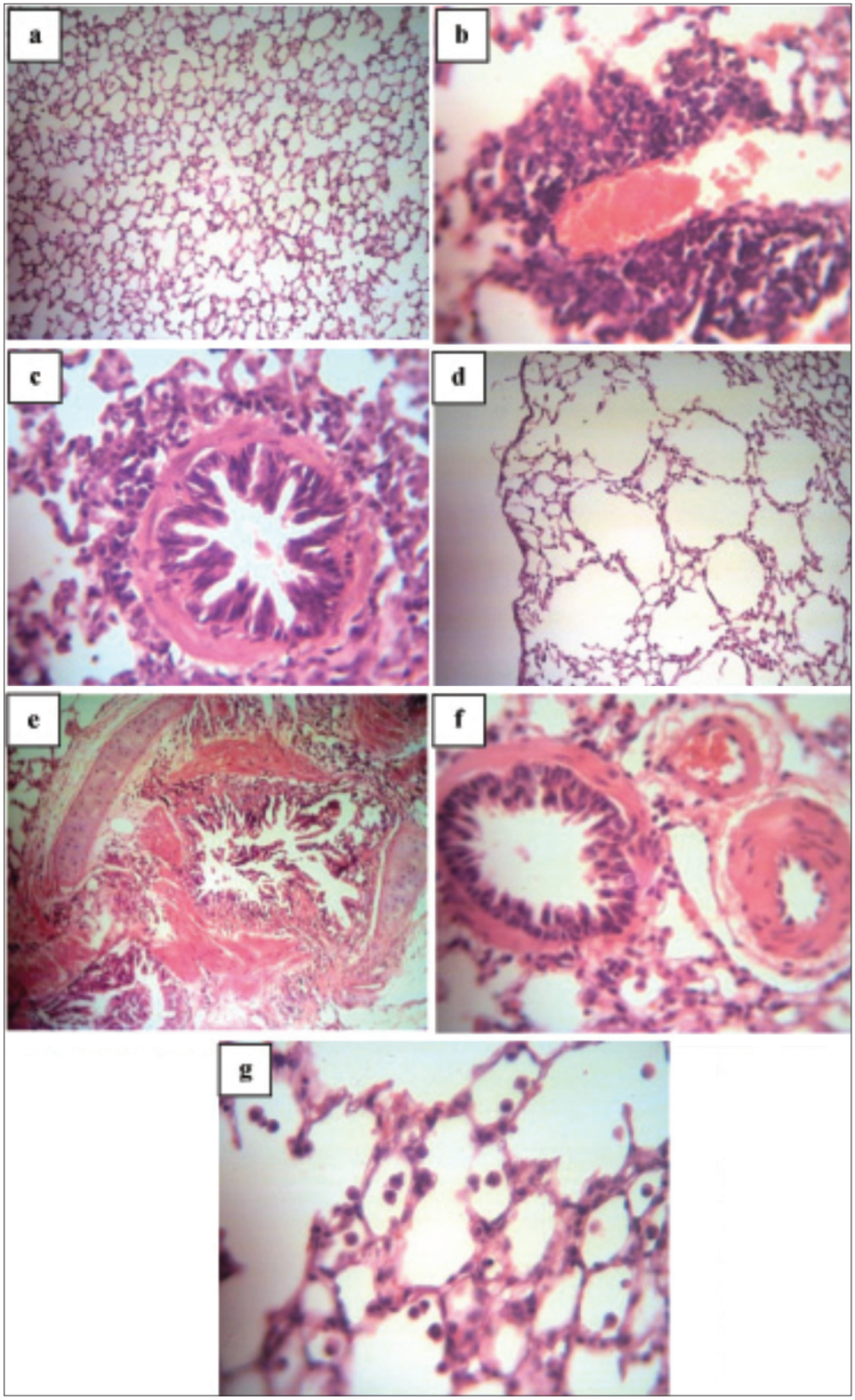

Fig 9. Photographs of lung specimen in guinea pigs: **a-** control normal lung tissues (C, 10x10), **b-** sensitized group (S, 10x40) with perivascular inflammatory infiltration, **c-** sensitized group pretreated with thymoquinone (S+TQ, 10x40) with airway smooth muscle hyperplasia and mild inflammatory infiltration, **d-** sensitized group pretreated with selective A_{2A} antagonist (S+Anta A_{2A},10x10) with emphysema and atelectasis, **e-** sensitized group pretreated with selective A_{2B} antagonist (S+Anta A_{2B}, 10x10) with respiratory epithelial denudation, **f-** sensitized group pretreated with selective A_{2A} antagonist and thymoquinone (S+TQ+Anta A_{2A}, 10x40) with vascular and airway smooth muscle hyperplasia and hypertrophy and **g-** sensitized group pretreated with selective A_{2B} antagonist and thymoquinone (S+TQ+Anta A_{2B}, 10x100) with inflammatory infiltration

Şekil 9. Gine domuzlarında akciğer örneklerinin fotoğrafları. **a-** Kontrol normal akciğer dokusu (C, 10x10), **b-**uyarılmış grup (S, 10x40), perivasküler yangısal infiltrasyon, **c-** timokinon verilmiş ve uyarılmış grup (S+TQ, 10x40), havayollarında düz kas hiperplazisi ve orta şiddette yangısal infiltrasyon, **d-** A_{2A} antagonisti uygulanmış ve uyarılmış grup (S+Anta A_{2A},10x10), amfizem ve atelektazi, **e-** A_{2B} antagonisti uygulanmış ve uyarılmış grup (S+Anta A_{2B},10x10), respiratorik epitel dökülmesi, **f-** A_{2A} antagonisti ile birlikte timokinon uygulanmış ve uyarılmış grup (S+TQ+Anta A_{2A}, 10x40), vasküler ve havayolu düz kas hiperplazi ve hipertrofisi ve **g-** A_{2B} antagonisti ile birlikte timokinon uygulanmış ve uyarılmış grup (S+TQ+Anta A_{2B}, 10x100), yangısal infiltrasyon

A_{2A} adenosine receptors are known as the anti-inflammatory mediators that are highly expressed on lymphocytes [26]. Extracellular adenosine increases during asthma and inflammation and this elevated plasma adenosine level could activate A_{2A} receptors.

As extracellular adenosine has anti-inflammatory effects by means of A_{2A} receptors, the activation of these receptors can change the level of blood cytokines [27]. The expression of A_{2A} receptors on lymphocytes correlates with adenosine levels in plasma and occupation of these receptors and does not have any relationship with the response of A_{2A} receptors [28]. In addition, the expression of A_{2A} receptors is slightly sensitive to changes in concentration of exogenous

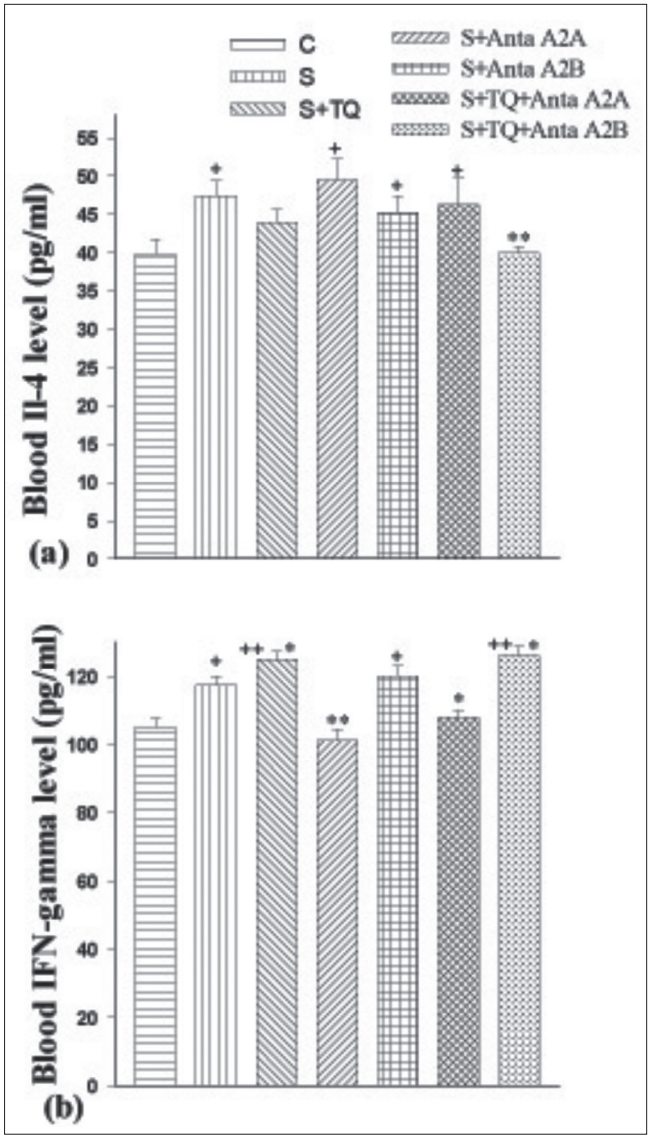

Fig 10. The blood IL-4 (a) and IFN-γ (b) levels (pg/ml) of control (C), sensitized (S), sensitized pretreated with thymoquinone (S+TQ), sensitized pretreated with selective A_{2A} antagonist (S+Anta A_{2A}), sensitized pretreated with selective A_{2B} antagonist (S+Anta A_{2B}), sensitized pretreated with selective A_{2A} antagonist and thymoquinone (S+Anta A_{2A}+TQ)and sensitized pretreated with selective A_{2B} antagonist and thymoquinone (S+Anta A_{2B}+TQ) groups (for each group, n=6). Statistical differences between control and different groups: + P<0.05, ++ P<0.01, Statistical differences between pretreated groups vs sensitized group: * P<0.05, ** P<0.01

Şekil 10. Kan IL-4 (a) ve IFN-γ (b) seviyeleri (pg/ml). Kontrol (C), uyarılmış (S), timokinon verilmiş ve uyarılmış (S+TQ), A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}), A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}), timokinon ile birlikte A_{2A} antagonisti verilmiş ve uyarılmış (S+Anta A_{2A}+TQ), timokinon ile birlikte A_{2B} antagonisti verilmiş ve uyarılmış (S+Anta A_{2B}+TQ) ginedonumuzu (Her grupta n=6). Kontrol ve diğer gruplar arasındaki istatistiksel farklar: + P<0.05, ++ P<0.01. Madde uygulanmış ve uyarılmış gruplar arasındaki istatistiksel farklar: * P<0.05, ** P<0.01

and endogenous factors involved in inflammation [29]. On the other hand, thymoquinone as an exogenous factor could change blood cytokine secretion (IL-4 & INF-γ) in the process of asthma; these changes were in accordance with previous studies [5,21]. So thymoquinone probably influences the secretion of cytokines of different types of lymphocytes (Th_2/Th_1) and changes the activity of A_{2A} receptors on the surface of lymphocytes and extracellular adenosine levels. Subsequently, these changes affect intracellular cAMP level and A_{2A} receptors mRNA expression.

A_{2A} adenosine receptors have intermediate expression in lung tissue [29] and are most relevant to lung disease [30]. Administration of thymoquinone caused enhanced A_{2A} receptor gene expression in lung tissue similar to the results of blood lymphocytes. A study in 2006 demonstrated that thymoquinone could affect IFN-γ level in bronchoalveolar lavage fluid [31]. So it is possible that thymoquinone might have affected lung cytokines secretion and induced intracellular mechanism of A_{2A} adenosine receptor mRNA expression in lung tissue; however, this effect was weaker than blood lymphocytes. This elevated A_{2A} receptor gene expression in lung tissue leads to wound healing and improving tissue destruction in group S + TQ during sensitization. Brown and his colleagues in 2008 showed that stimulation of wound healing in bronchial epithelial cells is the distinguished function of A_{2A} adenosine receptor expression [2].

The level of IL-4 increased significantly in S+Anta A_{2A} group although the level of IFN-γ decreased significantly in this group compared to the sensitized group. Administration of selective A_{2A} adenosine receptor antagonist, ZM241385, caused the elimination of A_{2A} adenosine receptor or the reduction of their numbers by decreasing cAMP level [32] and influencing secreted cytokines [33]. The previous study indicated that administration of this antagonist caused an increase in serum level of IL-4 and a decrease in IFN-γ [34].

As expression of pro-inflammatory cytokines can stimulate the gene expression of A_{2A} adenosine receptor [35], it has been suggested that A_{2A} adenosine receptor antagonist and increased serum IL- 4 probably cause improvement of A_{2A} receptors expression in lymphocytes. Although the administration of this antagonist suppresses the anti-inflammatory effect of A_{2A} receptors on the cell surface, but results of A_{2A} receptor mRNA expression were controversial. Nowadays it is accepted that the level of mRNA expression does not correlate exactly with proteins expressed on the cell surface because of some epigenetic factors [36].

The results of the present study indicate that all pathological criteria significantly increased in S+Anta A_{2A} group compared to controls. Spicuzza in 2006 demonstrated that ZM241385, selective A_{2A} adenosine receptor antagonist, could affect respiratory smooth muscle and blocking of these receptors exacerbated asthmatic symptoms [29].

The results of this investigation showed a significant reduction in gene expression of A_{2A} adenosine receptor in S+TQ+Anta A_{2A} group compared to the S + TQ and S

+ Anta A_{2A} groups. It is suggested that thymoquinone prevented the secretion of blood cytokines during the sensitization process [5,21] and the elevated level of IL-4 did not occur to stimulate A_{2A} adenosine receptor expression during administration of selective A_{2A} adenosine receptor antagonist.

There were not significant differences between A_{2A} adenosine receptor expression in lung tissue of group S+TQ+Anta A_{2A} and S+TQ group. According to the results of the present study, it is concluded that thymoquinone had a significant effect on A_{2A} receptor expression in respiratory epithelial cells since respiratory epithelial cells express A_{2A} adenosine receptors, although the A_{2A} receptors expression and its regulation under inflammatory conditions in epithelial cells have not been documented and the biological importance of increased expression on epithelial cells is unknown [35]. Our results suggest that the A_{2A} adenosine receptors probably play a role in the pathogenesis of inflammatory diseases. A previous study of ours showed that concurrent administration of thymoquinone and selective adenosine A_{2A} receptor antagonist improved tracheal smooth muscle contraction and WBC count in bronchoalveolar lavage fluid in comparison with the S+Anta A_{2A} group [37]. Polosa's study also showed that ZM241385, selective A_{2A} adenosine antagonist, affected bronchial smooth muscle [30].

The Effect of Thymoquinone on A_{2B} Adenosine Receptor: The second part of this study evaluated the effect of thymoquinone on blood cytokines, lung pathology and the gene expression of A_{2B} adenosine receptors, in the presence of MRS1706; selective A_{2B}receptor antagonist, in asthmatic guinea pigs.

The gene expression of A_{2B} receptors in sensitized animals (S group) and pretreated group with thymoquinone (S+TQ group) significantly increased compared to controls. This increase of expression in S group and S+TQ group was 86% and 72% respectively. In these groups, the blood IL-4 and IFN-γ levels and pathological changes were significantly raised in comparison to the control group. These findings were in accordance with results of our previous studies [19,34,38]. The increment in A_{2B} receptors gene expression, cytokines and pathological changes in S+TQ group compared with C group was due to inflammation process but there was statistically significant reduction in comparison with the S group. This supports the notion that thymoquinone can improve inflammation and relieve its symptoms.

This study showed that single dose administration of MRS1706, selective A_{2B} adenosine receptor antagonist, could improve lung pathological changes; however, it could not decrease to the control level. These results are in line with Mustafa's study [16]. In addition, the percentage of increase in A_{2B} receptor gene expression in S+Anta A_{2B} group significantly plummeted compared to the S group. There was not a statistically significant difference between A_{2B} receptors gene expression and pathological changes in this group and the control group. This indicated that administration of A_{2B} receptor antagonist could prevent asthma induced inflammatory changes.

It is demonstrated that the anti-inflammatory and pro-inflammatory effects of adenosine depend on their level in lung tissue. Lung inflammation causes a hypoxic environment in which adenosine is produced. At first, the low level of adenosine stimulates high-affinity receptors such as A_{2A} adenosine receptors which shows the protective effect of adenosine. However, in severe lung inflammatory conditions, the high level of produced adenosine affects low-affinity receptors such as A_{2B} receptors and exacerbates the inflammation [39].

The gene expression of A_{2B} receptor, pathological changes and blood IL-4 level in the sensitized group pretreated by thymoquinone and Anta A_{2B} (S+TQ+Anta A_{2B} group) were not statistically different from the control group. Also, the blood level of IFN-γ in S+TQ+Anta A_{2B} group rose significantly compared to the control and sensitized groups. Moreover, in the S+TQ+Anta A_{2B} group, the administration of thymoquinone and selective antagonist together caused more improvement than S+TQ and S+AntaA_{2B} groups. These results are in accordance with results of our current study [37] which is related to the effect of administration of thymoquinone and A_{2B} receptor antagonist together to relieve inflammation.

Cytokines can regulate the number of adenosine receptors in inflammatory environment [40] and our previous study demonstrated that IFN-γ level in sensitized group pretreated with A_{2B} receptor antagonist group has increased significantly compared to the S group and this change could influence the expression of adenosine receptors or T-cells [34]. These observations, therefore, support the hypothesis that blocking of A_{2B} receptor with selective antagonist probably causes changes in levels of secreted cytokines related to inflammation. These findings are in accordance with the results of Anvari's study which showed that in A_{2B} receptor-knockout mice, the expression and secretion of T-cell decreases significantly [41].

The results of gene expression of A_{2B} adenosine receptors in lung tissue are already similar to the findings in blood lymphocytes. The comparison of percentage of gene expression of A_{2B} receptor in blood lymphocytes and lung tissue in the analyzed groups showed that thymoquinone administration has more influence on blood lymphocytes while administration of the selective A_{2B} receptor antagonist was more effective in lung tissue. It was probably due to high expression of A_{2B} receptor in pulmonary resident cells; epithelial, bronchial smooth muscle and endothelial cells, because the pre-inflammatory effect of A_{2B} receptor stimulation was demonstrated in these cells [41]. On the other hand, this receptor could increase the expression of

pre-inflammatory mediators in different cells of chronic pulmonary diseases and over-expression of Th_2 cytokine in lung was associated with increasing levels of A_{2B} receptor [42]. While local cytokines played an important role in regulating pre/anti-inflammatory activities of adenosine, and while these cytokines could also regulate the levels of adenosine metabolic enzymes and adenosine receptors in inflammatory environment [40], MRS1706, selective A_{2B} receptor antagonist, probably inhibits their influence on the expression of pre-inflammatory mediators by blocking receptors. However, it is suggested that the effects of agonists of adenosine receptors are evaluated in future researches to determine the exact mechanism(s) of thymoquinone.

In conclusion, the results showed that thymoquinone could affect A_{2A} and A_{2B} adenosine receptors gene expression and some of the therapeutic effects of thymoquinone in reducing asthma symptoms might be partially mediated through A_2 adenosine receptors.

Acknowledgement

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Conflict of Interest: None declared.

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