

Effect Of Melatonin In A Rat Model Of Allergic Lung Inflammation

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Abstract

Aims: Asthma is an inflammatory lung disease characterized by bronchoconstriction and hyper responsiveness. Immuno stimulatory effects of melatonin have been reported. In this study, we investigated the impact of melatonin administration on allergic airway inflammation in a rat model. **Methods:** Forty five adult Wistar albino rats were equally divided into three groups: group I served as control; group II: rats sensitized to ovalbumin and challenged intranasally with ovalbumin to induce an allergic inflammatory response and group III, rats were sensitized and treated with intraperitoneally melatonin. The serum levels of IgE, IgG1 and ova-specific IgG were measured by ELISA. In the bronchoalveolar lavage fluid (BALF), the levels of IL-4, IL-5, IL-13, IL-10 were measured. IL-10 expression was measured by real time polymerase chain reaction. Histopathological examination of the lung tissues using H&E stain were done. **Results:** Melatonin administration inhibited allergen-induced lung eosinophilic infiltration and improved the pathological lesions of the lungs. It significantly decreased total serum IgE, IgG1 and OVA-specific IgG1 along with BALF levels of IL-4, IL-5, IL-13. Melatonin increased BALF levels of IL-10 and its mRNA expression. **Conclusion:** Melatonin administration exhibited a significant reduction in all the markers of allergic inflammation. The data suggests that inhibition of T-cell response and up regulation of IL-10 may be responsible for immunomodulatory effect of melatonin in the rat model of allergic airway inflammation.

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INTRODUCTION

Allergic inflammation associated with airway hyper reactivity is the main feature of allergic asthma. The inflammatory response is characterized by bronchial eosinophilia, mucus hyper secretion and activation of helper T type 2 (Th2) cells (1). The role Th 2 cells in the initiation of allergic airway inflammation and asthma is a very important role as they produce Th2-type cytokines (IL-4, IL-5, and IL-13) (2). In addition, the bronchial tissue produces also these inflammatory cytokines by mast cells, alveolar macrophages and epithelial cells which play a fundamental role in the patho physiology of airway inflammation. Eosinophils produce cytokines as well as several chemokines by which they maintain or even increase the airway hyper reactivity (3).

Interleukin-10 (IL-10) is an important immunosuppressive cytokine. IL-10 has been known to down regulate the production of proinflammatory cytokines (4).

The most important anti-inflammatory treatment for asthma is glucocorticosteroids. Several studies had reported many side effects for systemic glucocorticosteroids, such as suppression of adrenal gland function, decreased bone metabolism and decelerated growth in children. Therefore, many studies are being designed to develop novel, and safer therapies for asthma (5,6).

Melatonin has been shown to enhance the cytokines production, and has a modulatory role in lymphocyte proliferation(7). The biological activities of melatonin and its effect on inflammatory cells have been reported(8).

Melatonin appears to have an important immunomodulatory effect in allergic diseases (9). In addition, Shaji and colleagues (10) demonstrated that melatonin enhanced the proliferation of ovalbumin-specific CD4-positive T lymphocytes.

Considering the importance of the inflammatory response in the development of asthma, the hypothesis of this study was to investigate the impact of melatonin administration on inflammatory responses associated with the asthmatic airway in adult male rat model.

MATERIALS AND METHODS

Experimental Design:

The experimental protocol was approved by the Institutional Animal Research Committee of the Faculty of Medicine, Assiut University, Egypt and followed the published guidelines and regulations. Sixty male Wistar albino rats, 10 weeks of age (200- 220 gram) were weighed and maintained at animal house of Faculty of Medicine, Assiut University with free access to OVA-free food and water. Rats were familiarized with the laboratory environment and running on the treadmill, then were randomly assigned equally into four experimental groups of 15 rats each. The groups treated as follows:

Group I: the rats were sham sensitized and exposed to phosphate buffered saline (PBS), served as control.

Group II: the rats were sensitized and challenged with ovalbumin (OVA) (Sigma Chemical Co., USA),

Group III: the rats were sensitized and challenged with OVA and the rats in this group received a

daily intra peritoneal (i.p.) injection of melatonin 5 mg/Kg.

Group IV: the rats were injected with melatonin (5 mg/kg. i.p.) daily from the first day until the last day of the experiment.

Sensitization/Treatment protocol:

Induction of allergic airway inflammation was performed by intra peritoneal (i.p.) sensitization and airway challenge through nasal inhalation. Groups II and III were sensitized on day 0 by intra peritoneal injection of 10 µg of OVA per animal (Sigma–Aldrich, St. Louis, MO) mixed with aluminum hydroxide (10 mg per animal) as adjuvant, (Pierce Biotechnology, Rockford, IL) in a volume of 1 ml PBS (11).

Fourteen days after the first sensitization, the rats were exposed, for 15 min to an aerosol of OVA (1 mg/ml PBS). The period between the inoculation and the challenge was chosen based on the work of Coleman et al (12).

Melatonin treatment: melatonin was purchased from Sigma Chemical Co. (St Louis, MO, USA). It was freshly dissolved in absolute ethanol and further dilutions were made in saline. The final concentration of ethanol was 1%. An equal volume of ethanol, as used in the melatonin solution, was added to the saline solution. Considering the day of the immunization to OVA as day zero, melatonin administration to rats of group III started 15 days before and ended 15 days after immunization with OVA; (24 hr after the challenge procedure) (11).

Biochemical Analysis:

Twenty-four hours after completion of the sensitization/ treatment protocol, blood samples from all rats (15 per group) were collected from retro-orbital vein in micro tubes and centrifuged

immediately for 10 min at 5000 rpm to obtain clear sera which were stored at -20°C until analysis. Then, the rats were decapitated under anesthesia via i.p. injection of ketamine (8.7 mg/100 g body wt) mixed with xylazine (1.3 mg/100 g body wt).

The lungs were removed and bronchoalveolar lavage (BAL) was performed by instillation and withdrawal of 2 ml ice cold PBS through trachea and BAL fluid (BALF) was collected and stored at - 20 °C.

Parts of lung were fixed in neutral buffered formaldehyde and sectioned for histopathological studies. Remaining lung tissue was stored at -70 °C until processed.

Determination of Inflammatory cells in Bronchoalveolar lavage fluid (BALF)

Total cell numbers in BALF were determined with crystal violet stain using a hemocytometer (Veterinary Hematology Analyzer (Medonic CA 620, Sweden). For differential cell count, such as eosinophils, macrophages and lymphocytes, centrifuged preparations were fixed, stained with Giemsa Wright stain and counted according to differentiated morphology.

Estimation of BALF cytokines and serum levels of cortisol:

The levels of IL-4, IL- 5, and IL-10 of BALF were analyzed using ELISA kits (R &D Systems, Minneapolis, USA) as previously described (13). Serum cortisol levels were measured by radioimmunoassay (14).

Determination of OVA-specific IgG1:

The serum level of OVA-specific IgG1 was measured by ELISA. Briefly, the 96-well round-bottom micro titer plates were coated for overnight at 4 °C with 100 µl of 100 mg/ml OVA in

NaHCO₃ buffer (pH 9.6). The plates were washed three times with PBS–0.05% Tween 20, and then blocked by incubation with 200 ml 0.05% PBS–Tween and 2% BSA for 1 h at 37 °C. After washing, different dilutions of serum samples and standard rat IgG1 (AbD Serotec, Munich) was added and the plates were incubated for 1 h at 37 °C. OVA-specific IgG1 was detected with HRP-conjugated mouse anti-rat Kappa/Lambda chain (1 mg/ml) antibodies (AbD, Serotec) by incubation for 30 min at 37 °C. Then, TMB and H₂O₂ were added as a substrate and incubated at 37 °C for 20 min. The reaction was terminated with 2 N, H₂SO₄, and read at 450 nm by ELISA reader (Thermo Electron Corporation, Finland).

Total serum IgE and IgG1 levels:

The 96-well round-bottom microtiter plates were coated overnight at 4 °C with mouse anti-rat IgE and IgG1 heavy chain (AbD Serotec, Munich). Different dilutions of serum samples were added. Total serum IgE and IgG1 were measured with HRP conjugated mouse anti-rat Kappa/Lambda chain. The total IgE and IgG1 levels were expressed as mg/ml after comparing the optical density (O.D. at 450 nm) values to rat IgE and IgG1 standards.

RT-PCR for quantification of IL-10 in lung tissue:

After part of the right lung was dissected, immediately frozen in liquid nitrogen, and stored at - 80°C. Total RNA was extracted and isolated from lung tissue using TRIzol method. The mRNA expression using real-time RT-PCR was measured with a 7700 ABI Prism (Applied Biosystems, CA, USA). The RT-PCR response was determined using a QuantiTect(R) SYBR(R) Green PCR Kit (Qiagen, CA, USA). RT-PCR for IL-10, and

GAPDH consisted of 15 s of denaturation at 94 °C, 30 s of annealing at 57 °C, and 30 s of extension at 72 °C for 45 cycles. The following primers were used:

IL-10-Forward: 5' TGAATTCCCTGGGTGAGAAG-3', IL-10-Backward: 5' -TGGCCTTGTAGACACCTTGG-3'. GAPDH, Forward: TGCAGTGGCAAAGTGGAGATT-3', GAPDH-Backward: 5' ATTTGGCGTGAGTGGAGTCAT-3'.

The expression level was calculated using the $\Delta\Delta$ Ct (the comparative threshold cycle) method relative to the production of GAPDH (2).

Histopathological examination:

Lung specimens were fixed immediately in 10% buffered formalin, embedded in paraffin, prepared as 4- μ m-thick sections and stained with hematoxylin and eosin (HE). Stained sections were examined under light microscope (Olympus CX31, Japan) and photographed using digital camera (Olympus, Camedia C-5060, Japan) (15).

Statistical Analysis:

Data were expressed as mean \pm standard deviation [SD] for all parameters. The data were analyzed using Graph Pad Prism data analysis program [GraphPad Software, Inc., San Diego, CA, USA]. For comparison of statistical significance between different groups Student Newman-Keuls t-test for unpaired data were used. For multiple comparisons, one-way analysis of variance [ONE- WAY-ANOVA] test followed by the least Significant Difference [LSD]. A value of $P \leq 0.05$ was considered statistically significant (16).

RESULTS

Effects of melatonin administration on serum levels of total IgE, IgG1 and OVA-specific IgG1 levels:

The serum levels of total IgE, IgG1 and OVA-specific IgG1 of group II were significantly elevated compared to group I and IV ($p < 0.001$). However, melatonin administration decreased these elevated levels in the serum of

group III compared to control rats and group IV ($p < 0.05$) (Fig.1).

Effects of melatonin administration on inflammatory cells count in BALF:

To examine the effect of melatonin administration on lung inflammation in OVA sensitized rats, total and differential cell numbers in the BAL fluid were counted.

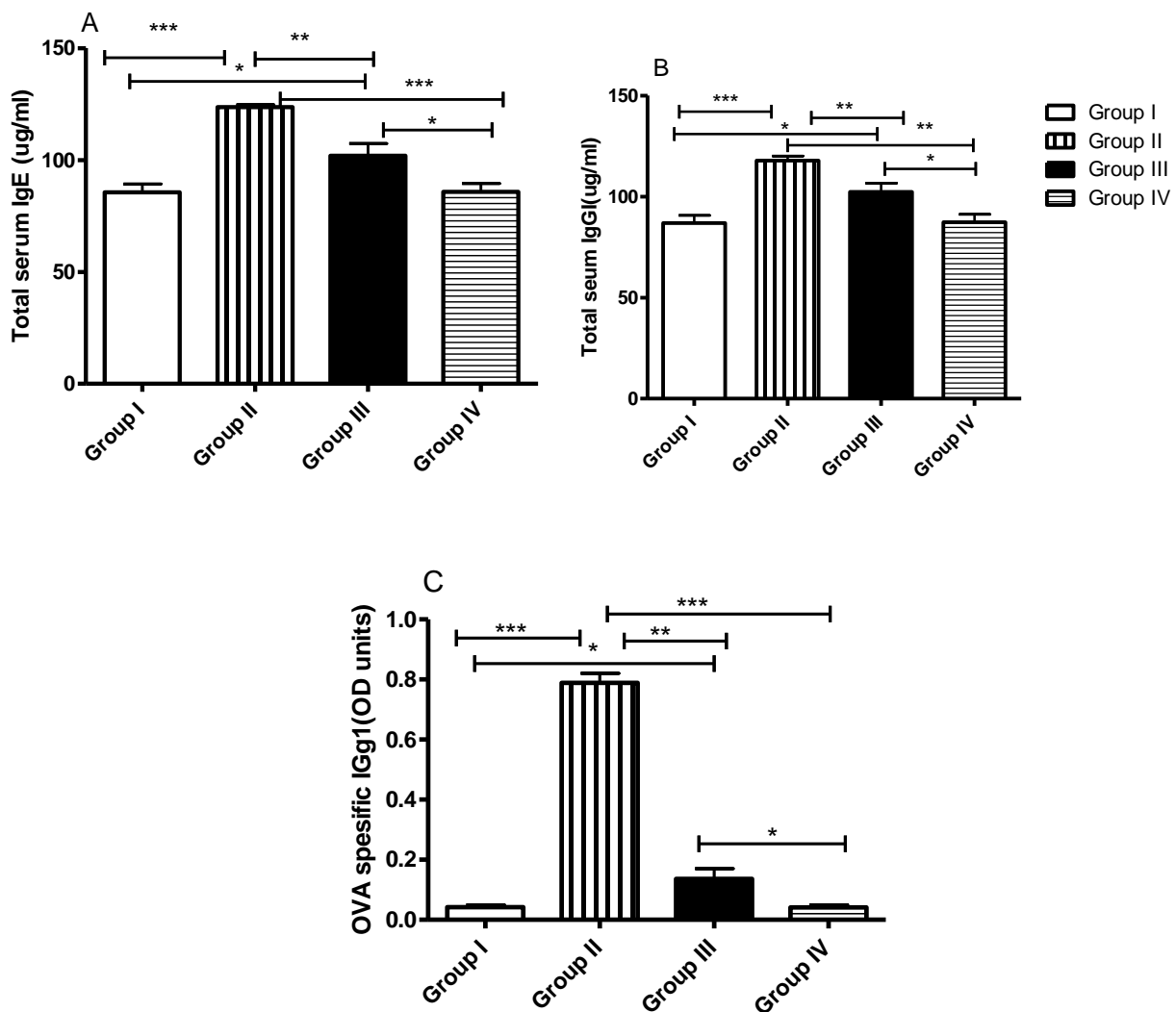


Fig.1: Total serum IgE, IgG1 and OVA-specific IgG1 in all studied groups (A) Levels of total serum IgE of rats in the four studied groups. Significant increased total Serum IgE in rats of group II as compared to control rats and group IV. Melatonin significantly ($P < 0.01$) reduced the elevated treated rats of group III. (B) Total serum IgG1 levels increased significantly in OVA sensitized rats of group II as compared to group I and IV ($P < 0.001$). Melatonin significantly lowered the level of total serum IgG1 in rats of group III compared to group I ($P < 0.01$) (C) OVA-specific IgG1 measured in the four groups. Melatonin significantly ($P < 0.001$) reduced the raised level of OVA-specific IgG1 in rats of group II. All data are represented as mean \pm SD for 15 rats each group. Significant levels are *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ comparing two groups.

Group II showed a substantial increase in the number of total leukocytes and the differential number of eosinophils ($P < 0.001$), macrophages ($P < 0.001$) and lymphocytes ($P < 0.001$) compared to group I and group IV. Rats of group III showed

significantly decreased the total ($P < 0.001$) and differential numbers of eosinophils ($P < 0.05$), macrophages ($P < 0.05$) and normalized the number of lymphocytes (Fig.2) compared to group I and group IV.

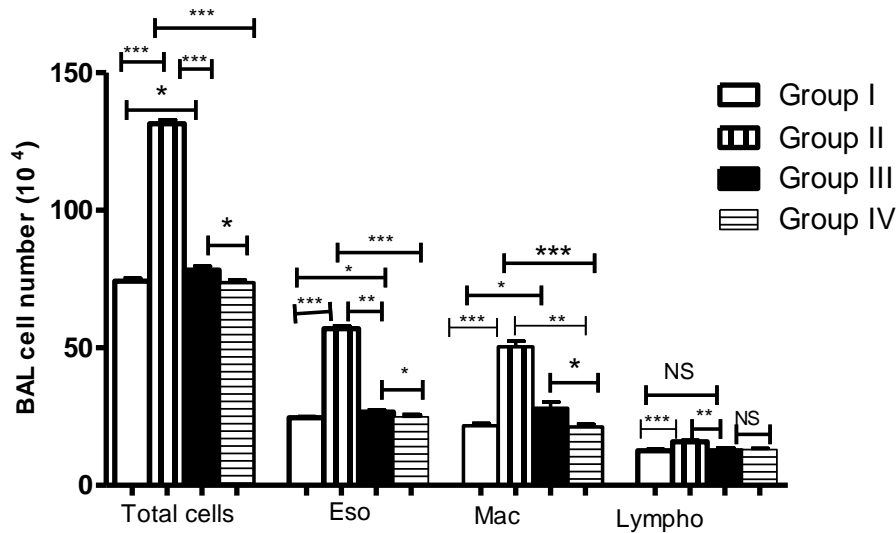


Fig.2: Mean number of total cells, eosinophil (Eos), macrophage (Mac) and lymphocyte (Lympho) in BALF from four different studied groups. Data presented as mean \pm SD for 15 rats each group. Significant levels are *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ comparing two groups

Effects of melatonin administration on production of Th2 cytokines in the studied groups:

The results presented in Table 1 showed that OVA sensitized rats in group II significantly elevated the BALF levels of IL-4, IL-5, IL-13 and decreased significantly the BALF levels of IL-10 compared to control rats and group IV ($p < 0.001$, respectively). Melatonin administration decreased the elevated levels of IL-4, IL-5 and IL-13 in the BALF of the rats of group III compared to control rats and group IV ($p < 0.05$). However, melatonin intake increased the lowered BALF levels of IL-10 in rats of group III. In addition, The whole BALF levels of IL-10 showed a significant negative correlation with BALF levels of IL-4 ($r = -0.881$,

$P < 0.001$), IL-5 ($r = -0.87$, $P < 0.001$) and IL-13 ($r = -0.909$, $P < 0.001$) (Table 2).

Effects of melatonin administration on the serum cortisol levels:

Table 1 showed that serum cortisol levels, assessed in all studied groups, indicated that neither the antigen challenge, nor melatonin treatment groups influenced serum cortisol levels when compared with that obtained from the control group.

Effects of melatonin administration on IL-10 mRNA expression:

Sensitization with OVA resulted in a significant decrease in IL-10 mRNA expression levels compared to control group. Interestingly, administration of melatonin to OVA-sensitized rats completely reversed the decrease in IL-10 mRNA expression to the control values (Fig.3).

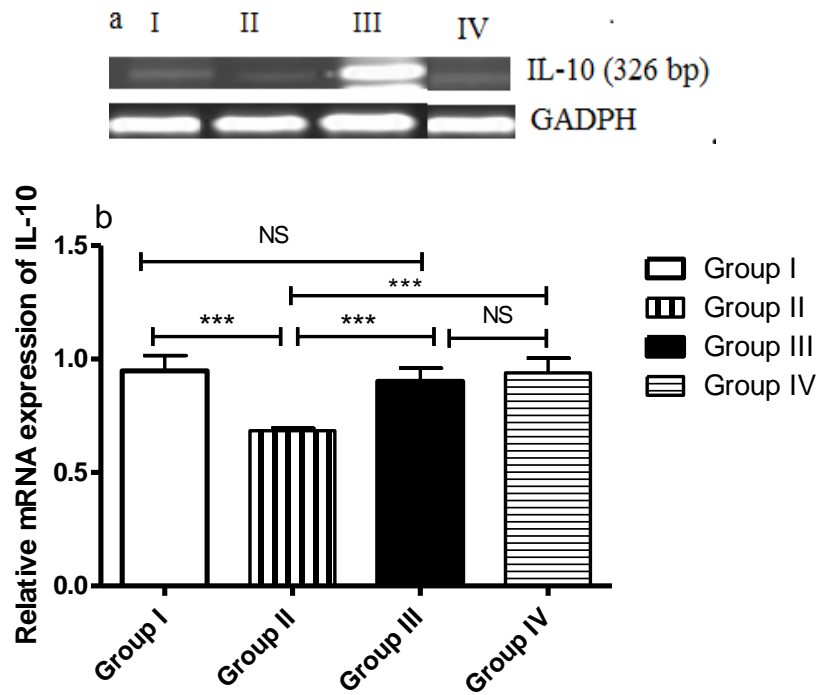


Fig.3: Effect of melatonin administration on IL-10 mRNA expression. (A) Total RNA was extracted and used in RT-PCR to assess IL-10 gene expression. GAPDH was amplified as an internal control. Representative bands from gel electrophoresis of PCR products are shown. Data is given for group I, II,III and group IV, respectively. (B) IL-10 mRNA expression in rat lungs expressed as a percent ratio to GAPDH mRNA determined by RT- PCR. Data is shown as mean \pm SD for 15 rats each group. A significant decrease in the mRNA expression for IL-10 was noted in OVA-sensitized rats (group II) as compared to control group. Melatonin significantly increased mRNA expression of IL-10 in group III compared to group II. Significant levels are ***P <0.001,**P < 0.01 and *P< 0.05 comparing two groups

Table (1): BALF Levels of IL- 4, IL- 5, IL-13,1L10 (pg/ml) and serum cortisol of the studied groups

	IL-5 (pg/ml)	IL-4 (pg/ml)	IL-10 (pg/ml)	IL-13 (pg/ml)	Cortisol (ng/ml)
Group I	31.26 \pm 4.09	16.4 \pm 1.69	88.51 \pm 5.22	10.65 \pm 0.81	8.66 \pm 0.87
Group II	87.93 \pm 7.16 ^a	51.92 \pm 10.32 ^a	59.18 \pm 6.08 ^a	107.2 \pm 12.47 ^a	8.71 \pm 0.94 ^{NS}
Group III	35.41 \pm 4.09 ^{a,b}	17.91 \pm 1.88 ^{a,b}	84.49 \pm 5.25 ^{a,b}	11.99 \pm 2.17 ^{a,b}	8.7 \pm 0.79 ^{NS}
Group IV	31.18 \pm 4.17 ^{NS, b,c}	16.28 \pm 1.75 ^{NS, b,c}	88.45 \pm 5.29 ^{NS, b,c}	10.61 \pm .83 ^{NS,b,c}	8.57 \pm 0.84 ^{NS, b,c}

Data are presented as mean \pm SD (15 rats in each group).a: significant as compared with group I , P < 0.05.b: significant as compared to group II, P < 0.05.c: significant as compared to group III, P < 0.05.NS: non-significant as compared to group I.

The IL-10 mRNA expression levels of group IV did not differ significantly than those of group I.

Histopathological results:

Histopathological examination of lung control rats and rats of group IV showed normal

appearance (Fig.4a). Lung sections of OVA treated rats revealed pathological changes (Fig. 4b). These changes were consisted of hyperplasia of the pneumocyte type II (Fig.5a), pulmonary eosinophilic infiltrations especially around the blood vessels (Fig. 5b), hyperplasia of the goblet

cells (Fig. 5c), and numerous lymphocytes and macrophages laden with hemosiderin pigment (Fig. 5d). Rats of group III revealed inhibition of

the infiltration of inflammatory cells, no hyperplasia of the pneumocyte type II and goblet cells (Fig. 4c).

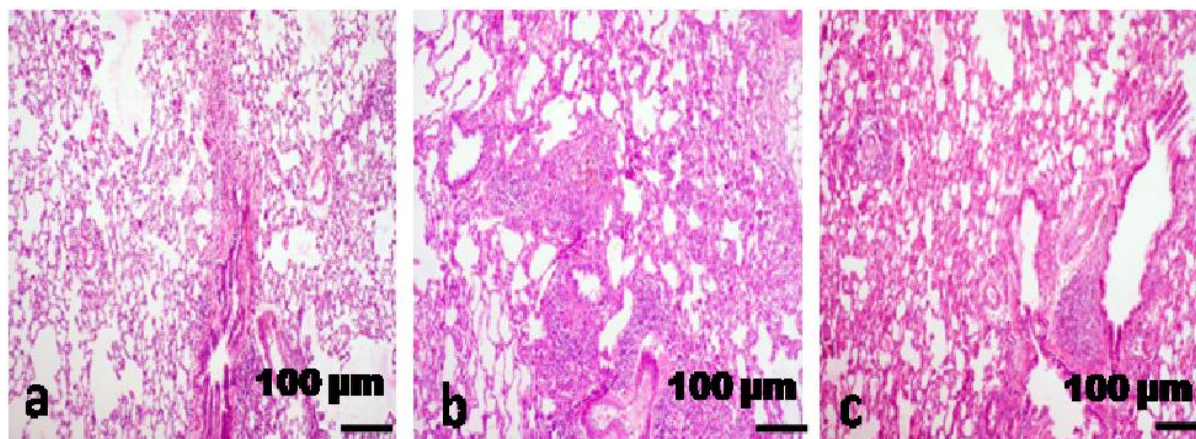


Fig. 4 (a-c): Representative micrograph of the lung from group I,II and III respectively. H&E stain. Bar= 100µm.

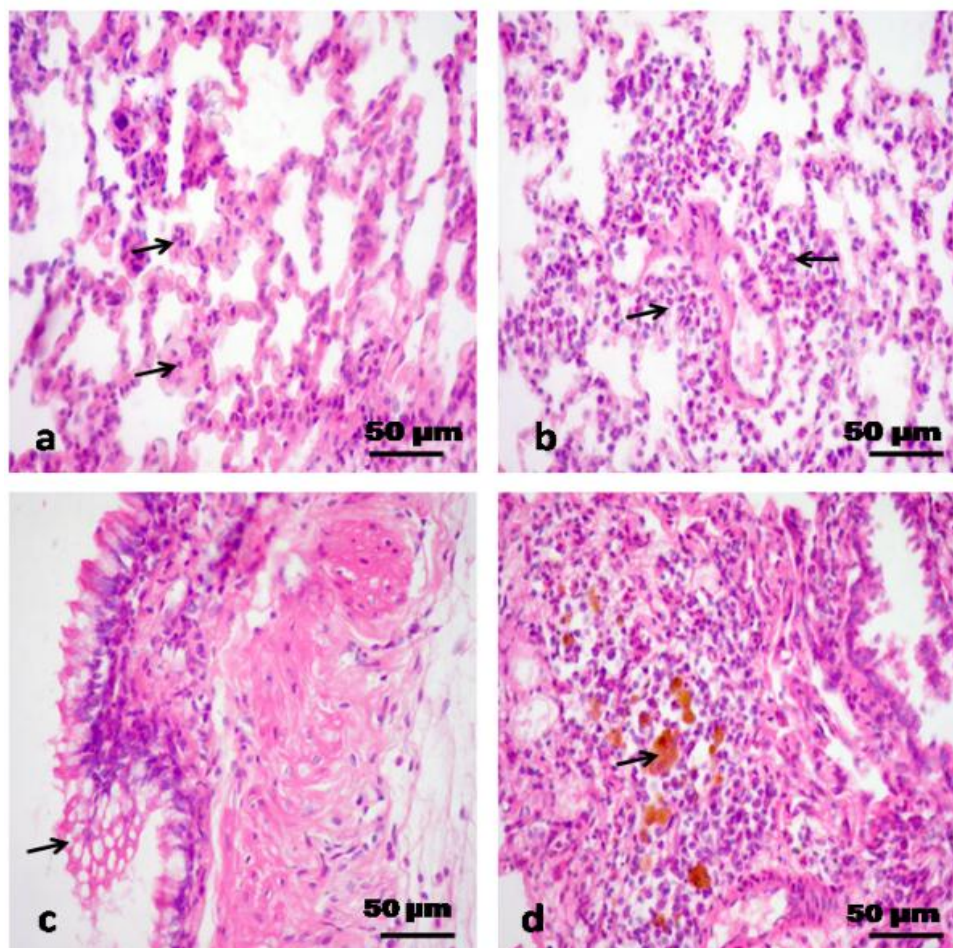


Fig.5: Representative micrograph of the lung from ovalbumin treated rats (group II) showing a) Hyperplasia of pneumocyte type II (arrows), b) eosinophils infiltration (arrows), c) goblet cell hyperplasia (arrow), d) macrophages laden with hemosiderin (arrow). H&E stain. Bar= 50µm.

DISCUSSION

The present study was designed to investigate the impact of melatonin administration on allergen-induced airway inflammation in a rat model of allergic asthma. Th2 cytokines play an impressive role in the pathogenesis of allergic airway inflammation through the production of cytokines (17). The increased production of IL-4, IL-5, and IL-13 has been recognized in different types of allergic disorders, including asthma (1). The present results showed that BALF levels of IL-4, IL-5 and IL-13 have been significantly elevated in rat of group II. On other hand, BALF levels of IL-10 decreased significantly in the same group. These results are consistent to other previous studies (18-20). Interestingly, melatonin administration significantly lowered the BALF levels of IL-4, IL-5, IL-13 and increased levels of IL-10 in treated rats of group III.

Table (2) Correlation between BALF levels of IL-10 and BALF levels of IL-4, 5 and 13

Parameters	IL-10	
IL-4	-0.881	P < 0.001
IL-5	-0.870	P < 0.001
IL-13	-0.909	P < 0.001

Increased serum levels of IgE and IgG1 are associated with airway inflammation and asthma. In this study, the immunological aspects of asthmatic airway rat model were confirmed by increased the serum levels of total IgE, IgG1 and OVA-specific IgG1. Other inflammatory parameters were demonstrated in rat of group II, including inflammatory cells infiltration such as eosinophils, lymphocytes, and macrophages. These finding may occur under the effect of

increased the measured Th2 cytokines. Wills-Karp (21) reported the ability of IL-4 and IL-5, in mediating IgE, IgG production and eosinophilia, which have been implicated in the pathogenesis of allergic asthma. Data of Pastva (18) study supported the present results as they found increased IL-4 and IL-5 in BALF secretions as well as increased IgE in the serum, suggesting that the employed OVA sensitization model is a valid one for the study of atopic asthma. Our histopathological results revealed that rats of group II showed hyperplasia of the pneumocyte type II, pulmonary eosinophilic infiltrations, hyperplasia of the goblet cells and numerous lymphocytes and macrophages. Rats of group III revealed inhibition of the infiltration of inflammatory cells, no hyperplasia of the pneumocyte type II and goblet cells.

In addition, It has been known that IL-4 and IL-13 are necessary for differentiation of T cells to the Th2 type and for isotype switching to IgE in B cells (22). However, decrease serum IgE levels and eosinophilic infiltration has been reported after blocking IL-4 with anti-IL-4 antibody in the airways of allergen-sensitized mice (23). Also IL-5 contributes for activation and survival of eosinophils the increased mucus production in the airways of allergic mice (24). These results may confirm the fact that Th2 type inflammatory events are mainly induced by the cytokines profile.

The present data revealed that melatonin administration to OVA sensitized rats reduced the serum levels of OVA-specific IgE, total IgE as well as IgG1. These results are supported with those of Martinz et al (11) who reported the ability

of melatonin to decrease the serum levels of IgE in OVA sensitized rats. These lowered levels in rats of group III may be due to the inhibition of Th2 cytokine levels occurred under the effect of melatonin administration.

The mechanisms responsible for the effects of melatonin on immune responses in the asthmatic lung remain ill defined; however, data of the present study suggest that such mechanisms may involve melatonin-induced changes in production of IL-10. Specifically, our results showed that melatonin increased significantly both the lowered BALF levels of IL-10 and its mRNA expression which were significantly decreased in rats of group II. In addition, there were significant negative correlations between the BALF levels of IL-10 and The BALF levels of IL-4, 5 and 13. IL-10 has been reported as an important key in downstream of the inflammatory cascade in the Th2 response to antigens and in the development of BALF eosinophilia and cytokine production in a murine model of asthma (25).

Many studies showed that IL-10 also down regulates Th2 clones and their production of IL-4 and IL-5 (26). In addition, IL-10 exerts various effects on the immune cells, such as the stimulation of B cell differentiation and IgG secretion (27). The observed low BALF levels of IL-4, IL-5 and IL-13 in rats of group III may be explained by increased levels of IL-10 induced by melatonin administration. Results of Kosaka (2) who found that when IL-10 was administered prior to OVA inhalation in OVA-sensitized mice, the IL-5 and IL-13 productions in the BALF were remarkably inhibited as well as the number of eosinophils, may support our explanation.

Further study of Wang et al (28) support our hypothesis that showed exogenous IL-10 administered in challenge phase alleviates nasal allergic inflammation in allergic rhinitis mice, most likely by reducing the number of eosinophils, decreases other cytokine levels in nasal lavage fluid. These suggested that the immunosuppressive properties of melatonin in allergic air way model may be through up regulation of IL-10.

Conclusions:

Melatonin inhibits allergen-induced lung inflammation by reducing airway eosinophilia, Th2 cytokines, and allergen-specific antibodies, thus supporting its anti-inflammatory role during the allergic response in the lung. Melatonin ameliorates allergic airway inflammation by up-regulation of IL-10 which controls Th2-type cytokine production and eosinophil infiltration. As for IL-10, the inhibition-related activities of this cytokine may be useful for the prevention and possible treatment of airway inflammation of humans

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