

Bufotalin ameliorates ovalbumin-induced allergic rhinitis by restoring the Tregs

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ABSTRACT

Background: Allergic rhinitis (AR) is one of the most common inflammatory diseases. IgE, inflammatory cytokine production and Th17/Tregs imbalance have been implicated in AR pathogenesis. Bufotalin, a component extracted from toad venom skin secretions and auricular glands, has anti-inflammatory activity and regulates Th17/Tregs balance. Here, the effects of bufotalin on AR were explored.

Methods: The AR mice model was established using ovalbumin (OVA). AR mice were treated with bufotalin started on Day 22 with various doses (1, 10, 100 µg or 1 mg per mouse) every day to Day 30. The sneezing and rubbing frequencies were counted. Serum levels of IL-1β, IL-10 and OVA-specific IgE were measured. The superficial cervical lymph nodes were harvested and the percentage of Tregs in lymph node was determined using CD4 and Foxp3 markers.

Results: OVA treatment successfully induced AR model in mice with significantly increased sneezing and rubbing frequency, elevated levels of serum histamine, IL-1β, IL-10 and OVA-specific IgE. Bufotalin treatment significantly ameliorated AR symptoms, with reduced histamine, IgE and IL-1β levels, as well as sneezing and rubbing frequency. Moreover, bufotalin treatment decreased the serum levels of IL-1β, IL-10 and OVA-specific IgE in AR mice.

Conclusion: Bufotalin ameliorated allergic rhinitis symptoms in AR mice by restoring Tregs in lymph node.

1. Introduction

Allergic rhinitis (AR), a nasal inflammation, is a common inflammatory disease [1]. The AR symptoms include running nose, sneezing, and itchy eyes. In the past decades, the incidence of AR increases steadily, which seriously affects life quality of patients all over the world.

AR is induced by various factors. However, the pathophysiology of AR is not entirely known. IgE is the key factor involved in pathophysiology of AR. AR patients who have severity symptoms have higher level of circulating allergen-specific IgE. Studies have found that IgE binds to allergen and further binds to Fc receptor on mast cells and basophils, which causes the degranulation of mast cells and basophils, together with histamine releasing. Treatments targeting IgE have been shown to attenuate AR symptoms [2]. As histamine is the direct factor cause of sneezing symptom, antihistamines which directly reduce histamine level alleviate AR symptoms and improve the life quality of AR patients [3]. Another factor which contributes to AR pathophysiology is the

infiltration of immune cells including T cells, basophils, and eosinophils in nasal tissues. These cells produce cytokines and chemokines and promote the inflammation [4]. The regulatory T cells (Tregs) is a subset of CD4⁺ cells which suppress immune response and maintain homeostasis. These cells suppress IgE and inflammatory cytokine production [5]. Adoptive transfer of Tregs attenuates allergic inflammation in animals, suggesting the protective roles of Tregs against allergic diseases [6]. Additionally, Th17 cells which are IL-17-producing cells and express transcription factor RORγt also play a role in AR induction [7]. Neutralization of IL-17 could markedly reduce AR symptoms, decreases eosinophil and neutrophil infiltrations, reduces Th2 and Th17 responses, and increases the Treg response in AR mice model [8]. Taken together, inhibition of inflammation which can target these factors could be a strategy to treat AR.

Bufotalin is a cardiotoxic bufanolide steroid and cardiac glycoside analogue extracted from skin secretions of toad venom. Bufotalin has several pharmacological activities including anti-tumor and anti-inflammation [9]. Bufotalin also suppresses Th17 population and

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inhibits the production of inflammatory cytokines in experimental Sjögren's syndrome [10]. Thus, we hypothesize that bufanolide could be beneficial to treat AR. Until now, the effects of bufotalin on AR have not been described yet. In present study, the protective effects of bufotalin on AR mice model were assessed.

2. Materials and method

2.1. Mice treatment

Female Balb/c mice with age of 6–8 weeks were obtained from CharlesRiver (Shanghai, China) and maintained in environment with controlled temperature and light. All mice had unrestricted access to food and water. AR model was induced according to the published protocol with necessary modifications [11]. As shown in Fig. 1A, to induce AR, mice were intraperitoneally injected with 75 µg OVA and 2 mg aluminum hydroxide in 200 µL phosphate-buffered saline (PBS) at Day 0, 7, 14 and 21. Started from Day 22, mice were sensitized with 500 µg OVA in 20 µL PBS by intranasal instillation every day to Day 30. Control group was sensitized with PBS instead. For bufotalin treatment, on Day 22, mice were treated with OVA together different amount of bufotalin (1, 10, 100 µg or 1 mg for each mouse, in 20 µL PBS) (purity >99.83%, MedChemExpress) every day to Day 30 by intranasal instillation. There were 8 mice in each group. The study protocol was approved by the institutional animal care and use committee of Sun-shine Union Hospital in Weifang (#wf-2020-008). The study was conducted in accordance with the NIH guidelines for the care and use of laboratory animals (8th edition, NIH). The mice have been euthanized by CO₂ inhalation after the experiments.

2.2. Nasal symptoms measurement

The numbers of sneezing and nose rubbing motions in 15 min were counted. In the control mice, sneezing is around 1 time per minutes while nose rubbing was around 5 times per 15 min. The OVA treatment would generate allergic behavior, the sneezing and nasal rubbing could reach more than 5 times and 20 times per 15 min, which indicates the success of the model generation.

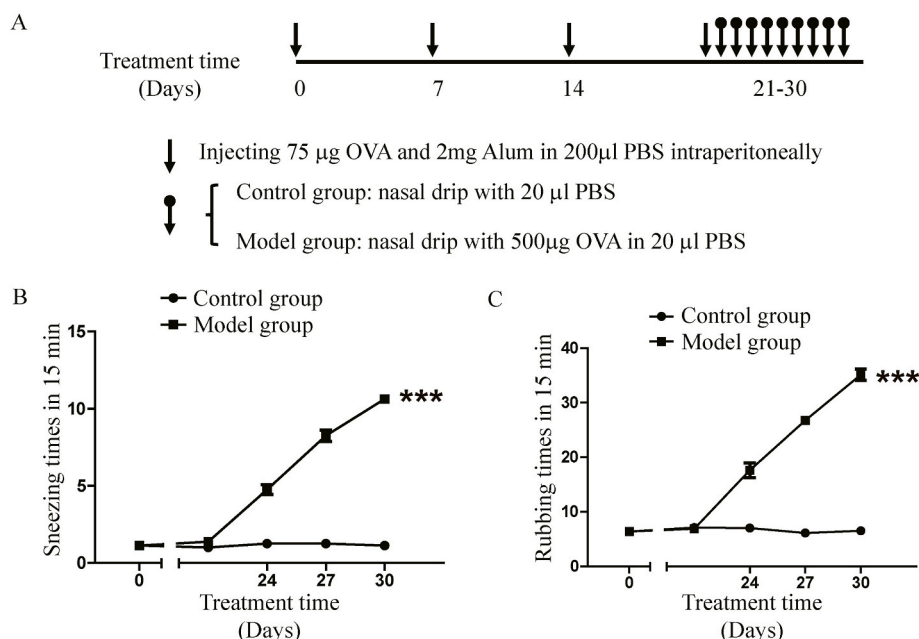


Fig. 1. Ovalbumin (OVA) and aluminum hydroxide could induce allergic rhinitis in mice model. (A) BALB/c mice (n = 8 for each group) were intraperitoneally injected with 75 µg OVA and 2 mg aluminum hydroxide at day 0, 7, 14, and 21. From day 22, the model group was sensitized with 500 µg OVA by intranasal instillation every day. Control group was treated with phosphate buffered saline instead. (B) Sneezing times in 15 min was significantly increased in model group. (C) Rubbing times in 15 min was significantly increased in model group. ***p < 0.001.

2.3. Measurement of histamine, IL-1β, IL-10 and IgE

Serum was collected through the eye socket with capillary tubes at different time points. Serum levels of histamine, IL-1β, IL-10 and OVA-specific IgE were measured using commercial ELISA kits (Abcam, China) following manufacturer's protocols.

2.4. Measurement of Treg cells with flow cytometry

The superficial cervical lymph nodes were harvested and the single cell suspension was prepared as describe previously [12]. Cells were stained with anti-CD4 and anti-Foxp3 using anti-Mouse/Rat Foxp3 Staining Set PE and anti-CD4-FITC (Thermo Fisher, USA) following manufacturer's protocols. Cells were subjected to BD FACSCalibur flow cytometer and the data were analyzed by FlowJo software.

2.5. Statistical analysis

Data were presented as mean ± standard deviation (SD). One-way ANOVA analysis with a Tukey's post hoc test, or two-way ANOVA with a Bonferroni post hoc test was used to calculate the p values. The statistical difference was significant when p < 0.05.

3. RESULTS

3.1. Establishment of OVA-induced AR mice model

First, we induced AR in mice using OVA and aluminum hydroxide to mimic AR symptoms in an animal model (Fig. 1A). We monitored the AR symptoms including sneezing and rubbing frequencies. As shown in Fig. 1B, after Day 22 (the first sensitization day), the sneezing times in 15 min of OVA sensitized mice were obviously increased when compared to PBS sensitized control mice. Similarly, the rubbing times in 15 min were remarkably increased in OVA sensitized mice (Fig. 1C). Therefore, we successfully established the OVA-induced AR mice model.

3.2. Measurement of AR related mediators

We then measured the serum level of AR related factors including histamine, IL-1β, IL-10 and OVA specific IgE (Fig. 2). We identified

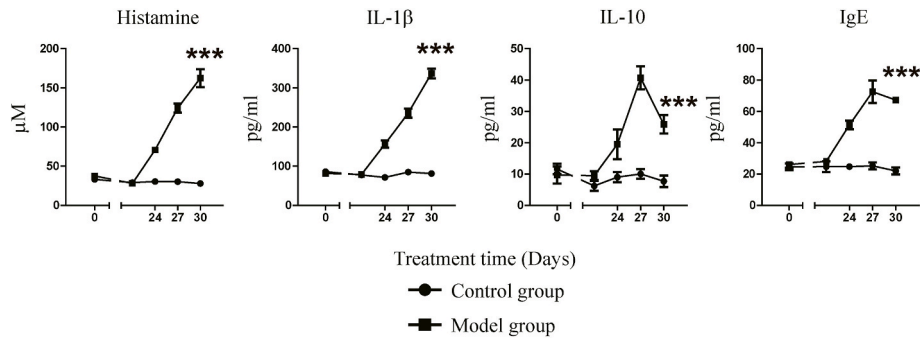


Fig. 2. The inflammatory factors were significantly induced in allergic rhinitis model mice.

The protein level of histamine, IL-1β, IL-10 and IgE were measured in the serum from the sacrificed mice by ELISA (n = 8 for each group). All these inflammatory factors were significantly upregulated in allergic rhinitis model mice. ***p < 0.001.

significantly increased serum histamine in mice sensitized with OVA on Day 24, 27 and 30, indicating elevated histamine was associated with AR. We also detected that OVA sensitized mice had significantly higher IL-1β, IL-10 and OVA-specific IgE than control mice.

3.3. Bufotalin ameliorated AR symptoms in mice

To evaluate the effects of bufotalin on AR, we administrated different amounts of bufotalin (1 μg, 10 μg, 100 μg and 1 mg) to mice at the time of OVA sensitization (Fig. 3A). No obvious side-effects were observed in the Bufotalin-treated mice, including the weight loss and abnormal behaviors (data not shown). We continued to monitor the AR symptoms in mice. As shown in Fig. 3B, AR mice had significantly increased sneezing times. AR mice treated with 1 μg and 10 μg bufotalin had similar sneezing times to AR mice. In contrast, AR mice treated with 100 μg and 1 mg bufotalin had obviously decreased sneezing times when compared to AR mice. AR mice treated with 1 mg bufotalin had less sneezing times than AR mice treated with 100 μg bufotalin. However, the AR mice treated with 100 μg and 1 mg bufotalin had more sneezing times when compared to control mice. These results indicated that 100 μg and 1 mg bufotalin treatment suppressed, but did not completely prevent, sneezing in AR mice. We also found that 100 μg and 1 mg, but not 1 μg and 10 μg bufotalin treatment decreased the rubbing times in AR mice (Fig. 3C). Taken together, these results demonstrated that bufotalin ameliorated AR symptoms in mice.

3.4. Bufotalin suppressed inflammation in AR mice

Next, we evaluated the effects of bufotalin on AR-related mediators in AR mice. As shown in Fig. 4, AR mice had significantly increased serum level of histamine. AR mice treated with 1 μg and 10 μg bufotalin had similar serum level of histamine to AR mice. In contrast, the serum level of histamine in AR mice treated with 100 μg and 1 mg bufotalin was lower than that in AR mice on Day 24, 27 and 30. Similarly, we found that 100 μg and 1 mg bufotalin treatments decreased the serum level of IL-1β, IL-10 and OVA-specific IgE in AR mice.

3.5. Bufotalin promoted Treg cell percentage in AR mice

Since Treg cells play important role in allergic diseases including AR [13], we explored the effects of bufotalin on Treg cells in AR mice. Compared to control mice, AR mice had decreased percentage of Treg cells in cervical lymph nodes (Fig. 5). AR mice treated with 1 μg and 10 μg bufotalin had decreased or similar percentage of Treg cells to AR mice. In contrast, AR mice treated with 100 μg and 1 mg bufotalin had remarkably increased Treg cells in cervical lymph nodes. Collectively, these results demonstrated that 100 μg and 1 mg bufotalin treatments enhanced Treg cell percentage in AR mice.

4. Discussion

AR is one of the most common inflammatory diseases over the world. Searching for new effective and efficient medicine targeting AR is still

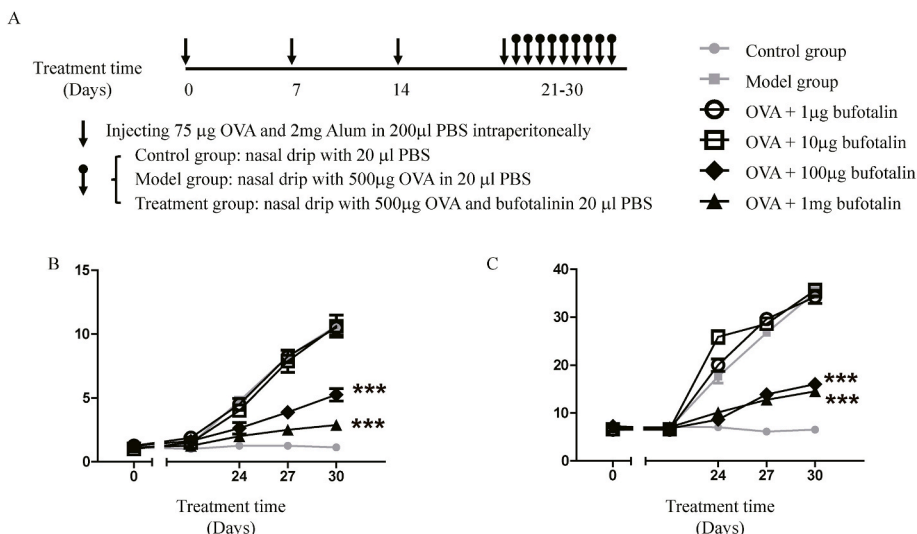


Fig. 3. Bufotalin improved OVA induced allergic rhinitis in mice model. (A) BALB/c mice (n = 8 for each group) were intraperitoneally injected with 75 μg OVA and 2 mg aluminum hydroxide at day 0, 7, 14, and 21. From day 22, the model group was sensitized with 500 μg OVA by intranasal instillation every day. Control group was treated with phosphate buffered saline instead. The treatment group was treated with 500 μg OVA and different dose of bufotalin by intranasal instillation every day. (B) Sneezing times in 15 min was improved in treatment group. (C) Rubbing times in 15 min was improved in treatment group. ***p < 0.001 versus model group.

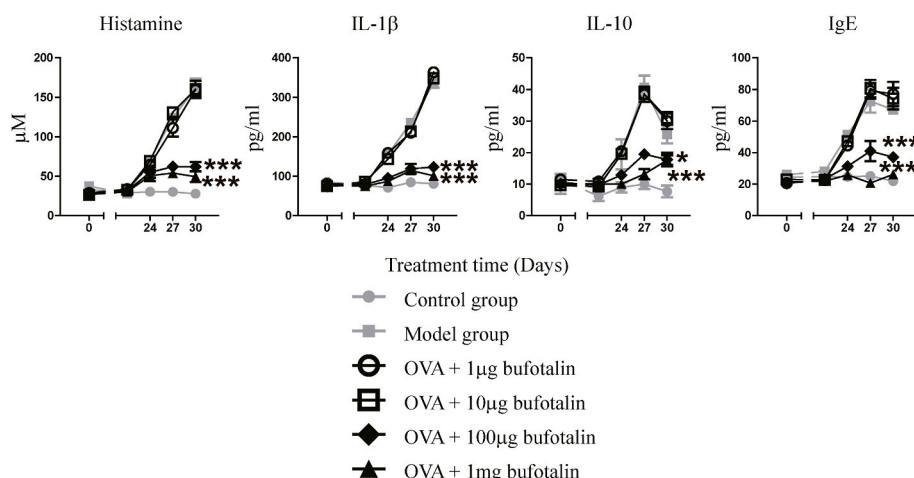


Fig. 4. Bufotalin decreased inflammatory factors induced in allergic rhinitis model mice. The protein level of histamine, IL-1 β , IL-10 and IgE were measured in the serum from the sacrificed mice by ELISA ($n = 8$ for each group). All these inflammatory factors were significantly improved by bufotalin in allergic rhinitis model mice. * $p < 0.05$, *** $p < 0.001$ versus model group.

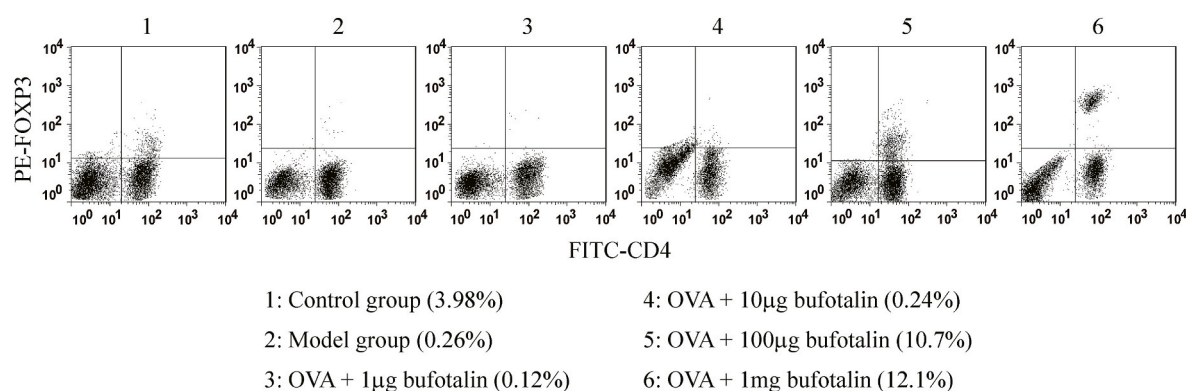


Fig. 5. Bufotalin restored Treg percentage to inhibit the inflammation in allergic rhinitis model mice. Representative flow cytometric profiles showing the population of Treg cells (determined by CD4 and Foxp3) in cervical lymph nodes of the mice in each group.

with great importance [14]. Here we reported that administration of bufotalin in ova-induced AR mice alleviated AR symptoms, decreased serum level of histamine, IL-1 β and OVA-specific IgE. Furthermore, bufotalin increased the Tregs in AR mice. These results demonstrated the protective effects of bufotalin against AR.

In AR, the binding of inhaled allergens to IgE promotes IgE binding to mast cells and basophils through Fc receptor, which results in degranulation of mast cells and basophils and releasing of histamine. Upregulated level of IgE and histamine is positively correlated with severity of AR [15]. Consistently, we also found elevated serum level of histamine and OVA-specific IgE in AR mice. Antihistamines are widely used to treat AR [3]. Inhibition/neutralization of IgE also ameliorates AR symptoms [2]. We found that in bufotalin-treated AR mice, the serum levels of histamine and OVA-specific IgE were significantly decreased, which contributed to the amelioration of AR symptoms by bufotalin. In addition, we found that bufotalin decreased the serum levels of pro-inflammatory cytokine IL-1 β in AR mice. IL-1 β has been shown to contribute to various inflammatory diseases including AR by promoting the activation of T cells and B cells [16,17]. The suppression of IL-1 β production by bufotalin could contribute to its protective effects on AR.

Although AR was characterized as a Th2 cell-mediated inflammatory disease [18], increased evidences suggest the Th17/Treg imbalance is critical in the pathogenesis of AR [19]. Increased Th-17 and serum level of IL-17 were associated with AR patients while AR patients had decreased TGF- β , a cytokine produced by Tregs. Inhibition of IL-17 using anti-IL-17 antibodies has been shown to alleviate AR symptoms, reduce

Th2 and Th17 responses, and increase the Treg response [8]. In present study, we found that bufotalin restored the Tregs cells in AR mice. This activity should contribute to bufotalin's effects on AR. However, it is not clear how bufotalin restored Tregs in AR mice. Since bufotalin inhibits Th17 differentiation and IL-17 production [10], it may function to inhibit Th17 response in AR mice. It is useful to determine the effects of bufotalin on Th17 cells in AR mice.

In the present study, we identified the protective effects of bufotalin on AR. However, several issues need to be further addressed in the future study. We administrated different amount of bufotalin to AR mice and only found the 100 μ g and 1 mg treatments had good protective effects. Since bufotalin displayed toxicity [20,21], it is necessary to determine the best dosage of bufotalin. In present study, although we demonstrated the effects of bufotalin, introducing a positive control such as antihistaminic or nasal corticosteroid should be useful to better understand bufotalin's effects. Both previous study and our present study demonstrated the regulatory activity of bufotalin on Th17 and Tregs [10]. It is also useful to test these activities in other inflammatory disease models. In addition, to further confirm the protective effects of bufotalin against AR, other AR animal models should be employed to examine its effects. In this study, there is another limitation. No detailed molecular mechanisms have been explored, which warrants further studies.

5. Conclusion

In this study, we successfully generated an AR mice model. Bufotalin

treatment was found to ameliorate AR symptoms in this mice model probably by the inhibition of inflammation through restoring Treg cells in cervical lymph node.

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CRediT authorship contribution statement

Qiang Ji: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Shoulei Ren:** Writing – review & editing, Software, Methodology, Investigation, Data curation. **Ling Li:** Writing – review & editing, Resources, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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