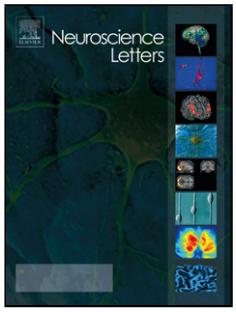

Title: Regulation of hypothalamic-pituitary-adrenal axis activity and immunologic function contributed to the anti-inflammatory effect of acupuncture in the OVA-induced murine asthma model

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Regulation of hypothalamic-pituitary-adrenal axis activity and immunologic function contributed to the anti-inflammatory effect of acupuncture in the OVA-induced murine asthma model

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Highlights

In this study, we evaluated the regulatory role of acupuncture in airway inflammation and the hypothalamic-pituitary-adrenal (HPA) axis activity in OVA-induced murine asthma model. Our results demonstrated that acupuncture was effective in suppression of AHR, inhibition of total leukocyte, neutrophil, lymphocyte and eosinophil counts in BALF. Acupuncture also attenuated airway inflammation and TNF-α, IL-1β, IL-5 and eotaxin secretion in serum. The HPA axis activity was regulated by acupucnture, which included promotion of adrenocorticotropic hormone and cortisol secretion in the plasma.

Our findings may provide support to better understand the contribution of acupuncture to the regulation of airway inflammation and HPA axis activity in asthma.

ABSTRACT

Asthma is a complex inflammatory disease of the airways and acupuncture is one of the effective therapies widely used to treat asthma in China. The aim of the study was to evaluate the regulatory role of acupuncture in airway inflammation and the hypothalamic-pituitary-adrenal (HPA) axis activity in OVA-induced murine asthma model. Our results demonstrated that acupuncture was effective in suppression of AHR, inhibition of total leukocyte, neutrophil, lymphocyte and eosinophil counts in BALF, attenuation of airway inflammation and TNF-α, IL-1β, IL-5 and eotaxin secretion. Furthermore, the HPA axis activity was also regulated by acupuncture, which included promotion of adrenocorticotropic hormone and cortisol secretion in the plasma. Our findings revealed that acupuncture could attenuate airway inflammation and regulate HPA axis and immunologic function in the OVA-induced murine asthma model, which may provide support to better understand the contribution of acupuncture to the regulation of airway inflammation and HPA axis activity in asthma.

Keywords

Acupuncture, Asthma, Inflammation, Immunologic function, HPA axis

1. Introduction

Asthma is a chronic inflammatory disorder affecting 300 million individuals worldwide[1]. Airway hyperresponsiveness (AHR) and chronic airway inflammation are two key features of asthma, and have been shown to be involved in the development of asthma pathogenesis. Both genetic and environmental factors are likely involved in asthma pathogenesis[2], which make the complexity and heterogeneity of asthma, and the latter in turn make both the clinical management and investigation of asthma challenging[3]. Inhaled corticosteroids (ICS) and β-adrenergic agonists are significantly effective in relieving inflammation and control symptoms in asthma[4], and most asthmatic individuals respond well to the currently available treatments of ICS and β-adrenergic agonists. However, side effects such as easy bruising, osteoporosis, cataracts and glaucoma, and adrenal suppression may occur in a population of asthmatics[1].

Acupuncture treatment of asthma has a long history, assured curative effects and is widely used in clinic in China. Previous studies have identified that acupuncture has the potential to regulate gene expression profiles in asthma, which include immune response and steroid hormone related pathways[5]. Furthermore, acupuncture is believed to elicit profound psychophysical responses by harmonizing or balancing the energy and blood flow through the body[6]. The hypothalamus-pituitary-adrenal (HPA) axis is considered to be a central integrative system being crucial in the successful physiological adaptation of the organism to stress[7], and HPA axis activation was reported to be associated with asthma pathogenesis[7, 8]. The activation of the HPA axis involves the release of corticotropin-releasing hormone (CRH) by the paraventricular nucleus of the hypothalamus, stimulating the production of adrenocorticotropic hormone (ACTH) in the anterior lobe of the pituitary gland. Secreted ACTH in the peripheral circulation stimulates the adrenal cortex, releasing glucocorticoids and, in particular, cortisol into the blood[9, 10]. It is commonly known that glucocorticoids inhibit the activity of the HPA axis by negative feedback

on CRH and ACTH secretion[11]. However, the data on the regulation of HPA axis by acupuncture is scarce and the potential role of acupuncture on the HPA axis in the OVA-induced murine asthma model has not been explored. In this study, we aimed to investigate the role of acupuncture in airway inflammation and the HPA axis activity in OVA-induced murine asthma model.

2. Materials and methods

2.1 Animals

All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Fudan University. Female BALB/c mice (six-week-old, 14-17g) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd and raised with food and water freely available.

2.2 Reagents

Ovalbumin (OVA), aluminum hydroxide (Al(OH)3), pentobarbital sodium and methacholine (Mch) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Adrenocorticotropic hormone (ACTH) and cortisol (CORT) ELISA kits were purchased from mybiosource (San Diego, California, USA) and cayman (Michigan, USA) separately. Inflammatory cytokine Bio-Plex kit was purchased from Bio-Rad (San Diego, California, USA). Sterile acupuncture needles (Hwato, 13mm long, 0.25mm in diameter) were purchased from Suzhou medical supplies co., LTD (Suzhou, Jiangsu, China). Trizol reagent and SYBR Green kit and RevertAid TM First Strand cDNA Synthesis kit were purchased from Thermo Fisher (Massachusetts, USA).

2.3 Asthma model establishment and acupuncture treatment

Forty mice were randomly divided into four groups (N=10 each group), including normal control group (NC), OVA-induced asthma group (A), asthma model combined with acupuncture treatment group (AA) and asthma model combined with sham

acupuncture treatment group (ASA). Murine asthma model was induced by multiple OVA sensitization and challenge. As shown in Fig 1, mice were sensitized with 20 μg OVA and 2 mg Al(OH)3 in 0.2 ml of saline solution by intraperitoneal injection on day 0 and day7. From day14 to day40, mice were challenged by inhalation of 3% (w/v) OVA solution for 30 min every other day for four weeks. Briefly, mice were placed in a plexiglas chamber and nebulized with an ultrasonic nebulizer (402AI, Yuyue medical equipment Co. Ltd., Jiangsu, China). Mice in NC group were sensitized and challenged with saline.

In traditional Chinese medicine (TCM), there are fourteen meridians which include 365 acupoints. According to the TCM theory, the acupuncture points, GV14 (Dazhui, between the C7 and T1 vertebrae), bilateral BL12 (Fengmen, foveola laterally between the T2 and T3 vertebrae), and bilateral BL13 (Feishu, foveola laterally between the T3 and T4 vertebrae) were selected for acupuncture treatment[5]. GV14 which has the function of regulating the vital Qi of the body belongs to the Governor Meridian. BL12 and BL13 which could regulate the local lung function, belong to the the Urinary Bladder Meridian of Foot-Taiyang. From day 14 and 1h before the OVA challenge, acupuncture treatment was performed with a fixation device in an awakened state. Sterile needles were inserted into the acupoints with a depth of approximately 3mm and withdrawn after the needle retaining time of 30 min. During the needle retaining time, manual manipulations were performed every 10 min. GB30 (Huantiao) belongs to the Gallbladder Meridian of foot-Shaoyang, and is considered to have no regulatory effect on the lung function. Therefore, distal irrelevant acupoints of bilateral GB30 was selected as sham acupuncture treatment in the same way.

2.4 Measurement of AHR

Within 24h of the last OVA challenge, AHR was investigated with an invasive method under anaesthesia using Buxco pulmonary system (Buxco Electronics Inc., NY, USA). Tracheostomy was performed and the tracheal tube was inserted. Mice were put into

the body plethysmograph chamber and the inserted tracheal tube was connected to the ventilator. After a stable baseline airway pressure (<5% variation over 2.5 min) was reached, PBS and Mch (3.125, 6.25, and 12.5mg/mL) were administrated to mice by nebulization. The airway resistance (R_L) and lung dynamic compliance (Cdyn) were recorded. Results were expressed as the percentage change in baseline.

2.5 Hematoxylin and eosin (HE) staining of the lung tissue

The right lung of mice were harvested after bronchoalveolar lavage (BALF) collection, fixed in 4% paraformaldehyde, dehydrated in alcohol gradient, embedded in paraffin and sliced at 4μm of thickness. Lung sections were then stained with H&E. Six mice from each group and six arbitrarily selected fields in each mouse were photographed at 200× magnification using an optical microscope (ECLIPSE 80i, Nikon, Japan).

2.6 Inflammatory cell counts in BALF

BALF was performed by lavaging the left lung through the tracheal cannula using 0.3ml aliquots of sterile PBS twice (total volume 0.6 ml), then centrifuged at 500g for 10 min, and the cell pellet was resuspended with 100 μl PBS for inflammatory cell counts using hemavet 950 instrument (Drew scientific group).

2.7 Inflammatory cytokines and HPA axis activity assay

To measure inflammatory cytokines in serum, the TNF-α, IL-1β, IL-5 and eotaxin level were detected by Bio-Plex assay using BioPlex 2200 analyzer according to the manufacturer's instructions. Briefly, our amples are added to reaction vessels which contain bead reagent and sample diluent. After two incubations and washing steps, the beads were read by a flow-based detector. Data were calculated as relative fluorescence intensities and converted to a fluorescence ratio (FR) using an internal-standard bead. The FR is compared to an assay-specific calibration curve to determine the concentration of the cytokines. The ACTH and CORT level in the

plasma were tested by ELISA according to the manufacturer's instruction.

2.8 Real‑time quantitative PCR (qPCR) analysis

The hypothalamus in each group was collected for PCR assay. Total RNA was isolated from the hypothalamus using Trizol reagent according to the manufacturer's instructions. The first strand cDNA was then synthesized, and the CRH and GAPDH were then amplified by PCR Amplifier (ViiA7, life technologies, Carlsbad, CA, USA). The final data was normalized to GAPDH and presented as fold induction compared with the expression level of mice in NC group. The primers' sequences used for CRH and GAPDH were shown in Table 1.

2.9 Statistical analysis

All results were expressed as mean±SD. Statistical analysis was conducted by ANOVA and followed by post hoc for multiple comparisons. A p-value of less than 0.05 was accepted as significant.

3. Results

3.1 Acupuncture suppressed AHR in OVA-induced murine asthma model

Mesurement of AHR to Mch was performed and our results demonstrated that Mch challenge induced a significant increase in R_L and decrease in Cdyn (Fig 2, $p<0.05$ or $p<0.01$). After 4 weeks acupuncture treatment, the R_L decrease and the Cdyn increase markedly (Fig 2, $p<0.05$ or $p<0.01$). However, sham acupuncture did not improve the AHR prominently (Fig 2, $p>0.05$). With the increase of the Mch dosage, the AHR exacerbated more obviously, and acupuncture had better effect on the improvement of AHR. What's more, there were significant differences between the acupuncture and sham acupuncture groups in the suppression of AHR (Fig 2, $p<0.05$).

3.2 Acupuncture attenuated airway inflammation in OVA-induced murine asthma model

We investigated the effect of acupuncture on the inflammation in each group. An increase of inflammation around the lung tissue was noted in the OVA-induced murine asthma model (Fig.3). There were a large number of inflammatory cells infiltrating in and around the airways. Furthermore, we also observed increased mucus secretion inside the airways in the A group. Histopathologic analysis demonstrated that acupuncture resulted in reduced inflammatory cell infiltration in and around the airways. Although sham acupuncture alleviated inflammation of the lung tissue, there were still a number of inflammatory cells around the airways.

We also observed the changes of inflammatory cells in BALF of mice in each group. Total leukocyte (Total), neutrophil (Neu), lymphocyte (Lym) and eosinophil (Eos) were determined, and these cell types were all found to be increased significantly compared with the NC group (Fig 4, $p<0.01$ or $p<0.05$). Mice in the acupuncture treatment group showed a marked decrease in these cell counts (Fig 4, $p<0.05$). In contrast, there was no significant decrease in the total leukocyte, Neu and Lym cells by sham acupuncture (Fig 4, $p>0.05$). Additionally, a reduction in Eos was observed in mice of the ASA group compared with mice in the A group (Fig 4, $p<0.05$).

3.3 Acupuncture inhibited the secretion of inflammatory cytokines in OVA-induced murine asthma model

We analysed the effect of acupuncture on the inflammatory cytokines in the serum of mice in each group. A marked increase of the TNF-α, IL-1β, IL-5 and eotaxin was observed in the OVA-induced murine asthma group and ASA group compared with the NC group (Fig 5, $p<0.01$ or $p<0.05$). Acupuncture had a beneficial effect on the inhibition of these inflammatory cytokines (Fig 5, $p<0.05$). In particular, acupuncture also induced significant inhibitory effect on TNF-α and IL-1β compared with sham acupuncture (Fig 5, $p<0.05$).

3.4 Acupuncture regulated the HPA axis function in OVA-induced murine asthma model

To identify the role of acupuncture in the HPA axis function, we evaluated the CRH mRNA expression in the hypothalamus and the ACTH and CORT level in plasma. There was no significant increase in CRH mRNA expression in the hypothalamus of the OVA-induced asthma model compared with mice in NC group (Fig 6A, $p>0.05$). However, acupuncture induced significant elevation of CRH mRNA expression compared with the A group (Fig 6A, $p<0.01$), and sham acupuncture did not exert such effect (Fig 6A, $p>0.05$). Mice in the A and ASA groups had reduced ACTH level (Fig 6B, $p<0.05$) and increased CORT level (Fig 6C, $p<0.05$) compared with the NC mice. Acupuncture induced a marked promotion of ACTH (Fig 6B, $p<0.05$) and CORT (Fig 6C, $p<0.05$) level compared with the OVA-induced murine asthma model. Additionally, acupuncture exerted dramatic promotion of ACTH and CORT level than sham acupuncture ($p<0.05$).

4. Discussion

Asthma is a common disease that affects approximately 300 million persons worldwide, accounts for about one out of every 250 deaths worldwide and has profound healthcare costs in terms of emergency room visits and hospitalisations[12], and brings heavy burden to the society and family[1]. In our study, we explored the potential of acupuncture in the regulation of airway inflammation and HPA axis function in OVA-induced murine asthma model. We have demonstrated that acupuncture was capable of reducing AHR in OVA-induce murine asthma model, indicating the efficacy of acupuncture in asthma.

Inflammatory cells infiltration into the airways is a hallmark of asthmatic inflammation during asthma pathogenesis. Chronic OVA inhalation resulted in a large amount of inflammatory cells accumulating in the airways, and acupuncture exerted a significant suppression of these cells aggregation. Infiltration of eosinophils, neutrophils and lymphocytes contributed to the progression of asthma. What’s more, acupuncture also induced inhibition of the total leukocyte, Eos, Lym, and Neu in BALF, indicating the anti-inflammatory function by acupuncture. It has been shown

that eosinophils interact with both innate and adaptive immune cells and play a central role in the inflammatory response associated with bronchial asthma[13]. Previous reports have identified that strategies aimed at optimizing eosinophil counts in sputum with corticosteroids significantly reduce the frequency of asthma exacerbations[14]. Eosinophilic inflammation was reported to be associated with the whole spectrum of asthma severity, ranging from mild-to-moderate to severe uncontrolled disease, whereas neutrophilic inflammation occurs mostly in more severe asthma[15]. Our data on the inflammatory cell counts in BALF may partly demonstrate the anti-inflammatory efficacy of acupuncture treatment.

Cytokines were thought to be responsible for airway inflammation in asthma. To clarify the cytokines interfered by acupuncture, we detected the TNF-α, IL-1β, IL-5 and eotaxin level in serum. TNF-α has been implicated as a pro-inflammatory cytokine in asthma and blood monocytes from patients with refractory asthma have increased expression of TNF-α[16]. A human monoclonal anti-TNF-α antibody (adalimumab) has been shown to reduce airway inflammation[17]. In line with previous research results, we have shown that acupuncture reduced TNF-α level in serum. IL-1β is an important inducer of the pathological progression of allergic asthma[18], and IL-1β has been shown to significantly increase AHR, bradykinin and neutrophil counts in the BALF in OVA-sensitized rats[19]. Inhalation of anti-IL-1β IgY inhibits pathological responses in the pulmonary tissues of guinea pigs with allergic asthma[18]. Eotaxin plays a prominent role in developing eosinophilic inflammation, and is involved in the regulation of the retardation of eosinophil apoptosis[20]. Eotaxin is not only a potent chemoattractant for eosinophils but also acts as a multifunctional enhancer for eosinophils[21, 22]. Acupuncture was shown to suppress eotaxin secretion, which was in consistent with the data on the BALF cell counts. IL-5 is critical for terminal differentiation of Eos and for eosinophilic inflammation. Recent reports demonstrated that antibody to IL-5 (mepolizumab) had been shown to significantly reduce the frequency of asthma exacerbations in patients with refractory asthma[23]. Reduction of IL-5 in serum was observed by acupuncture

intervention, these in all demonstrated that acupuncture was an important therapy to alleviates inflammation in asthma.

HPA axis was reported to be associated with asthma pathogenesis[7, 8]. To explore the relationship between acupuncture and HPA axis activity, we detected the CRH gene expression in the hypothalamus and the ACTH and CORT level in plasma. The HPA axis is activated when neurons in the paraventricular nucleus of the hypothalamus secrete CRH, and CRH travels to the anterior pituitary gland to induce the secretion of ACTH. Next, the ACTH signal is carried through the peripheral circulation to the adrenal glands, which synthesize and release cortisol[10]. A growing number of studies have suggested an altered HPA axis function to stress in allergic children, and dysfunctional HPA axis in response to stress may facilitate immunological aberrations and increase the risk for allergic sensitization and exacerbation especially under stressful conditions[7]. The expression of CRH mRNA was increased by acupuncture. In particular, an inhibition of ACTH level in plasma was observed in OVA-induced asthma model, and acupuncture reversed such alteration. This finding is also supported by data from the literature that as time passes, chronic stressors become associated with reduced HPA axis output[24], which was in consistent with our results of the ACTH level in plasma. It has been proposed that in chronic inflammatory conditions, persistently increased levels of endogenous cortisol in response to proinflammatory cytokine release may lead to hypo-responsiveness of the HPA axis probably due to increased negative feedback mechanisms[25]. In the OVA induced mouse asthma model, the proinflammatory cytokines increased significantly, which resulted in the increased levels of endogenous cortisol, and the increased levels of endogenous cortisol led to hypo-secretion of ACTH in the asthma model group. In the acupuncture treatment group, acupuncture had regulatory effect both on inflammation and HPA axis function, which both resulted in the attenuation of inflammatory cytokines secretion. Acupuncture had inhibitory effect on the counts of immune cell such as lymphocyte and proinflammatory cytokine secretion, which may shift the hypo-responsiveness of the HPA axis to hyper-responsiveness, and

induce increase of CRH. Previous data revealed that changes in HPA activity could worsen asthma, which was shown that exposure to high doses of cortisol can bias the immune system towards an excessive Th2 cytokine response[10, 26]. Acupuncture treatment both relieved the Th2 cytokine IL-5 and other inflammatory cells and cytokines associated with Th2 immune process. Furthermore, the HPA axis function was also enhanced by acupuncture. In consideration of these results, acupuncture could be an important therapeutic method to treat asthma.

In summary, the present study showed that acupuncture played an important role in the attenuation of airway inflammation and regulation of HPA axis function in the OVA-induced murine asthma model, which may provide support to better understand the contribution of acupuncture to the regulation of airway inflammation and HPA axis activity in asthma.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Figures and legends

Table 1 Primer sequences used for qPCR amplification

Gene	Primer sequence
CRH (NM_ 205769.3)	Forward primer: 5'-ATGCGGCTGCGGCTGCTGGT-3' Reverse primer: 5'-GGCCGGCTTGCGCCGCGGCT-3'
GAPDH (NM_008084.2)	Forward primer: 5'-GTGGAGTCTACTGGTGTCTTC-3' Reverse primer: 5'-CTCAGTGTCCCACCACCCT-3'

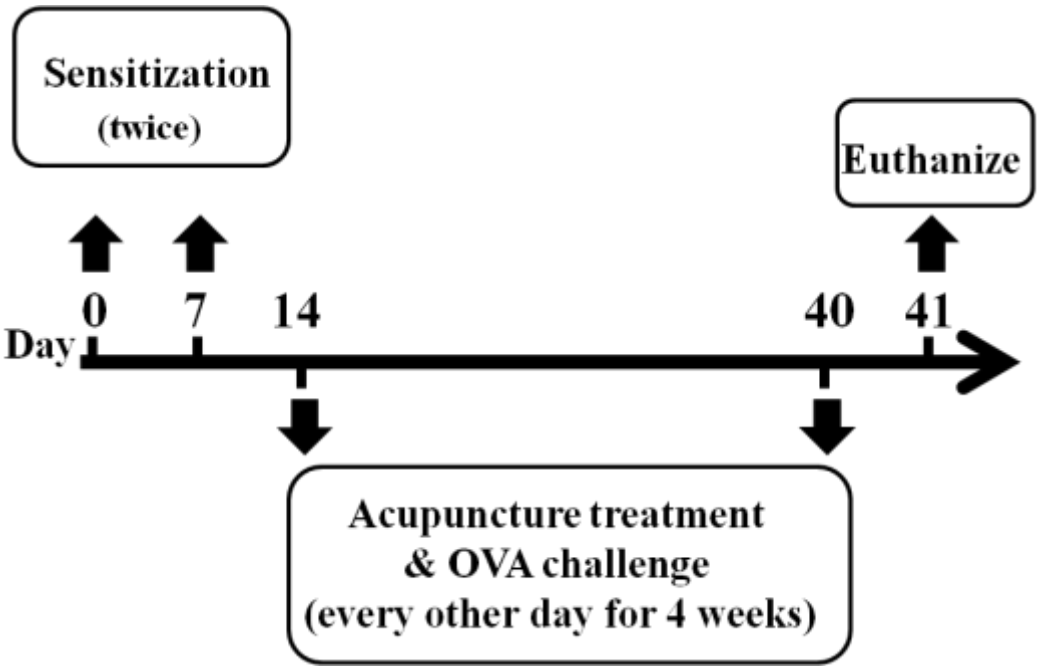

Fig.1. Protocol for murine asthma model establishment and acupuncture treatment

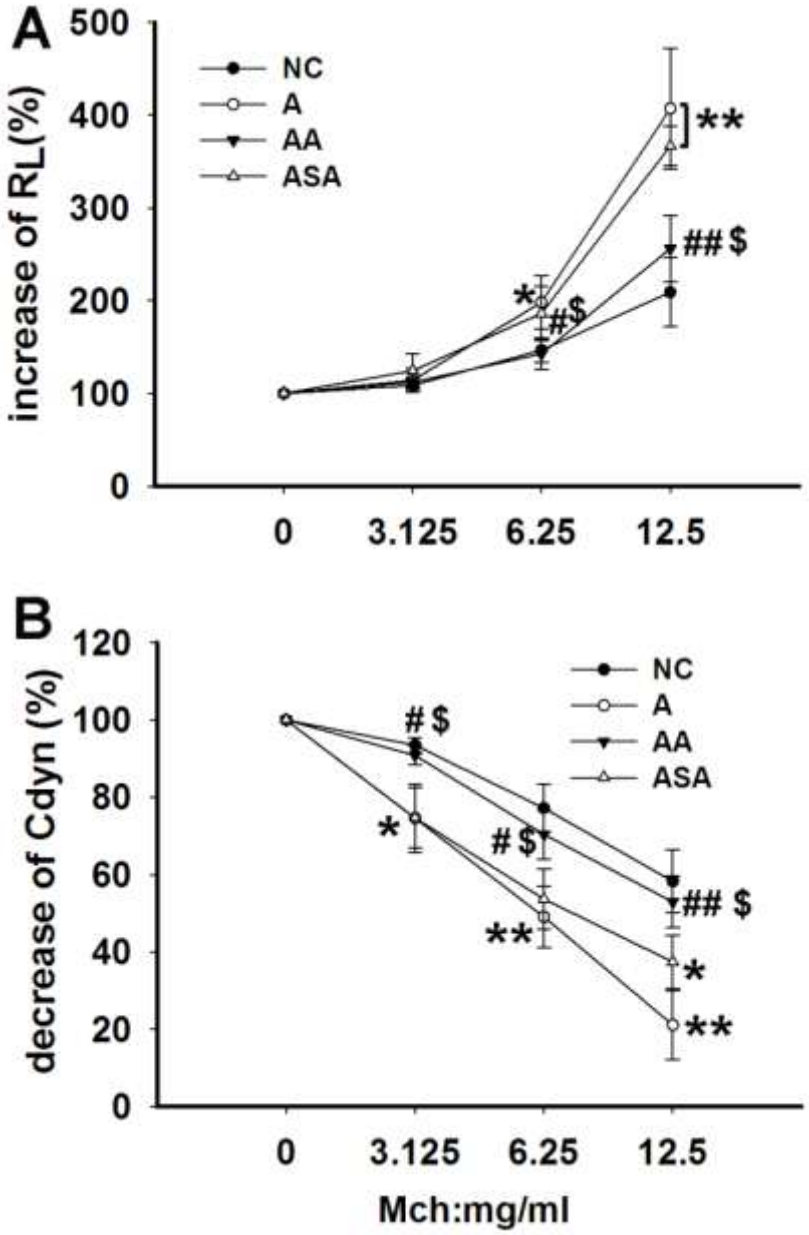

Fig.2. Acupuncture suppressed AHR in OVA-induced murine asthma model. Results were expressed as means±SD values (N=6 each group). * p< 0.05 vs NC group, ** p< 0.01 vs NC group, # p < 0.05 vs A group, ## p < 0.01 vs A group, $ p < 0.05 vs ASA group.

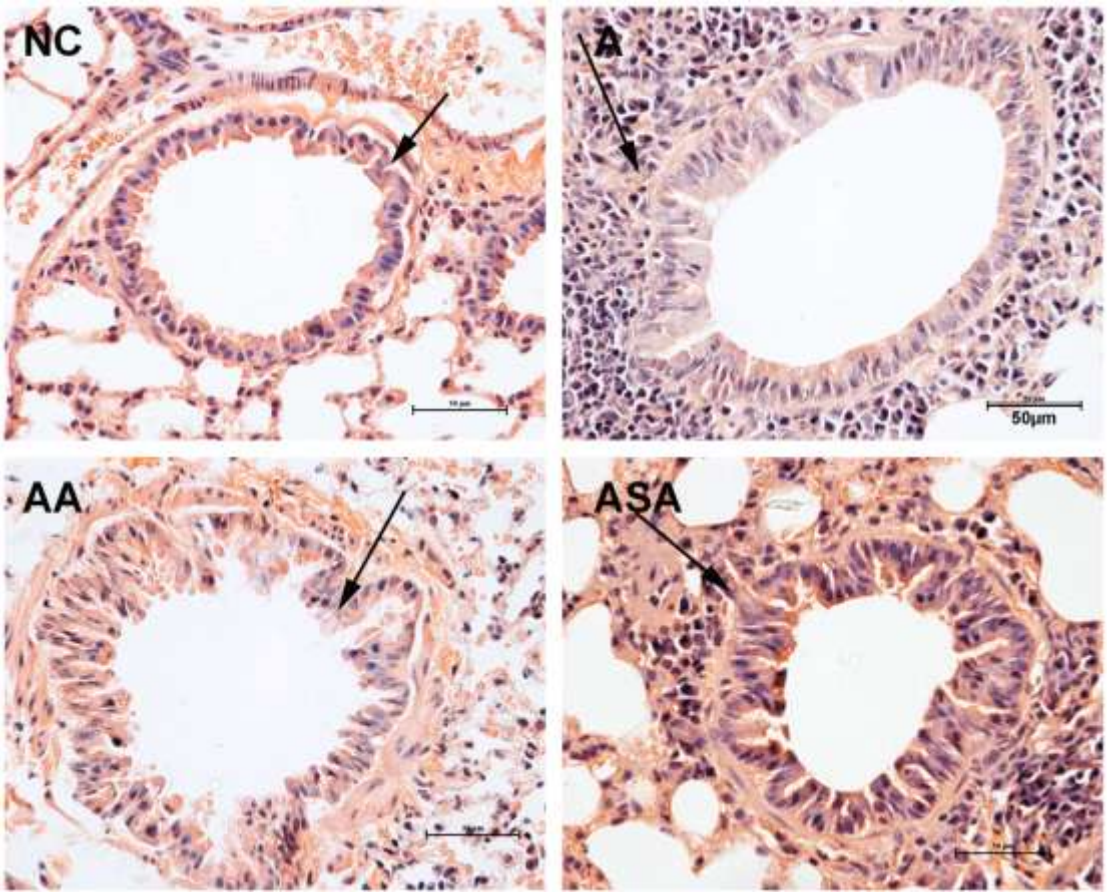

Fig.3. Acupuncture attenuated airway inflammation in OVA-induced murine asthma model (N=6 each group). Bar = 50μm. The arrows directed to the inflammatory changes around the airways.

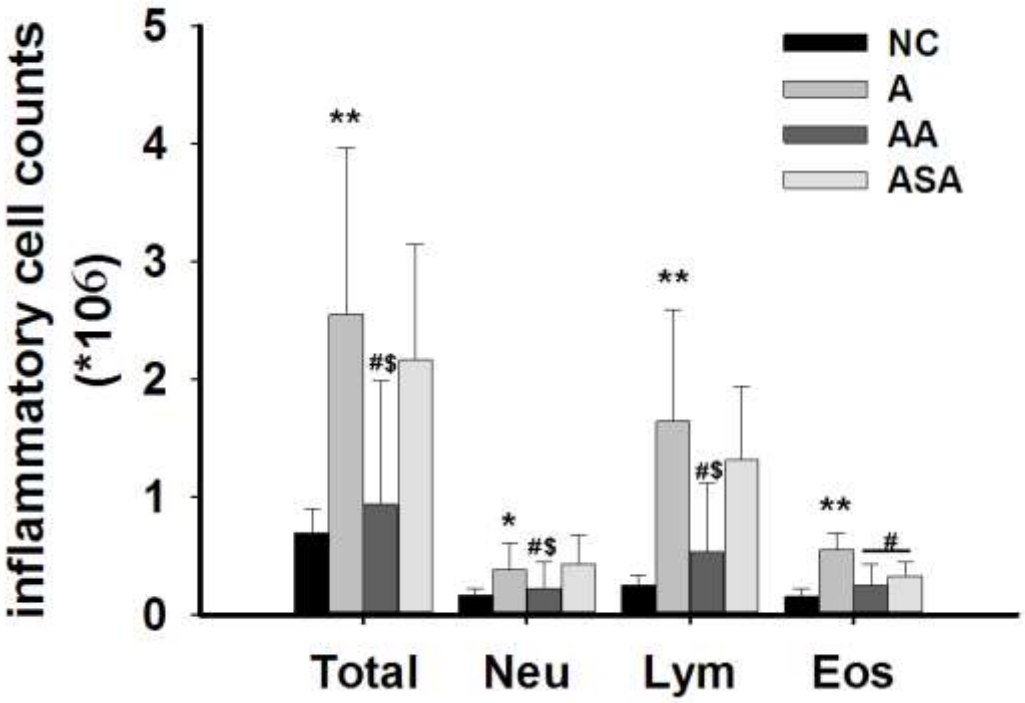

Fig.4. Acupuncture inhibited inflammatory cells in BALF of OVA-induced murine asthma model. Total leukocyte (Total), neutrophil (Neu), lymphocyte (Lym) and eosinophil (Eos) were counted. Results were expressed as means±SD values (N=10 each group). * $p < 0.05$ vs NC group, ** $p < 0.01$ vs NC group, # $p < 0.05$ vs A group, $ $p < 0.05$ vs ASA group.

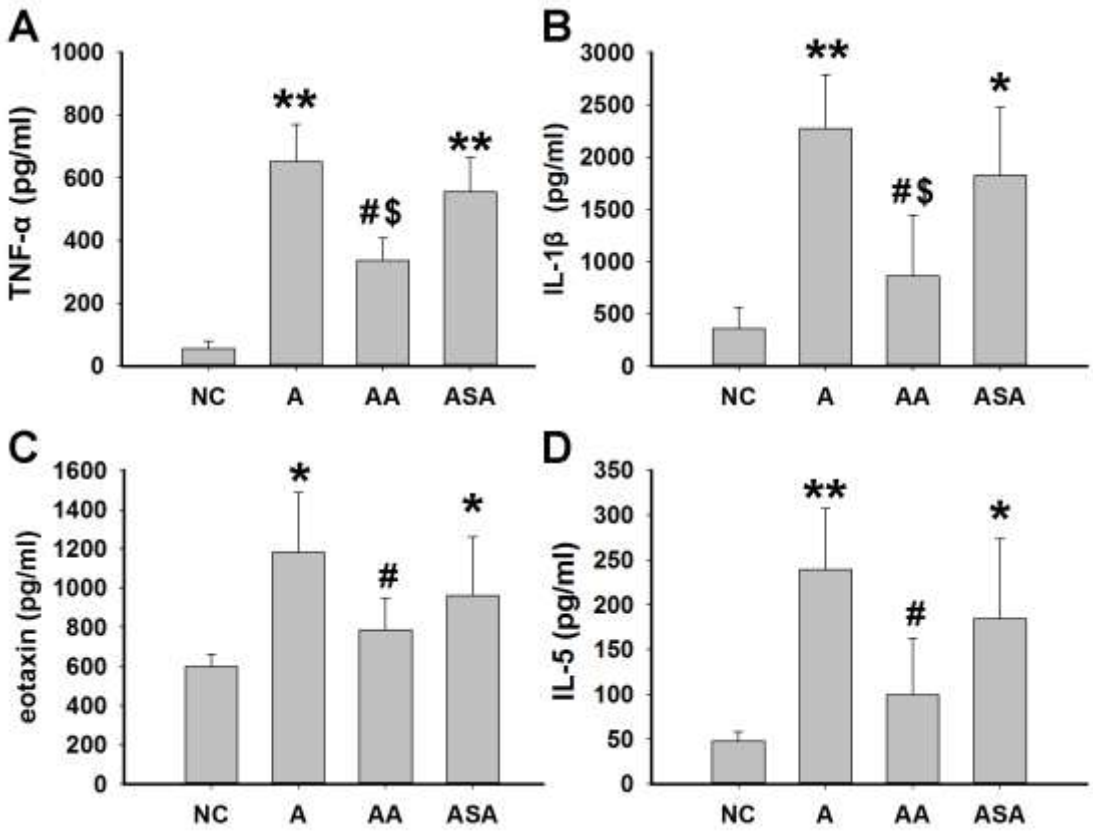

Fig.5. Acupuncture inhibited inflammatory cytokines in serum of OVA-induced murine asthma model. Results were expressed as means±SD values (N=10 each group). * $p< 0.05$ vs NC group, ** $p< 0.01$ vs NC group, # $p < 0.05$ vs A group, $ $p < 0.05$ vs ASA group.

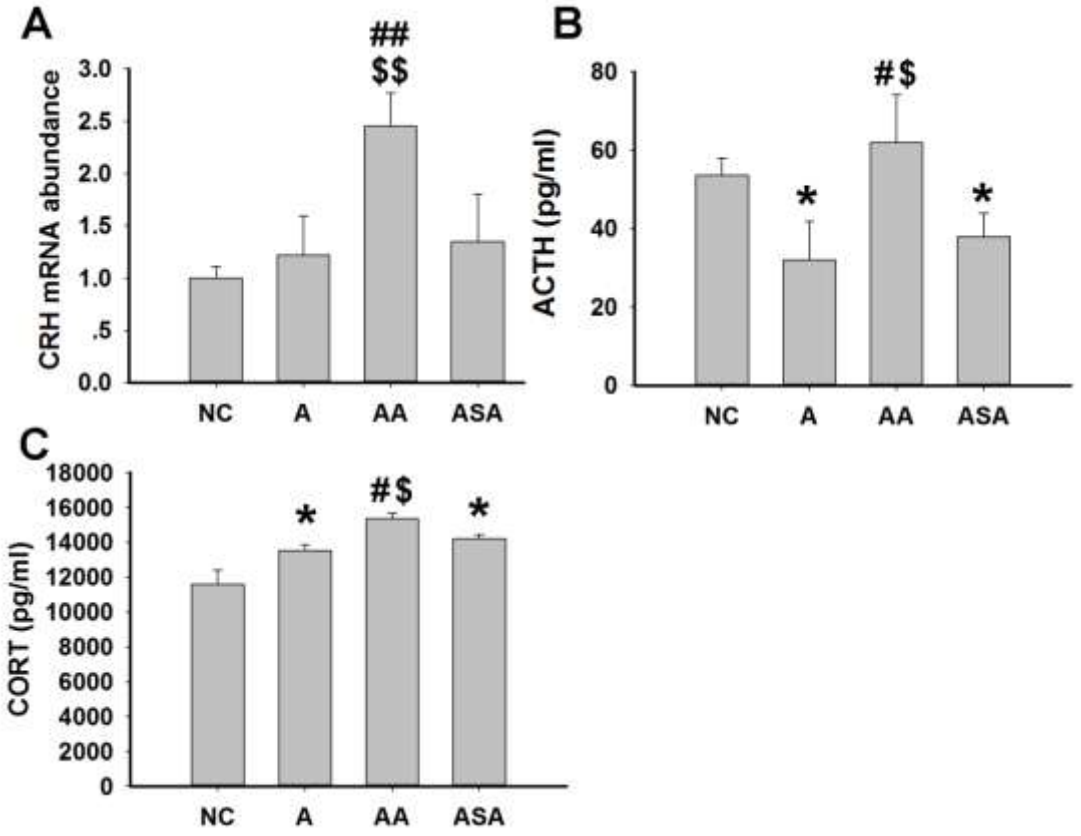

Fig.6. Acupuncture regulated the HPA axis function in OVA-induced murine asthma model. Results were expressed as means±SD values (N=10 each group). * $p < 0.05$ vs NC group, # $p < 0.05$ vs A group, ## $p < 0.01$ vs A group, $ $p < 0.05$ vs ASA group, $$ $p < 0.01$ vs ASA group.