Effect of dexamethasone injection into Zusanli (ST 36) acupoint on ovalbumin-induced allergic rhinitis

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Abstract

OBJECTIVE: To investigate the effects of acupuncture with dexamethasone (A. Dex) on allergic rhinitis (AR) by injecting dexamethasone into the Zusanli (ST 36) acupoint.

METHODS: Thirty 6-week-old female BALB/c mice were sensitized on days 1, 5, and 14 by intraperito-neal injection of 100 µg of ovalbumin (OVA). The mice were then randomly divided into six groups (n = 5 in each group). Five groups were sensitized intranasally with 2 µL of 1.5 mg of OVA for 10 consecutive days, while one group was sensitized intranasally with PBS in a similar manner as a negative control group. One hour before each administration of intranasal OVA, two groups were orally administered either a control vehicle (distilled water; AR control group) or 200 µg/kg Dex (O. Dex group), while three groups received A. Dex at Zusanli (ST 36) with Dex concentrations of 2, 20, and 200 µg/kg for each group, respectively. AR symptoms were evaluated by measuring the rubbing score, which comprised the number of nose, ear, and eye rubs that occurred in the initial 10 min after OVA intranasal provocation on the 10th day. We isolated spleen, serum, and nasal mucosal tissue after measuring the rubbing score. Spleen weight was measured using an electronic microbalance. The levels of IgE, thymic stromal lymphopoietin, tumor necrosis factor-α, intercellular adhesion molecule-1, and macrophage-inflammatory protein-2 in serum or nasal mucosal tissue were measured using enzyme-linked immunosorbent assays. The serum histamine levels of OVA-sensitized AR mice were measured using O-phthaldialdehyde spectrofluorometry. Western blot analysis was performed on nasal mucosal tissue extracts.

RESULTS: A. Dex significantly reduced the rubbing score, spleen weight, serum IgE, and serum histamine in OVA-sensitized mice. A. Dex significantly decreased the serum levels of inflammatory cytokines (thymic stromal lymphopoietin and tumor necrosis factor-α) in OVA-sensitized mice. A. Dex sig-
niﬁcantly reduced the nasal mucosal levels of inﬂammatory markers (intercellular adhesion molecule-1 and macrophage-inﬂammatory protein-2) in AR mice. A. Dex effectively attenuated the expression of caspase-1 and receptor interacting protein-2 in nasal mucosal tissue.

CONCLUSION: A. Dex may be a new and useful therapy for AR.

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Keywords: Dexamethasone; Point ST36 (Zusanli); Pharmaceutical research; Acupuncture; Rhinitis, allergic; Ovalbumin; Thymic stromal lymphopoietin

INTRODUCTION
Allergic rhinitis (AR) is a common inﬂammatory disease that affects the nasal mucosa.1 AR includes symptoms such as nasal congestion, nasal itching, sneezing, and rhinorrhea, which can reduce quality of life and involve considerable costs to society.2 These clinical manifestations result from the release of histamine and other active substances from mast cells, which stimulate blood vessel dilatation, irritation of nerve endings, and tear secretion.3 AR involves inﬂammatory IgE-mediated responses, activation of mast cells and eosinophils, and release of inﬂammatory mediators, such as thymic stromal lymphopoietin (TSLP) and tumor necrosis factor-α (TNF-α).4 Inﬂammatory cell inﬁltration is induced by the expression of intercellular adhesion molecule-1 (ICAM-1) in mucosa during persistent inﬂammation.5 Macrophage-inﬂammatory protein-2 (MIP-2) is a potent chemoattractant for immune cells.6 ICAM-1 and MIP-2 are considered to be critical markers of allergic inﬂammation.7

Traditional Chinese Medicine (TCM) therapy is a popular treatment for patients with allergic diseases. Acupuncture is a therapeutic TCM modality in which ﬁne needles are used to pierce speciﬁc acupoints, and this method has improved clinical results in patients with AR.8 Pharmaceutical acupuncture or herbal acupuncture (a combination of acupuncture and herbal therapies) is a new acupuncture therapy in TCM.9 Pharmaceutical acupuncture is reportedly much more effective than acupuncture alone, and its effect varies with the herb used.10

One of the most commonly used acupoints is Zusanli (ST 36).11 Zusanli (ST 36) is located on the stomach meridian, and is the main point for regulation of gastrointestinal function, promotion of detoxiﬁcation, and protection of mucosal barriers.12 Moreover, the anti-inﬂammatory and anti-allergic properties of Zusanli (ST 36) intervention have been recently reported.13

Various medical treatments are available for AR, including oral decongestants, anti-histamine drugs, mast cell stabilizers, and corticosteroids.14 Corticosteroids are the most effective AR treatment.15 Dexamethasone (Dex) is a synthetic glucocorticosteroid that has anti-inﬂammatory and immunosuppressant properties.16 As Dex has glucocorticoid effect that is 25 times more potent than cortisol, Dex has been widely used in treating AR.17 We hypothesized that using a relatively novel acupuncture method of injecting small amounts of medicine into Zusanli (ST 36) could beneﬁt patients with AR, especially those who experience side effects from oral Dex or cannot take oral Dex.

In the present study, Dex was injected into the Zusanli (ST 36) acupoint of mice with ovalbumin (OVA)-induced AR to investigate the effects of acupuncture with Dex (A. Dex) on AR.

MATERIALS AND METHODS
Materials
Dex, OVA, aluminum hydroxide, avidin peroxidase, bicinchoninic acid, and O-phthalaldehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-mouse IgE/TNF-α antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Anti-mouse TSLP/ICAM-1/MIP-2 antibodies were purchased from R & D Systems (Minneapolis, MN, USA). Antibodies for caspase-1, receptor interacting protein 2 (RIP-2), and actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

The ovalbumin-induced allergic rhinitis animal model
All procedures involving experimental animals were approved by the animal care committee of Kyung Hee University [approval No. KHUASP (SE)-15-118]. We maintained 6-week-old female BALB/c mice (Charles River Laboratories, Inc., Wilmington, MA, USA) under pathogen-free conditions. After 1 week of acclimatization, the 30 mice were randomly assigned to six groups (n = 5 in each group). The mice were sensitized on days 1, 5, and 14 by intraperitoneal injection of 100 μg of OVA emulsified with 20 mg of aluminum hydroxide in 100 μl of phosphate-buffered saline (PBS). Five groups were then sensitized intranasally with 2 μl of 1.5 mg of OVA once daily for 10 consecutively,6 while one group was sensitized intranasally with PBS in a similar manner as a negative control group (normal mice). One hour before each administration of intranasal OVA, two groups were orally administered either a control vehicle (distilled water; AR control group) or 200 μg/kg Dex (O. Dex group), while three groups received A. Dex (2, 20, and 200 μg/kg) at Zusanli (ST 36). The location of Zusanli (ST 36) was deﬁned as 2 mm lateral to the anterior tubercle of the tibia and 3 mm below the knee joint.20 AR symptoms were evaluated by measuring the rubbing score, which
comprised the number of nose, ear, and eye rubs that occurred in the initial 10 min after intranasal provocation on the 10th day. The total numbers of rubs were assessed on video recordings.

**Spleen weight**
Spleen weight was analyzed using an electronic microbalance, as spleen enlargement usually indicates the presence of an immune response.  

**Enzyme-linked immunosorbent assay**
Pro-inflammatory cytokines are critical factors in the pathogenesis of AR. Thus, we investigated the regulatory effect of A. Dex on the serum inflammatory cytokines levels of AR mice. ICAM-1 and MIP-2 are chemotactic molecules for inflammatory cells. Hence, we examined the regulatory effect of A. Dex on the levels of ICAM-1 and MIP-2 in the nasal mucosal tissues of AR mice. The IgE, TSLP, TNF-α, ICAM-1, and MIP-2 levels in serum or nasal mucosal tissue were measured using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (BD Biosciences Pharmingen; R & D Systems). The absorbance was measured using an ELISA reader at 405 nm. The cytokine levels in nasal mucosa were presented as the value divided by the total protein concentration. The protein concentrations were estimated using a bicinchoninic acid protein assay.

**Histamine assay**
The serum histamine levels of OVA-sensitized AR mice were measured by the O-phthalaldehyde spectrofluorometric method. A spectrofluorometer was used to analyze the fluorescent intensities at 440 nm (excitation at 360 nm).

**Western blot analysis**
Caspase-1 plays a very important role in inflammatory responses, while RIP-2 interacts with caspase-1 and is involved in immune response signaling. Thus, the caspase-1 and RIP-2 levels in the nasal mucosal tissue of AR mice were analyzed using Western blot analysis. The nasal mucosal tissue extracts were heated at 95 °C for 5 min, and then cooled on ice. After centrifugation, 50 μg aliquots were resolved by 12% sodium dodecyl sulphate-polyacrylamide gelelectrophoresis. The resolved proteins were electrophoretically transferred to nitrocellulose membrane that had been presoaked with transfer buffer (25 Mm Tris, pH 8.5, 200 mM glycerin, and 20% methanol). The blots were blocked in blocking buffer (PBS containing 0.05% Tween 20 and 5% nonfat dry milk) for at least 2 h, and then incubated with primary caspase-1 and RIP-2 antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibodies, and detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA).

**Statistical analysis**
Statistical analyses were performed by one-way analysis of variance via Tukey’s multiple range tests using SPSS statistical software (SPSS 11.5; SPSS Inc., Chicago, IL, USA). Experimental values represent the mean ± standard error of mean (SEM).  

**RESULTS**

**Rubbing score**
Dex did not affect the rubbing score on the 3rd day after the OVA intranasal provocation (Figure 1A). However, the rubbing score in OVA-sensitized mice was significantly higher than that in PBS-sensitized mice on the 10th day of intranasal provocation (Figure 1A; P = 0.000). This elevation of the rubbing score was significantly inhibited by A. Dex (P = 0.000) or O. Dex (P = 0.025; Figure 1A). The mean rubbing score of A. Dex-treated AR mice was significantly lower than that of O. Dex-treated AR mice (Figure 1A; P = 0.008).
**Spleen weight**

The mean spleen weights after OVA sensitization in the five OVA-sensitized groups were significantly higher than that in the PBS-sensitized mice (Figure 1B; *P = 0.000*). This increase in spleen weight was inhibited by treatment with A. Dex (20 μg/kg, *P = 0.003*; 200 μg/kg, *P = 0.000*; Figure 1B). The mean spleen weight of O. Dex-treated AR mice was significantly lower than that of A. Dex-treated AR mice (Figure 1B; *P = 0.000*).

**Serum biomarkers**

As shown in Figure 2A, the serum IgE levels were significantly higher in AR mice than in PBS-sensitized mice (*P = 0.000*). The increased serum IgE levels in AR mice were significantly reduced by A. Dex (*P = 0.000*) or O. Dex (*P = 0.000*). The serum histamine levels were also significantly decreased by A. Dex (20 μg/kg, *P = 0.006*; 200 μg/kg, *P = 0.000*) and O. Dex (Figure 2B; *P = 0.000*). A. Dex (*P = 0.000*) and O. Dex (*P = 0.000*) significantly suppressed the serum levels of TNF-α in AR mice (Figure 2D). The serum levels of TNF-α of O. Dex-treated AR mice was significantly lower than those of A. Dex-treated AR mice (Figure 2D; *P = 0.042*).

**Inflammatory cytokines and chemokines levels in nasal mucosal tissue**

The protein levels of TSLP (*P = 0.000*) and TNF-α (*P = 0.049*) in the nasal mucosal tissues of AR mice were significantly increased compared with those of the normal mice (Figure 3A, 3B). The levels of TSLP that were upregulated by OVA sensitization were significantly downregulated by A. Dex (20 μg/kg, *P = 0.000*; 200 μg/kg, *P = 0.000*; Figure 3A). The levels of TNF-α that were upregulated by OVA sensitization were significantly downregulated by A. Dex (200 μg/kg, *P = 0.010*; Figure 3B). The ICAM-1 levels that were increased by OVA sensitization were significantly downregulated by A. Dex (*P = 0.000*; Figure 3D). O. Dex also significantly decreased the levels of TSLP (*P = 0.000*), TNF-α (*P = 0.012*), ICAM-1 (*P = 0.000*), and MIP-2 (*P = 0.000*) in the nasal mucosal tissue (Figure 3). The serum levels of ICAM-1 of O. Dex-treated AR mice was significantly lower than those of A. Dex-treated AR mice (*P = 0.006*; Figure 3D).

**Caspase-1/RIP-2 expression in nasal mucosal tissue**

As shown in Figure 4, A. Dex inhibited the OVA-induced expression of caspase-1 and RIP-2 in the nasal mucosal tissue. O. Dex also suppressed the expression of caspase-1 and RIP-2 in the nasal mucosal tissue. O. Dex had a greater regulatory effect on caspase-1 expression than A. Dex (Figure 4).
DISCUSSION

The present study showed that A. Dex suppressed the levels of IgE, histamine, inflammatory cytokines, caspase-1, and RIP-2 in the serum or nasal mucosal tissue of mice with OVA-induced AR. AR is an upper airway disease resulting from IgE-mediated inflammatory reactions to allergen exposure. \(^1\) Allergen-IgE-dependent activation of mast cells causes the production of active mediators, such as histamine and cytokines, which induce rhinorrhea, sneezing, and itching in the early phase of the inflammatory response. \(^2\) Epithelial accumulations of mast cells, eosinophils, and basophils occur in the late phase of AR. \(^3\) The recruitment of inflammatory cells results in the further release of histamine, as well as releases of pro-inflammatory cytokines and chemokines, which sustain the allergic response and promote the late phase response. \(^4\) Pro-inflammatory cytokines released from mast cells, such as TNF-\(\alpha\), participate in allergic inflammation; \(^6\) TSLP and TNF-\(\alpha\)-induce inflammatory reactions in the nasal mucosal tissue in AR. \(^5\) ICAM-1 and MIP-2 have been proposed as chemotactic factors and markers of allergic inflammation, as the expressions of ICAM-1 and MIP-2 are increased in the nasal mucosal epithelium of patients with AR. \(^7\) The symptoms of AR can be relieved using medications. Oh \textit{et al.} \(^8\) reported that rosmarinic acid has a beneficial effect on AR, as it inhibits the levels of IgE, histamine, and inflammatory cytokines, and reduces the number of nasal, ear, and eye rubs in OVA-sensitized mice. The anti-inflammatory effects of glucocorticosteroids in AR are reportedly mediated by inflammatory cells, such as mast cells. \(^9\) Smith \textit{et al.} \(^10\) reported...
that glucocorticosteroids down regulate a function of mast cells and reduce the production of inflammatory cytokines and recruitment of inflammatory cells. Corticosteroids also directly affect the epithelial synthesis of ICAM-1, resulting in a reduced number of eosinophils or neutrophils in the nasal mucosal tissue in AR. MIP-2 levels in tissue injury of AR were suppressed by glucocorticosteroid administration. In the present study, A. Dex inhibited the levels of IgE, histamine, TSLP, TNF-α, ICAM-1, and MIP-2 in AR mice. Therefore, we suggest that A. Dex has an anti-allergic inflammatory effect, and we speculate that these effects of A. Dex in AR may be induced through a mechanism similar to that of glucocorticosteroid administration. Dex has direct inhibitory effects on human mast cell maturation and on mature mast cell function. Consequently, we can speculate that the inhibition of mast cell development by Dex would lead to fewer mast cells in tissues and this could lead to a reduced allergic inflammatory reaction. In addition, the capacity of glucocorticosteroids to inhibit cytokine production in mature mast cells suggests that they might reduce the recruitment, activation and survival of cells involved in the late phase of allergic reactions or in airway remodelling, such as eosinophils. Caspase-1 mediates inflammatory responses. Caspase-1 levels are significantly increased in patients with allergic asthma compared with normal individuals, and caspase-1-deficient mice show decreased TNF-α levels during inflammatory reactions. TSLP is expressed and produced through caspase-1 activation in mast cells, while RIP-2 promotes caspase-1 activation and induces cytokine stimulation. Furthermore, RIP-2 is reported to be associated with the development and severity of allergic airway inflammation in a mouse model. In the present study, A. Dex inhibited caspase-1/RIP-2 expression in AR mice. Thus, we suggest that the inhibitory effect of A. Dex on inflammatory cytokine production might be derived from the blockade of the caspase-1/RIP-2 pathway. Acupoint treatment reportedly ameliorates the allergic reaction in mice. Furthermore, acupuncture modulates immune responses in the upper airways to improve clinical results in patients with AR, and significantly reduces AR symptoms in patients with AR. In particular, stimulation of Zusanli (ST 36) decreases inflammation and edema in an inflammation-induced rat model, and acupuncture at Zusanli (ST 36) suppresses inflammation in OVA-induced allergic reaction. In the present study, stimulation of Zusanli (ST 36) with Dex reduced the allergic inflammatory reaction in AR mice. A. Dex had an inhibitory effect similar to that of O. Dex on AR. Therefore, we suggest that A. Dex can be used as a new drug delivery system. However, further study is needed to compare the effect of stimulation at Zusanli (ST 36) with the effect of pharmaceutical acupuncture with Dex in AR. In conclusion, our findings indicate that A. Dex has an anti-allergic inflammatory effect in OVA-induced AR mice by ameliorating the number of nasal itching motions and inhibiting the release of allergic mediators. Furthermore, A. Dex suppressed the expression of caspase-1 and RIP-2. The present results suggest that A. Dex might be useful as a new drug administration method for the treatment of allergic inflammatory diseases, such as AR. However, further studies are needed to identify the distinct mechanisms of A. Dex prior to clinical use in humans.

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