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## Role of Zinc Sulphate in Immune Regulation in *Artemisia annua* Pollen-challenged P815 Mastocytoma Cells

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### ABSTRACT

**Objective:** This study aimed to investigate the role of zinc sulphate in immune regulation in *Artemisia annua* pollen-challenged P815 mastocytoma cells.

**Methods:** P815 mastocytoma cells were treated with various concentrations of zinc sulphate and *Artemisia annua* pollen. Cell proliferation was measured using the Cell Counting Kit-8. The amount of ST2 and p38 in the cells were measured using Western blotting. The level of interleukins (IL)-33 in the supernatant was determined using the enzyme-linked immunosorbent assay. The levels of IL-2, IL-4, IL-5, interferon- $\gamma$ , and tumor necrosis factor were measured using the cytometric bead array.

**Results:** *Artemisia annua* pollen at a concentration  $>0.001 \mu\text{g/mL}$  induced allergic response in the P815 mastocytoma cells. Expressions of IL-33, IL-4, ST2, and p38 increased along with higher concentrations of *Artemisia annua* pollen. Zinc sulphate of 50–200  $\mu\text{mol/L}$  promoted the proliferation of P815 mastocytoma cells. Zinc sulphate attenuated the upregulation of IL-33, IL-4, ST2, and p38 caused by *Artemisia annua* pollen.

**Conclusion:** Zinc sulphate can promote the proliferation of P815 mastocytoma cells. It can also attenuate allergic response in the P815 mastocytoma cells induced by *Artemisia annua* pollen, which might provide a new treatment method for allergic diseases.

### KEYWORDS

Zinc sulphate; P815 mastocytoma cells; *Artemisia annua* pollen; p38

## Introduction

Allergic diseases are caused by inflammatory mediators released from the mastocytes and the eosinophilic granulocytes. The prevalence of allergic rhinitis is increasing in recent years, affecting about 40% of the global population (Meltzer 2016). In China, the prevalence of this disease showed a significant increase during 2005–2011 and has reached 32.4% (Wang et al. 2018; Zhang and Zhang 2019). One in every three people with allergic rhinitis also has bronchial asthma, which causes extra disease burden (Shaaban et al. 2008). It was previously thought allergic diseases are immune responses mediated by immunoglobulin E (IgE). More recent studies have shown that non-IgE-

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mediated immune responses also play roles in the development of allergic diseases (Thathiah et al. 2011; Woolhiser et al. 2004). For example, the surface interleukins (IL)-33 receptors and Toll-like receptors can activate the mast cells in a non-IgE-mediated manner, leading to degranulation and inflammation.

Th1/Th2 balance is central to immune responses. IL-33 is a new member of the Th2-like cytokines and plays a dual role of cytokine and nuclear factor. When functioning as a cytokine, IL-33 binds to the ST2 receptor, activating myeloid differentiation factor 88 and tumor necrosis factor (TNF)-associated factor 6. TNF-associated factor 6 then activates mitogen-activated protein kinases and downstream signals, leading to the release of Th2-like cytokines and the final inflammatory response (Schmitz et al. 2005). It has been shown that IL-33/ST2 plays an important role in mastocyte-mediated inflammatory responses (Allakhverdi et al. 2007).

Pollen is one of the most important allergens that can cause allergic diseases. *Artemisia annua* is a widely distributed plant species and its pollen is often found to be the allergen for patients. Zinc plays important roles in the regulation of T cells and its deficiency may result in infection, immune disorders, and allergy (Haase and Rink 2009; Kahmann et al. 2008; Kaltenberg et al. 2010). Our study aimed to investigate the effect of zinc sulphate on the proliferation of P815 mastocytoma cells and its role in immune regulation in *Artemisia annua* pollen-challenged P815 cells.

## Materials and methods

### Cell culture

The P815 mastocytoma cells (Cell Bank of Shanghai Institutes for Biological Sciences, Shanghai, China) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum at 37°C with 5% CO<sub>2</sub>. The cells were passaged every other day. A total of  $2 \times 10^5$  cells was cultured for 24 h and treated with various concentrations of zinc sulphate (blank control, 50 µmol/L, 100 µmol/L, 150 µmol/L, 200 µmol/L, and 300 µmol/L), *Artemisia annua* pollen (blank control, 0.001 µg/mL, 0.01 µg/mL, and 0.1 µg/mL; Macro-Union Pharmaceutical, Beijing, China), or both for 12 and 24 h.

### Cell proliferation assay

P815 cells in the exponential phase were collected and centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in DMEM and its density was adjusted to  $1 \times 10^5$  cells/mL. The cells were transferred to a 96-well plate with 100 µL per well and cultured for 24 h. The experiment was performed in triplicate. Culture medium without cells served as the blanks. The cells were treated with various concentrations of zinc sulphate (0, 50, 100, 150, 200, and 300 µmol/L) for 24 and 48 h with a final well volume of 200 µL. Each well was added with 20 µL Cell Counting Kit-8 solution (TransGen Biotech, Beijing, China) for 1 h. Optic absorbance was measured at 450 nm.

### Western blotting

The P815 cells were collected and lysed to extract the total protein. A total of 20 µg protein was loaded for each well. The proteins of p38, *p*-p38, and ST2 were separated using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a membrane. The membrane was blocked with 5% defatted milk for 2 h and incubated with the primary antibodies (p38 MAPK 1:1000, phospho anti-p38 1:1000, ST2 1:1000, and beta-actin 1:5000, Proteintech, Fisher Scientific, USA) at 4°C overnight. The secondary antibody (goat anti-rabbit IgG, Proteintech, Fisher Scientific, USA) was added at room temperature for 2 h. The bands were exposed and analyzed using the ImageJ software (Schneider et al. 2012).

### Enzyme-linked immunosorbent assay

A mouse anti-IL-33 enzyme-linked immunosorbent assay kit (RayBiotech, USA) was brought to room temperature for 30 min. Standard protein solution (500 pg/mL, 200 pg/mL, 80 pg/mL, 32 pg/mL, 12.8 pg/mL, 5.12 pg/mL, 2.05 pg/mL) was prepared according to the manufacturer's instruction. The standard solution and the culture supernatant were added to a 96-well plate for 2.5 h with 100 µL per well. The experiment was performed in duplicate. The plate was incubated with the primary antibody for 1 h, horseradish peroxidase for 45 min, tetramethyl benzidine for 30 min, each after washing for 4 times. Each well was added with 50 µL stop solution. The optical density was read at 450 nm.

### Cytometric bead array

The reference cytokine solutions were prepared according to the manufacturer's instruction (BD, USA) and were mixed with the cytokine capture beads. The reference tubes were added with 50 µL serial dilutions of the reference solutions. The test tubes were added with 50 µL culture supernatant. All tubes were incubated with corresponding antibodies against IL-2, IL-4, IL-5, interferon (IFN)-γ, and tumor necrosis factor (TNF) at room temperature in dark for 3 h. Then the tubes were centrifuged at 200 g for 5 min. The cell pellet was resuspended in 300 µL washing solution and analyzed using the FCAP Array v1.0 software.

### Statistical analysis

All data were presented as means and standard deviations. Comparisons were made using the Student's *t*-test. All statistical analyses were performed using the SPSS 19.0 software (SPSS, Chicago, IL, USA). *P* < .05 was considered statistically significant.

## Results

Treating the P815 mastocytoma cells with *Artemisia annua* pollen of various concentrations for 12 h increased the expression of IL-33 in a concentration-dependent manner (Figure 1a). The increase in the expression of IL-33 was more prominent at 24 h (Figure 1b). The supernatant levels of TNF were decreased and that of IL-4 were increased (Figure 2). No significant difference

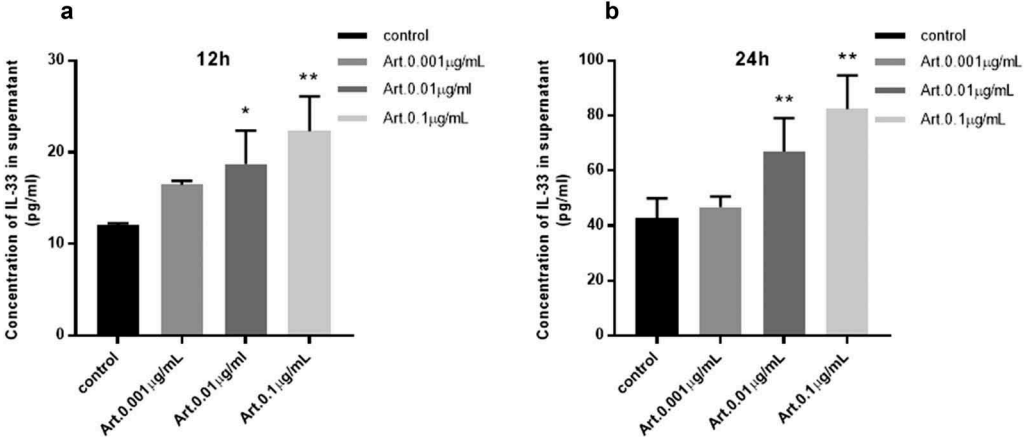


**Figure 1.** Levels of IL-33 (pg/mL, n = 5) in the supernatant of P815 mastocytoma cells treated with various concentrations of *Artemisia annua* pollen. \*P < .05, \*\*P < .01, versus control.

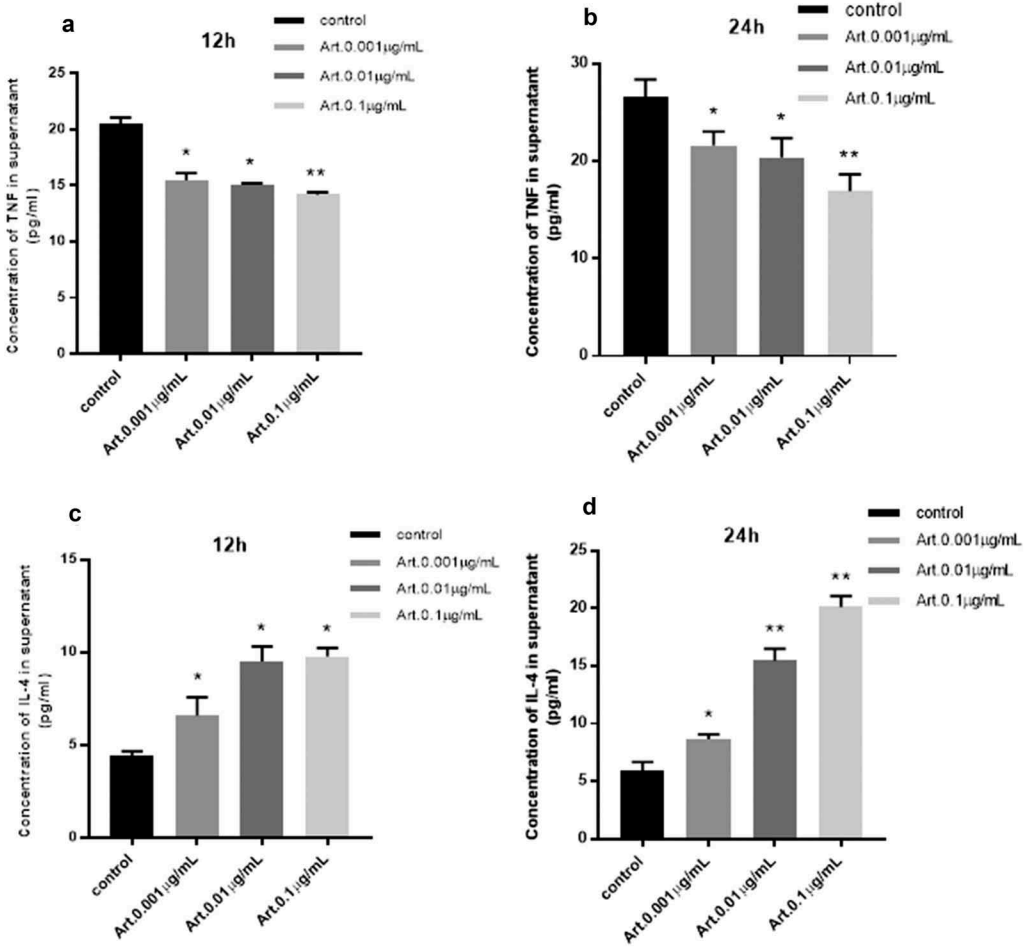


**Figure 2.** Levels of TNF and IL-4 (pg/mL, n = 3) in the supernatant of P815 mastocytoma cells treated with various concentrations of *Artemisia annua* pollen. \*P < .05, \*\*P < .01, versus control.

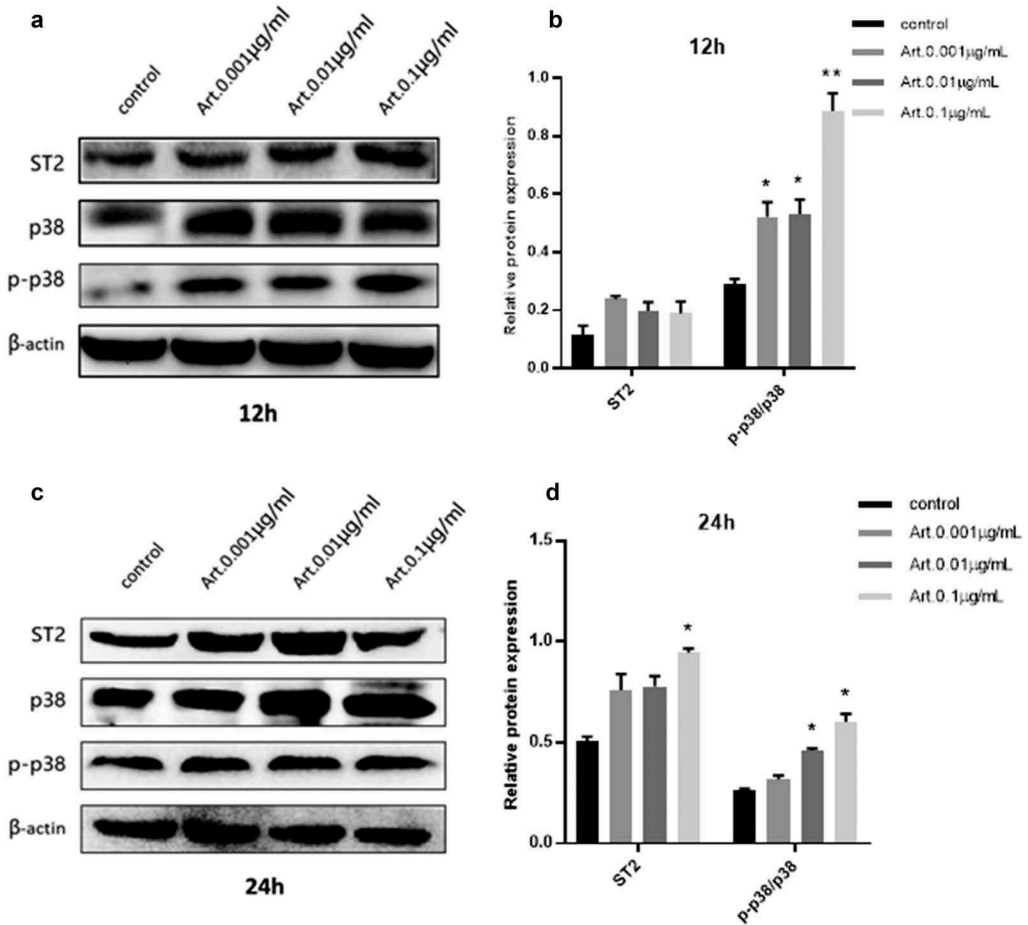


**Figure 3.** Western blotting of p38 and ST2 in the P815 mastocytoma cells treated with various concentrations of *Artemisia annua* pollen. \* $P < .05$ , versus control.

was noticed in the supernatant levels of IL-2, IL-5, and IFN- $\gamma$ . In addition, the expression of ST2 and p38 was significantly upregulated at 12 h and 24 h (Figure 3). These results suggested that the effective concentration of *Artemisia annua* pollen to trigger allergic response in the P815 mastocytoma cells was at least 0.001  $\mu$ g/mL.

Treating the P815 mastocytoma cells with zinc sulphate at 200  $\mu$ mol/L for 12 h, or at 100  $\mu$ mol/L for 24 h, yielded the strongest cell proliferation effect (Figure 4). Therefore, zinc sulphate of 50, 100, and 200  $\mu$ mol/L was used for the following experiments.

With *Artemisia annua* pollen maintained at 0.01  $\mu$ g/mL, zinc sulphate of 200  $\mu$ mol/L significantly downregulated IL-33 (Figure 5a,b), IL-4 (Figure 6c,d), ST2, and p38 (Figure 7a,c), but significantly upregulated TNF (Figure 6a,b). These effects were more prominent at 24 h. With zinc sulphate maintained at 100  $\mu$ mol/L, its inhibition effect decreased along with higher concentrations of *Artemisia annua* pollen (Figures 5–7).

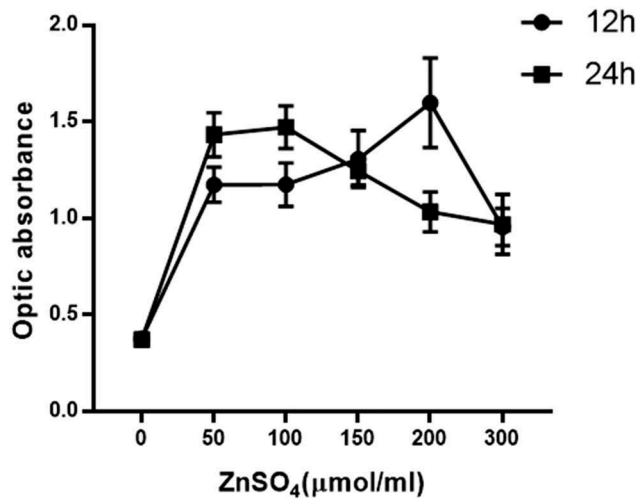


**Figure 4.** Proliferation of the P815 mastocytoma cells treated with various concentrations of zinc sulphate (n = 5).

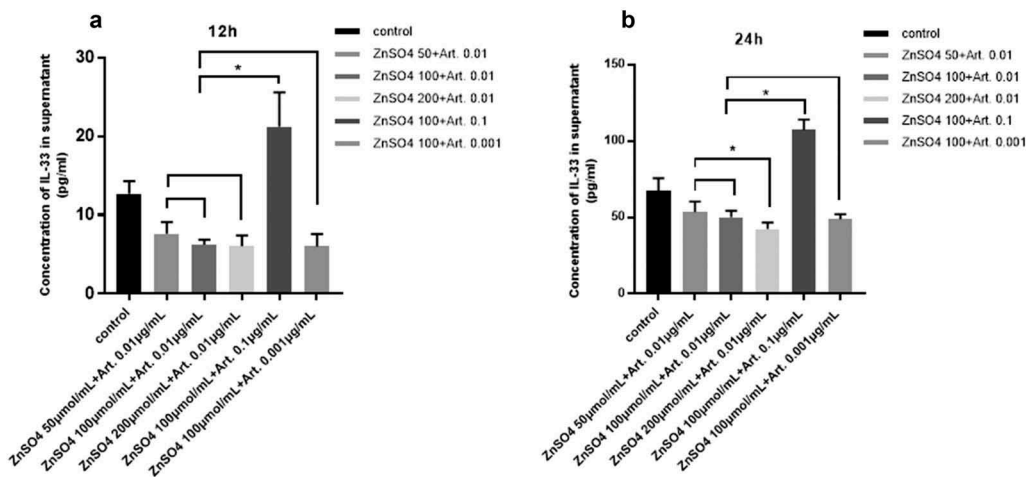


**Figure 5.** Levels of IL-33 (pg/mL, n = 5) in the supernatant of P815 mastocytoma cells treated with various concentrations of *Artemisia annua* pollen and zinc sulphate. With *Artemisia annua* pollen maintained at 0.01 μg/mL, the levels of IL-33 decreased along with higher concentrations of zinc sulphate. With zinc sulphate maintained at 100 μmol/L, *Artemisia annua* pollen of 0.1 μg/mL upregulated the expression of IL-33, while *Artemisia annua* pollen of 0.01 μg/mL and 0.001 μg/mL downregulated the expression of IL-33. \*P < .05.

These results indicated that zinc sulphate attenuated allergic response in the P815 mastocytoma cells that was induced by *Artemisia annua* pollen.

## Discussion

Our study found that treatment with *Artemisia annua* pollen significantly increased the levels of ST2 and p38 in the P815 mastocytoma cells. Treatment with *Artemisia annua*

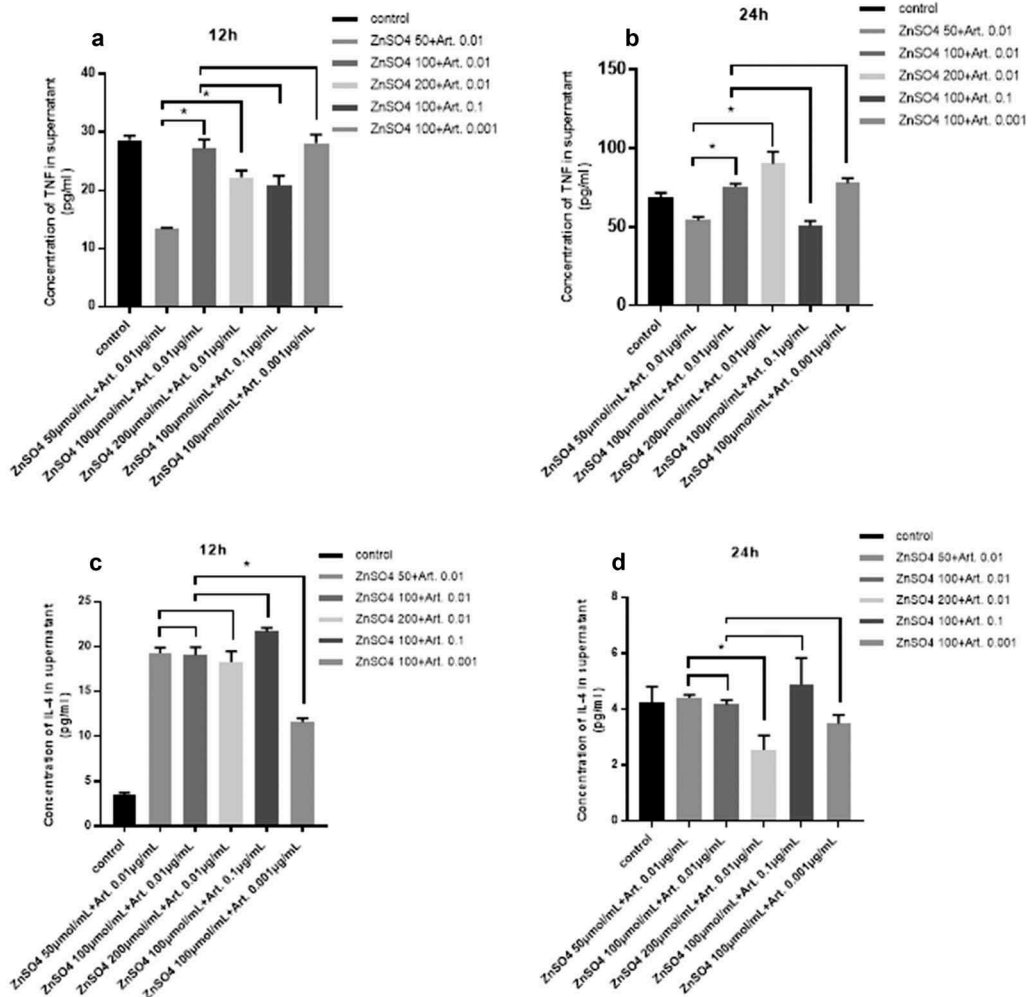


**Figure 6.** Levels of TNF and IL-4 (pg/mL, n = 3) in the supernatant of P815 mastocytoma cells treated with various concentrations of *Artemisia annua* pollen and zinc sulphate. With *Artemisia annua* pollen maintained at 0.01 μg/mL, the levels of TNF increased along with higher concentrations of zinc sulphate (a,b), while that of IL-4 decreased (c,d). With zinc sulphate maintained at 100 μmol/L, *Artemisia annua* pollen of 0.1 μg/mL downregulated the expression of TNF (a,b) but upregulated IL-4 (c,d), and *Artemisia annua* pollen of 0.01 μg/mL and 0.001 μg/mL upregulated the expression of TNF (a, b) but downregulated IL-4 (CD).

pollen for 24 h resulted more significant changes than treatment for 12 h. The levels of IL-33, IL-4, and IL-5 increased significantly after treatment with *Artemisia annua* pollen, but that of IL-2, IFN-γ, and TNF did not change significantly. Our findings suggest that *Artemisia annua* pollen induced allergic responses in the P815 cells.

A previous study found that IL-33 induced Th2-like immune response in vitro in the mastocytes (Mccarthy et al. 2019). Our study showed that *Artemisia annua* pollen at a concentration greater than 0.001 μg/mL upregulated the expression of IL-33 and IL-4 but downregulated TNF in the P815 cells, which looks like a Th2 cytokine-

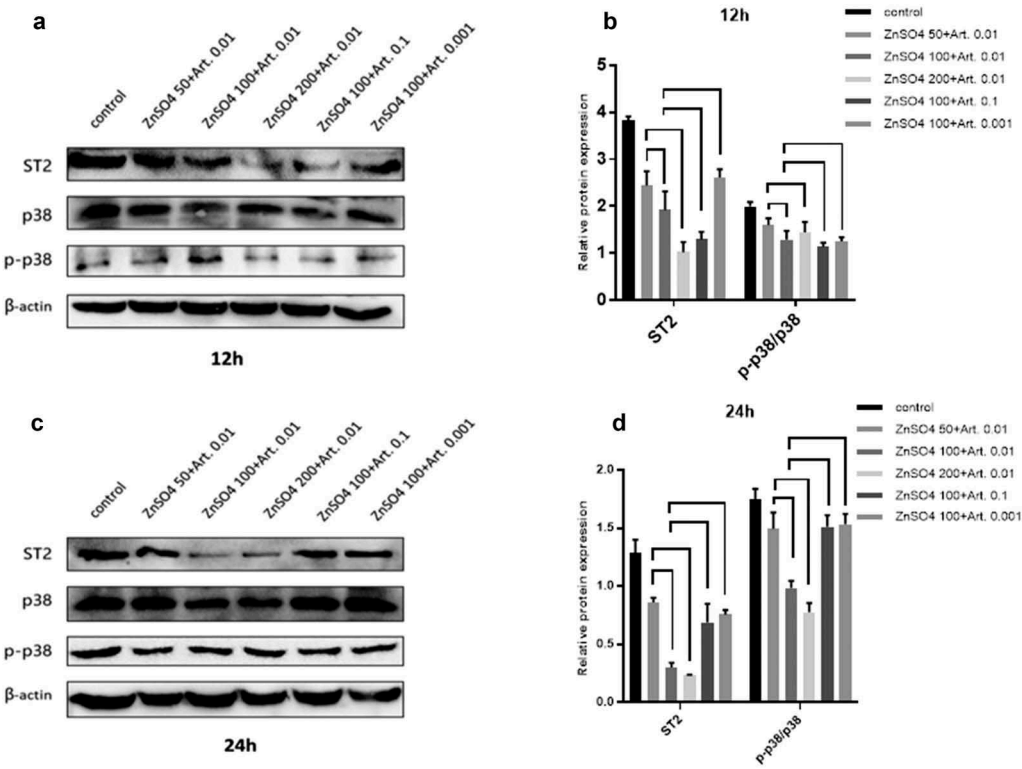


**Figure 7.** Western blotting of p38 and ST2 in the P815 mastocytoma cells treated with various concentrations of *Artemisia annua* pollen and zinc sulphate. With *Artemisia annua* pollen maintained at 0.01  $\mu$ g/mL, the levels of ST2 and p38 decreased along with higher concentrations of zinc sulphate (50–200  $\mu$ mol/L). With zinc sulphate maintained at 100  $\mu$ mol/L, *Artemisia annua* pollen of 0.01  $\mu$ g/mL, 0.1  $\mu$ g/mL, and 0.001  $\mu$ g/mL decreased the expression of p38 and ST2 in comparison with the blank control, but not reaching statistical significance.

induced immune response. In addition, the downstream p38 MAPK was upregulation. All these results suggested that *Artemisia annua* pollen induced allergic response in the P815 cells in a concentration-dependent manner.

Zinc is a trace element in human bodies and is involved in many aspects of physiology, such as cell proliferation, protein synthesis, immunity, and endocrine. Its deficiency may result in growth retardation, immune disorders, inflammatory responses, and increased risks of infection, allergic diseases, and autoimmune diseases (Basnet et al. 2015; Chasapis et al. 2012; Ibs and Rink 2003). Children are more vulnerable to zinc deficiency (Nurmatov et al. 2012). Increasing intake of antioxidative vitamins, zinc, and selenium during pregnancy has been found to be associated with less risks of childhood asthma and eczema (Patelarou et al. 2011).

Our study found that treatment with *Artemisia annua* pollen and zinc sulphate significantly reduced the production of ST2 and p38 in the P815 cells. Greater reduction was noticed when higher concentrations of zinc sulphate were used. In addition, the Th2-like cytokines such as IL-4 and IL-5 were also downregulated. In our study, treatment with *Artemisia annua* pollen induced allergic responses in the P815 cells mediated by the Th2-

like cytokines. IL-33 plays a role in the regulation of various inflammatory responses (Choi et al., 2019; da Silva et al. 2019; Morrow et al. 2019). It has been shown that IL-33 can upregulate the Th2-like cytokines (Han et al. 2017; Mccarthy et al. 2019). Our study found that *Artemisia annua* pollen upregulated IL-33 in the P815 cells, while addition of zinc sulphate downregulated IL-33. These results suggest that zinc sulphate may inhibit the allergic response induced by *Artemisia annua* pollen.

In conclusion, zinc sulphate can promote P815 cell proliferation and attenuate the allergic response in the P815 cells induced by *Artemisia annua* pollen. Our findings may provide new insight in understanding the mechanisms of *Artemisia*-associated allergic diseases and inspire novel treatment methods. Further investigation is needed to fully illustrate the association between zinc sulphate and *Artemisia annua* pollen-induced allergy.

### Authors' contribution

FXY and RXM contributed to the conception and design of the study; FXY, LH, WLW and XLS contributed to the acquisition of data; FXY, LH, XLS and NYF performed the experiments; FXY and NYF contributed to the analysis of data; FXY wrote the manuscript; All authors reviewed and approved the final version of the manuscript.

### Availability of data and material

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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