



Xinqin exhibits the anti-allergic effect through the JAK2/STAT5 signaling pathway



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ABSTRACT

Ethnopharmacological relevance: Xinqin, a polyherbal medicine, is an important traditional Chinese herbal formula used in traditional oriental medicine for treatment of allergic rhinitis (AR). The formula is based on the Chinese Pharmacopoeia

Aim of the study: Previously, Xinqin exhibited potent anti-allergic effect in a guinea pig model of AR. In this study, we explored the molecular mechanism of the anti-allergic effect mediated by Xinqin.

Materials and methods: AR was induced in guinea pigs (Hartley) with toluene-2, 4-diisocyanate (TDI) in vivo and in HMC-1 mast cells with A23187/phorbol 12-myristate-13-acetate (PMA) in vitro. The releases of allergic inflammatory mediators such as histamine, leukotriene (LT) D₄, immunoglobulin (Ig) E, TNF- α , and IL-6 were analyzed for allergy. The mast cell degranulation was displayed in HMC-1 mast cells. The activities of janus protein kinase 2 (JAK2), signal transduction and activator of transcription 5 (STAT5) and suppressor of cytokine signaling 3 (SOCS3) were evaluated by Western blot.

Results: Treatment with Xinqin resulted in AR symptoms and decreases in levels of histamine, LTD₄, IgE, TNF- α , and IL-6 in serum of guinea pig model of AR and in A23187/PMA-stimulated HMC-1 mast cells. Treatment with Xinqin also inhibited cell degranulation in A23187/PMA-stimulated HMC-1 mast cells. The JAK2/STAT5 signaling pathway could play an important role in the anti-allergic activity mediated by Xinqin.

Conclusions: Xinqin exerts the anti-allergic effect by modulating mast cell-mediated allergic responses by down-regulating JAK2/STAT5 signaling pathway. Results from this study provide a mechanistic basis for the application of Xinqin in the treatment of AR.

1. Introduction

Allergic rhinitis (AR) is characterized by inflammation of the nasal mucosa with hypersensitivity resulting from seasonal or perennial responses to specific environmental allergens. Environmental and lifestyle changes have resulted in a rapid increase in AR prevalence (Turner et al., 1988; von Mutius et al., 1994). Epidemiological data indicate that AR affects up to 17% of the global population (Bousquet et al., 2012). Clinical symptoms, including nasal itching, nasal congestion, sneezing and clear nasal discharge flow, significantly impede patient quality of life and work performance (Lee and Arriola, 1999).

The allergic responses in AR are associated with a series of cellular interactions (Bousquet et al., 1996). Antigens stimulate the host to activate type-2 T helper (Th2) cells to produce Th2 cytokines, which promote specific immunoglobulin (Ig) E production as well as the infiltration of immune cells in sensitized patients (Benson et al., 2001).

Among the immune cells, mast cells play crucial roles in the pathogenesis of allergic responses in AR. Immunoglobulin Fc epsilon receptor 1 (Fc ϵ RI), a mast cell surface receptor, induces specific IgE responses to certain antigens. After specific antigen-antibody reactions, allergens cross-link to IgE–Fc ϵ RI complexes on the surface of mast cells (Okuda, 1990). Subsequently, mast cells are activated (mast cell degranulation) and release allergic mediators, such as histamine, leukotriene (LT), α -hexosaminidase, cytokines, chemokines and arachidonic acid derivatives, mediating acute and chronic inflammation (Church and Levi-Strauss, 1997). However, most current drug treatments have limited clinical applications due to the adverse side effects of long-term use. Consequently, it is necessary to develop safe and affordable alternative therapeutic agents for allergic disorders.

Recently, various medicinal plants and their bioactive compounds have been identified as possible treatments for allergic diseases because of their safety and efficacy (Hon et al., 2015; Jung et al., 2012). For

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example, Xinqin, a polyherbal medicine, has been successfully used for the treatment of AR in China (Sayin et al., 2013; Yan, 2004). Previously, we reported that Xinqin exhibits potent anti-allergic effects in a guinea pig model of AR (Zhang et al., 2005). However, the molecular mechanism underlying the anti-allergic effect of Xinqin remains unclear.

In this study, we evaluated the effect of Xinqin on allergic inflammatory reactions in a guinea pig model of toluene-2, 4-diisocyanate (TDI)-induced AR and in HMC-1 mast cells stimulated by A23187 combined with phorbol myristate acetate (A23187/PMA). We found that Xinqin effectively alleviated AR symptoms in the AR guinea pig model and mast cell degranulation in mast cells by suppressing allergic and inflammatory mediators, such as histamine, leukotriene D₄ (LTD₄), IgE, TNF- α , and IL-6. The mechanistic study revealed that Xinqin inhibited the phosphorylation of both janus protein kinase 2 (JAK2) and signal transduction and activator of transcription 5 (STAT5) and induced suppressor of cytokine signaling 3 (SOCS3) in the sensitized mast cells. These findings provide a novel mechanistic basis for the use of Xinqin in the treatment of AR.

2. Materials and methods

2.1. Cells and reagents

HMC-1 cells were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS). The 10 raw herbs used in Xinqin were purchased from Chongqing Tong Jun Ge CO., LTD. (Chongqing, China), with batch number and producing area as follows: Radix Et Rhizoma Asari, 130925, Hubei, China; Radix Scutellariae, 130201, Gansu, China;

Herba Schizonepetae, 131023, Beijing, China; Radix Saposhnikovia, 131012, Shangdong, China; Radix Angelica Dahurica, 131016, Beijing, China; Fructus Xanthii, 131023, Jiangsu, China; Radix Astragali, 134016, Shanxi, China; Rhizoma Atractylodis Macrocephalae, 131016, Chongqing, China; Ramulus Cinnamomi, 131012, Hainan, China and Rhizoma Acori Tatarinowii, 130925, Yunnan, China. Loratadine was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). TDI was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The calcium ionophore A23187 and PMA were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The following primary antibodies were used for Western blot analysis: an antibody against SOCS3 (1:1000) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against phospho-JAK2 (Tyr1007/1008, 1:500), JAK2 (1:1000), phospho-STAT5 (Tyr694, 1:200), and STAT5 (1:1000) were obtained from Cell Signaling Technology (Beverly, MA, USA) and an antibody against GAPDH (1:5000) was obtained from Beyotime (Shanghai, China). Horseradish peroxidase-conjugated species-specific secondary antibodies were purchased from Kirkegaard and Perry Laboratories (MD, USA).

2.2. Preparation of Xinqin

Xinqin consisted of ten different herbs: *Asarum sieboldii* Miq., *Scutellaria baicalensis* Georgi, *Schizonepeta tenuifolia* (Benth.) Briq., *Saposhnikovia divaricata* (Turcz.) Schischk., *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav., *Xanthium sibiricum* Patr. ex Widder, *Astragalus membranaceus* (Fisch.) Bunge, *Atractylodes macrocephala* Koidz., *Cinnamomum cassia* (L.) J. Presl and *Acorus tatarinowii* Schott (Table 1). The voucher specimens of the 7 herbs were deposited at Herbarium, Institute of Botany, Chinese Academy Science, Beijing, China, with voucher number as follows: *Asarum sieboldii* Miq., 01901615; *Schizonepeta tenuifolia* (Benth.) Briq., 00697456; *Saposhnikovia divaricata* (Turcz.) Schischk., 01575428; *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex

Franch. & Sav., 01575391; *Xanthium sibiricum* Patr. ex Widder, 01917625; *Atractylodes macrocephala* Koidz., 01837432 and *Cinnamomum cassia* (L.) J. Presl, 00700263. The voucher specimens of the 2 herbs were deposited at Northwest Agriculture & Forestry University Herbarium, Xianyang, China, with voucher number as follows: *Scutellaria baicalensis* Georgi, 0190382 and *Astragalus membranaceus* (Fisch.) Bunge, 520248. *Acorus tatarinowii* Schott was deposited under no. 1251800 at Herbarium, Kunming Institute of Botany, Chinese Academy Science, Kunming, China. The Xinqin prescription was composed of 10 raw herb, with dose in the formula as follows: Radix Et Rhizoma Asari, 50.0 g; Radix Scutellariae, 50.0 g; Herba Schizonepetae, 50.0 g; Radix Saposhnikovia, 50.0 g; Radix Angelica Dahurica, 50.0 g; Fructus Xanthii, 50.0 g; Radix Astragali, 50.0 g; Rhizoma Atractylodis Macrocephalae, 50.0 g; Ramulus Cinnamomi, 50.0 g and Rhizoma Acori Tatarinowii, 50.0 g (Table 2). Xinqin was prepared by decocting the dried prescription of the above herbs with boiling distilled water for 2.5 h (two times). The extract was filtered, condensed, and spray-dried. The yield of dried extracts from starting crude materials was about 22.3%.

2.3. Mast cell degranulation examination

Mast cell degranulation was observed using microscopy. HMC-1 cells were pretreated with Xinqin (0.25, 0.50, or 1.0 mg/ml) or loratadine (5 μ M) for 1 h and stimulated with A23187 (1 μ M) and PMA (25 nM) for another 1 h. Then, the cells were stained with toluidine blue and viewed under a microscope.

2.4. Measurement of histamine, LTD₄, TNF- α and IL-6 release from the mast cells

HMC-1 cells were pretreated with various concentrations of Xinqin (0.25, 0.50, or 1.0 mg/ml) or with loratadine (5 μ M) for 1 h and were then stimulated with A23187 (1 μ M) and PMA (25 nM) for 6 h. The cell culture supernatants were collected and filtered through a 0.22- μ m Steriflip filter (Millipore, MA, USA). The histamine, LTD₄, TNF- α and IL-6 concentrations were quantified using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (Santa Cruz, CA, USA).

2.5. Ethics statement

The animal experiment was conducted with an approval from and under the supervision of the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University. Animal surgeries were performed under sodium pentobarbital anesthesia with minimal suffering.

2.6. TDI-induced AR animal model and evaluation of nasal symptoms

Male guinea pigs (Hartley guinea pigs, n=40, 300–350 g, Hualan Biological Engineering, Inc., Chongqing, China) were randomly divided into five groups: control group, TDI-sensitized group, TDI-sensitized group with Xinqin treatment at a dose of 25 or 50 mg/kg and TDI-sensitized group with loratadine treatment at a dose of 50 mg/kg (8 guinea pigs per group). Briefly, TDI olive oil solution (5 μ l, 10% w/v) was dripped bilaterally on the nasal vestibule of AR model groups once a day for 14 days. Control group were administered olive oil alone on the same schedule. Nasal symptoms were evaluated including the frequency of nasal scratching (nasal itching), sneezes and the extent of watery rhinorrhea that occurred in 15 min at the 10-d mark before the intranasal TDI provocation on a scale ranging from 0 to 3 according to the criteria presented in Table 3. The total scores that is greater than or equal to 5 points indicated the successful establishment of AR model.

The AR guinea pigs were then intragastric administration with

Table 1
Active compounds of Xinqin.^a

Plant name	Active compound ^b
<i>Asarum sieboldii</i> Miq.	Asarinin (PubChem CID: 11869417)
<i>Scutellaria baicalensis</i> Georgi	Baicalin (PubChem CID: 64982)
<i>Schizonepeta tenuifolia</i> (Benth.) Briq.	Pulegone (PubChem CID: 6988)
<i>Saposhnikovia divaricata</i> (Turcz.) Schischk.	Prim-O-glucosylcimifugin (PubChem CID: 14034912); 5-O-methylvisammoside (PubChem CID: 21670038)
<i>Angelica dahurica</i> (Hoffm.) Benth. & Hook. f. ex Franch. & Sav.	Imperatorin (PubChem CID: 10212)
<i>Xanthium sibiricum</i> Patrín ex Widder	Carboxyatractyloside (PubChem CID: 36403)
<i>Astragalus membranaceus</i> (Fisch.) Bunge	Astragaloside (PubChem CID: 45006101); Calycosin-7-O-beta-D-glucoside (PubChem CID: 5318267)
<i>Atractylodes macrocephala</i> Koidz.	Atractylenolide I (PubChem CID: 5321018); Atractylenolide III (PubChem CID: 155948)
<i>Cinnamomum cassia</i> (L.) J. Presl	Cinnamaldehyde (PubChem CID: 637511)
<i>Acorus tatarinowii</i> Schott	Shyobunone (PubChem CID: 5321293); Acorenone (PubChem CID: 12480741); Calarene (PubChem CID: 15560278)

^a Atractylenolide III (PubChem CID: 155948).^b The number has been checked at the PubChem CID from NCBI PubChem Compound database.**Table 2**
Herbal constituents of Xinqin.^a

Plant name	Raw herb	Dose in the formula, g
<i>Asarum sieboldii</i> Miq.	Radix Et Rhizoma Asari	50.0
<i>Scutellaria baicalensis</i> Georgi	Radix Scutellariae	50.0
<i>Schizonepeta tenuifolia</i> (Benth.) Briq.	Herba Schizonepetae	50.0
<i>Saposhnikovia divaricata</i> (Turcz.) Schischk.	Radix Saposhnikoviae	50.0
<i>Angelica dahurica</i> (Hoffm.) Benth. & Hook. f. ex Franch. & Sav.	Radix Angelica Dahurica	50.0
<i>Xanthium sibiricum</i> Patrín ex Widder	Fructus Xanthii	50.0
<i>Astragalus membranaceus</i> (Fisch.) Bunge	Radix Astragali	50.0
<i>Atractylodes macrocephala</i> Koidz.	Rhizoma Atractylodis Macrocephalae	50.0
<i>Cinnamomum cassia</i> (L.) J. Presl	Ramulus Cinnamomi	50.0
<i>Acorus tatarinowii</i> Schott	Rhizoma Acori Tatarinowii	50.0

^a The prescription is from Chinese Pharmacopoeia 2015.**Table 3**
Criteria for grading the severity of TDI-induced nasal symptoms.

Nasal symptoms	Score			
	0	1	2	3
Scratching frequency	(–)	1–2	> 2	/
Sneezes frequency	(–)	1–3	4–10	≥11
Watery rhinorrhea	(–)	At nostril	Between 1 and 3	Drops of discharge from nose

Xinqin (25 mg/kg or 50 mg/kg body weight) or loratadine (50 mg/kg body weight) once a day for 10 days (from days 15–24). After 14 days, TDI intranasal provocation was used to stimulate AR guinea pigs every other day to maintain the allergy. Control and TDI-sensitized group were administered olive oil alone on the same schedule. Guinea pigs were given 200 µl of pentobarbital sodium (1%) by intraperitoneal injection 30 min before sacrifice at the termination of the experiment. Blood were collected and nasal mucosa tissues were lysed and subjected to Western blot analysis.

2.7. Measurement of IgE, histamine, LTD4, TNF-α and IL-6 serum levels

Blood samples were taken to measure the levels of IgE, histamine, LTD4, TNF-α and IL-6 using ELISA. The serum samples were prepared using centrifugation at 3000 rpm for 15 min at 4 °C. The concentrations of histamine, IgE, LTD4, TNF-α and IL-6 were measured using an ELISA Kit (Santa Cruz, CA, USA) according to the manufacturer's protocol.

2.8. Western blots

HMC-1 cells were pretreated with various concentrations of Xinqin (0.25, 0.50, or 1.0 mg/ml) or loratadine (5 µM) for 1 h and then stimulated with A23187 (1 µM) and PMA (25 nM) for 6 h. After treatment, the cells were harvested.

Total protein of the nasal mucosa tissue samples and HMC-1 cellular samples was extracted using lysis buffer containing 1 mM PMSF (Beyotime, Shanghai, China). Protein concentrations were determined using a BCA Protein Assay Kit (Beyotime, Shanghai, China); 15–60 µg of sample protein was separated using SDS-PAGE and transferred to PVDF membranes (Millipore, MA, USA). The membranes were blocked with 5% fat-free dry milk in 1×Tris-buffered saline (TBS) and incubated with primary antibodies. Protein bands were detected after incubation with horseradish peroxidase-conjugated secondary antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), which were visualized using an enhanced chemiluminescence reagent (Millipore, MA, USA).

2.9. Statistical analysis

The data are expressed as the mean plus or minus the standard deviation (mean ± S.D.). The statistical analysis was performed by one-way analysis of variance (ANOVA) with Dunnett's Multiple Comparison test using GraphPad Prism 5.0 statistical analysis software (GraphPad Software, Inc., San Diego, CA, USA). The significance of differences between the normal and control groups was evaluated using Student's *t*-tests. **P* < 0.05, ***P* < 0.01 or ****P* < 0.001 were considered significant.

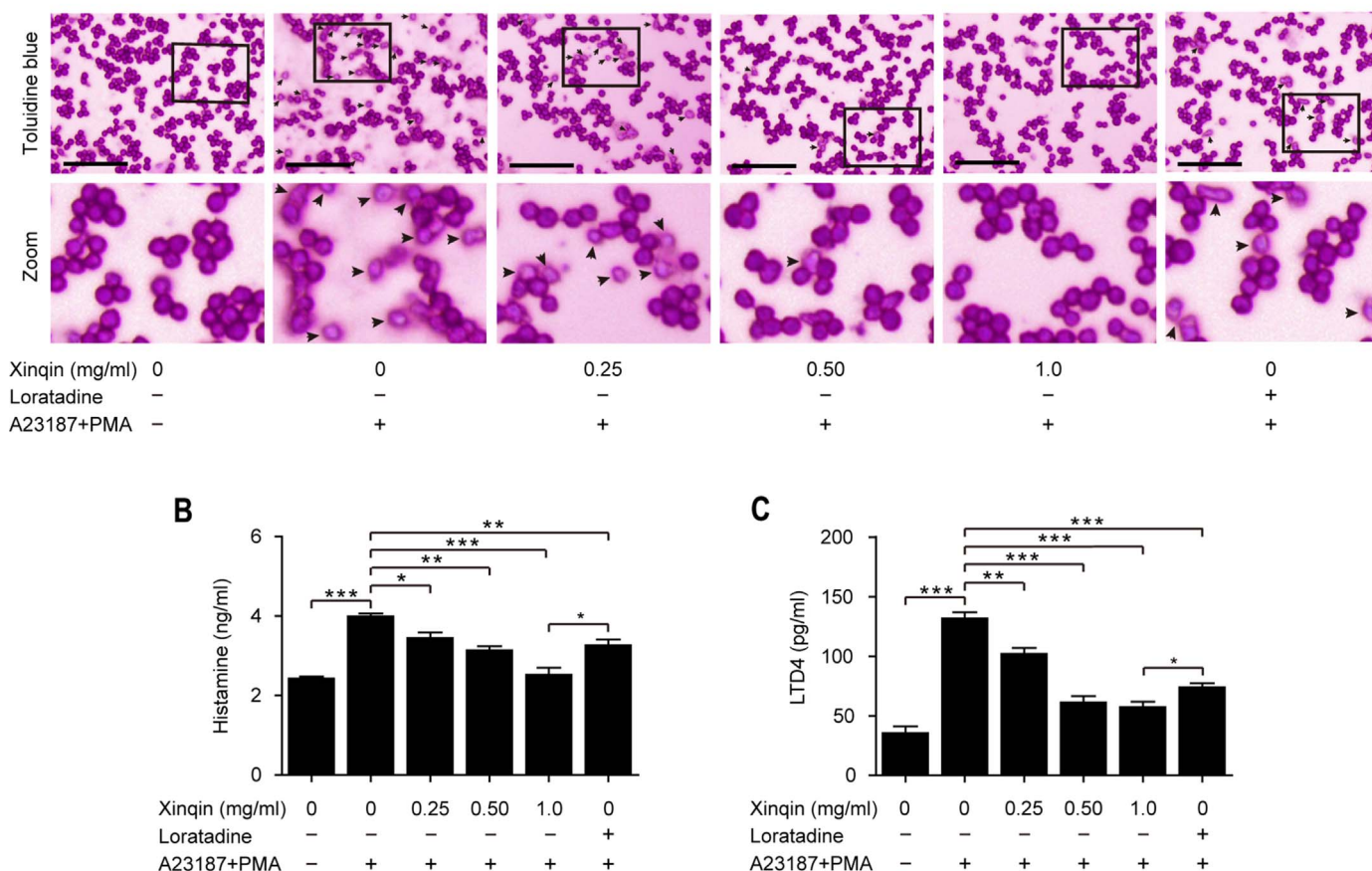
A

Fig. 1. Inhibitory effect of Xinqin on A23187/PMA-induced HMC-1 mast cell degranulation and histamine and LTD4 release. (A) Mast cell degranulation was observed using a microscope 1 h after A23187/PMA sensitization. The cells were stained with toluidine blue. The images are representative of three independent experiments. Black arrows indicate the activated mast cells. The scale bar represents 200 μ m. (B) Histamine and (C) LTD4 concentrations in the culture medium were measured using ELISA. Three independent experiments were performed. The data are presented as the mean $\pm$ S.D. of three independent experiments. A difference was considered significant when * P < 0.05, ** P < 0.01, or *** P < 0.001.

3. Results

3.1. Inhibitory effect of Xinqin on A23187/PMA-induced HMC-1 mast cell degranulation and histamine and LTD4 release

Mast cell degranulation and subsequent release of allergic molecules are implicated in initiating and maintaining AR (Baraniuk, 2001; Poole and Rosenwasser, 2005). Firstly, we examined changes in mast cell degranulation using HMC-1 cells by toluidine blue staining and microscopy. As shown in Fig. 1A, the cytoplasm of normal mast cells was dominated by closely packed secretory granules, while the cells stimulated with A23187 plus PMA clearly showed extensive degranulation and multiple extruding granules. To determine whether Xinqin treatment could inhibit mast cell degranulation, mast cells were pretreated with various doses of Xinqin (0.25, 0.50 and 1.0 mg/ml) and then incubated with A23187/PMA. Treatment with Xinqin markedly prevented mast cell degranulation stimulated by the combined treatment with A23187 and PMA.

Within allergen exposure, sensitized mast cells exhibited degranulation, releasing performed and newly synthesized mediators including histamine and LTD4. We next determined the effect of Xinqin on the release of histamine and LTD4 in media collected from untreated control, stimulated with A23187 and PMA, and Xinqin-treated mast cells by ELISA. As shown in Fig. 1B and C, combined treatment with A23187 and PMA resulted in a significant increase in levels of histamine and LTD4 in media of HMC-1 cells compared to that in untreated control cells. Pretreating HMC-1 cells with Xinqin significantly

attenuated the levels of histamine and LTD4 induced by the combination of A23187 and PMA. Additionally, Xinqin at high dose (1.0 mg/ml) has a stronger ability to inhibit histamine and LTD4 release stimulated by the combination of A23187 and PMA than loratadine. Thus, these findings indicate that Xinqin treatment represses mast cell degranulation and histamine and LTD4 release stimulated by the combination of A23187 and PMA.

3.2. Xinqin inhibits the expression of TNF- α and IL-6 stimulated by the combination of A23187 and PMA in HMC-1 cells

Since the pro-inflammatory cytokines are also important factors of allergic inflammations, we next determined whether Xinqin could inhibit the expression of TNF- α and IL-6 stimulated by the combination of A23187 and PMA using ELISA. As shown in Fig. 2A and B, stimulating cells with the combination of A23187 and PMA resulted in increases in levels of TNF- α and IL-6 in media of HMC-1 cells compared to that in untreated control cells. Pretreatment of cells with Xinqin significantly attenuated the secretion of TNF- α and IL-6 stimulated by the combination of A23187 and PMA. In addition, Xinqin at high dose (1.0 mg/ml) exhibited a more potent inhibition of TNF- α and IL-6 expression stimulated by the combination of A23187 and PMA than loratadine. Thus, these data indicate that Xinqin treatment inhibits the expression of A23187/PMA-sensitized TNF- α and IL-6 in HMC-1 cells.

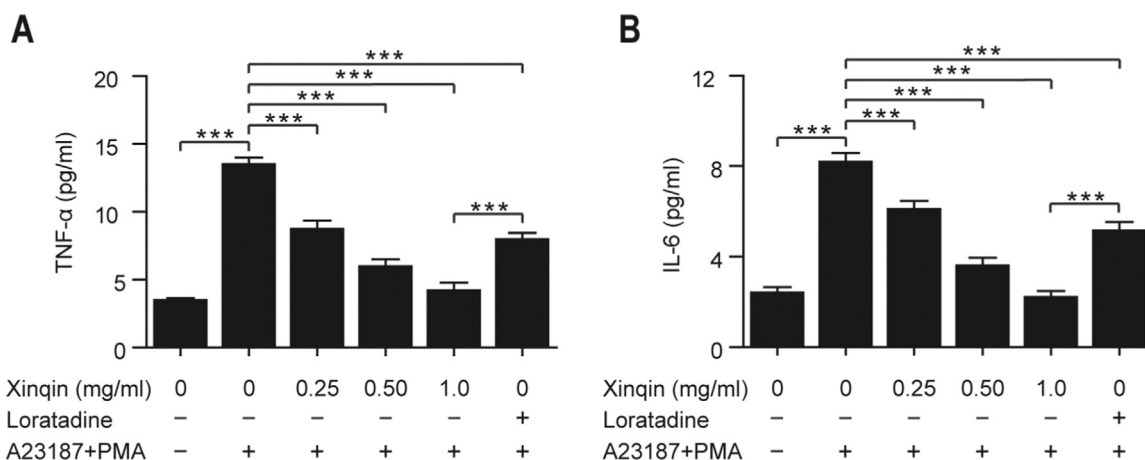


Fig. 2. Xinqin inhibits the expression of TNF- α and IL-6 stimulated by the combination of A23187 and PMA in HMC-1 cells. The cells were pretreated with Xinqin (0.25, 0.50 and 1.0 mg/ml) or loratadine (5 μ M) for 1 h and were then stimulated with A23187 (1 μ M) combined with PMA (25 nM) for 24 h. The secretion levels of (A) TNF- α and (B) IL-6 were measured using ELISA. The data are presented as the mean $\pm$ S.D. of three independent experiments. A difference was considered significant when *** P < 0.001.

3.3. Xinqin suppresses the phosphorylation of JAK2 and STAT5 and induces activation of SOCS3 in A23187/PMA-induced HMC-1 cells

The effects of Xinqin in HMC-1 cells were examined in relation to changes in the signal transduction pathway implicated in the regulation of mast cell degranulation and allergic inflammation. Western blot analysis showed that stimulating cells with the combination of A23187 and PMA resulted in increases in levels of phospho-JAK2 and phospho-STAT5, and decrease in level of SOCS3 compared to that in untreated control cells (Fig. 3). Pretreating cells with Xinqin markedly attenuated the phosphorylation of JAK2 and STAT5, and suppression of SOCS3 mediated by the combination of A23187 and PMA in a dose-dependent manner (Fig. 3). In contrast, either Xinqin or the stimulation of A23187 combined with PMA had no effect on the expression of total JAK2 and STAT5. In addition, Xinqin at high dose (1.0 mg/ml) exhibited more potent regulatory effect on the JAK2/STAT5 signaling pathway than loratadine. Thus, these results suggest that the JAK2/STAT5 signaling pathway may play a critical role in Xinqin-mediated the inhibition of mast cell degranulation and allergic inflammation.

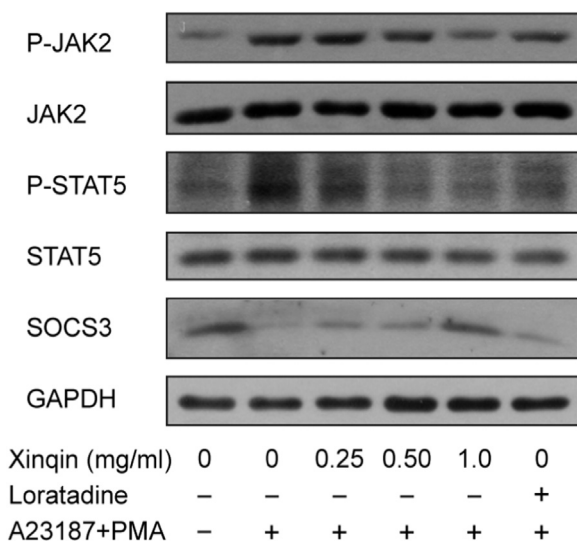


Fig. 3. Xinqin suppresses the phosphorylation of JAK2 and STAT5 and induces activation of SOCS3 in A23187/PMA-induced HMC-1 cells. The cells were pretreated with Xinqin (0.25, 0.50 and 1.0 mg/ml) or loratadine (5 μ M) for 1 h and then stimulated with A23187 (1 μ M) combined with PMA (25 nM) for 6 h. Total cellular protein was used for the detection of the phosphorylated or total forms of JAK2 and STAT5, as well as the negative regulator SOCS3. The protein bands are representative of three independent experiments.

3.4. Xinqin alleviates rhinitis symptoms in the TDI-induced AR model

The ability of Xinqin to inhibit mast cell degranulation and allergic inflammation in vitro led us to evaluate the anti-allergic activity in vivo. The AR guinea pig model sensitized by intranasal TDI challenge was employed, and rhinitis symptoms were evaluated. Various doses of Xinqin (25 mg/kg and 50 mg/kg) or loratadine (50 mg/kg), which is a positive control for the treatment of AR, were administered by intragastric administration before the intranasal TDI challenge from days 15–24 (Fig. 4A). As shown in Fig. 4B, AR guinea pigs in TDI group exhibited remarkable clear nasal discharge flow, sneezing, nasal rubbing, congestion, and edema symptoms. Treating TDI guinea pigs with Xinqin obviously alleviated the rhinitis symptoms, which was mediated by TDI.

To determine the effect of Xinqin on nasal symptoms, symptom scores were evaluated by counting the instances and the severity of nasal itching, sneezing and watery rhinorrhea that occurred in 15 min after the intranasal TDI challenge. As shown in Fig. 4C, the significant increases in scores for clear nasal discharge flow, sneezing and nasal rubbing were observed in TDI-induced AR guinea pigs compared to that in untreated control guinea pigs. Treatment with Xinqin significantly decreased AR nasal symptom scores mediated by TDI in a dose-dependent manner. Additionally, Xinqin at high dose (50 mg/kg) exhibited a more potent inhibition of AR symptoms than loratadine (50 mg/kg). Taken together, these findings indicate that Xinqin efficiently alleviates rhinitis symptoms in the AR guinea pig model.

3.5. Xinqin inhibits the expression of IgE, histamine, LTD4, TNF- α , and IL-6 in serum of TDI-challenged guinea pigs

AR is triggered by the interaction of ubiquitous environmental proteins and specific inflammation-related protein IgE in sensitized Patients (Bousquet et al., 2001; Togias, 2000), which result in mast cells activation and allergic mediators releases, such as histamine, LT and cytokines (Church and Levi-Schaffer, 1997). Pro-inflammatory cytokines such as TNF- α and IL-6 are potent multifunctional cytokines that play an important role in the pathogenesis of allergic rhinitis (Grzelewska-Rzymowska and Pietrkowicz, 2004). Since AR is triggered by the production of allergic and inflammatory mediators, we next determined the effects of Xinqin on the expression of IgE, histamine, LTD4, TNF- α , and IL-6 in serum of TDI-challenged guinea pigs by ELISA. As shown in Fig. 5A, the significant increases in levels of IgE were observed in serum of TDI-sensitized guinea pig compared to that in untreated control animals. Treatment with Xinqin at doses of 25 and 50 mg/kg significantly decreased the IgE levels in the serum of

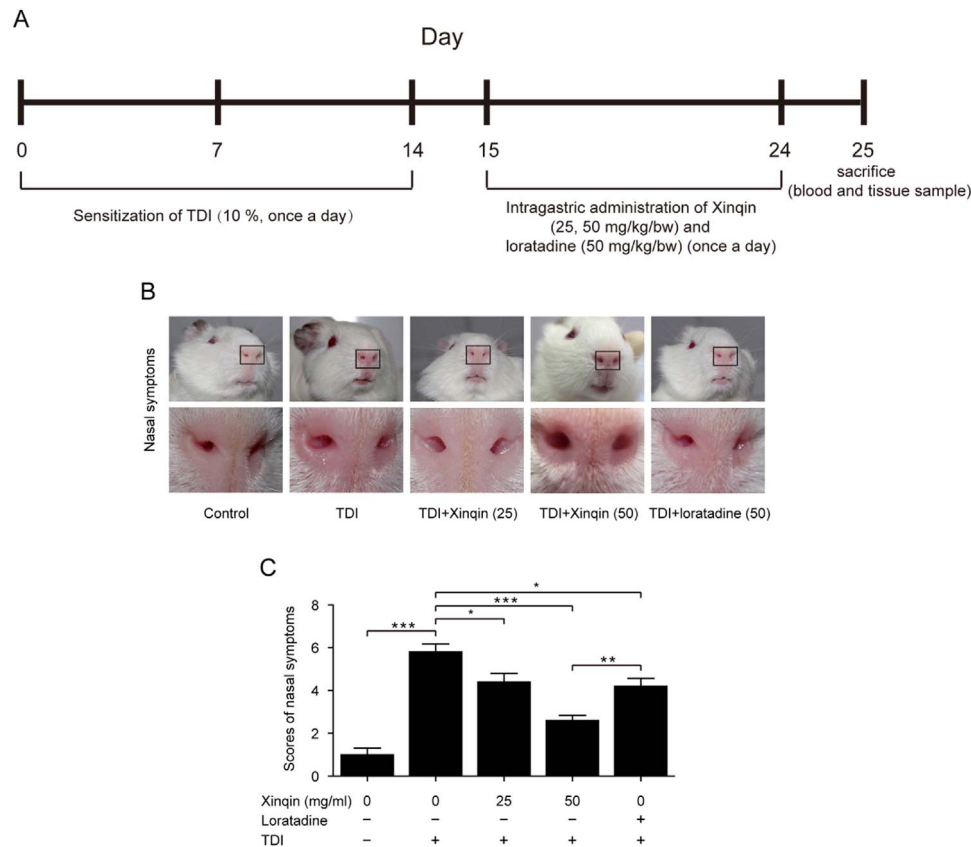


Fig. 4. Xinqin alleviates rhinitis symptoms in the TDI-induced AR model. (A) Schematic protocol for the experimental guinea pig in vivo model. (B) The effects of Xinqin on nasal symptoms in the TDI-induced AR guinea pigs. The images are representative of three independent experiments. (C) Nasal symptoms were measured by clinical scoring. The data are presented as the mean $\pm$ S.D. (n=8). A difference was considered significant when $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$.

TDI-sensitized guinea pigs compared to that in TDI-challenged guinea pigs alone. Similarly, treatment of TDI-challenged guinea pigs with

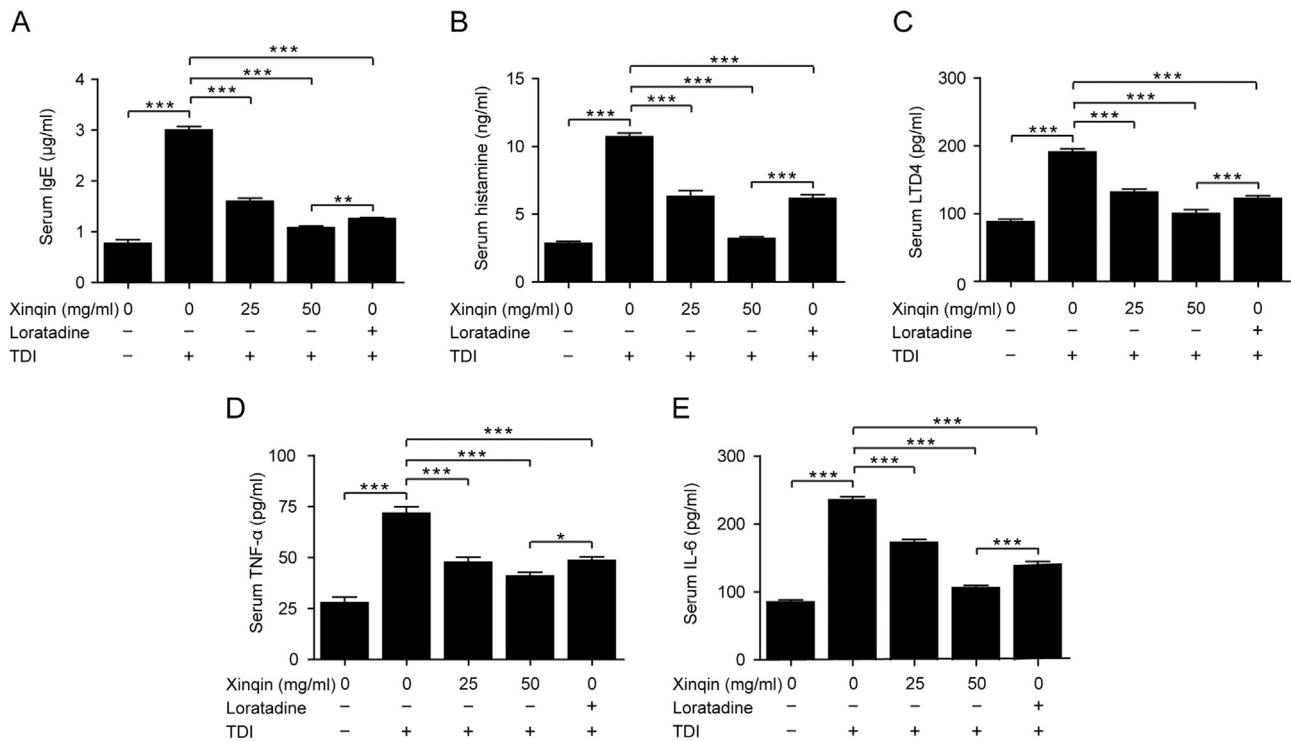


Fig. 5. Xinqin inhibits the expression of IgE, histamine, LTD4, TNF- α , and IL-6 in serum of TDI-challenged guinea pigs. The levels of (A) TDI-specific IgE, (B) histamine, (C) LTD4, (D) TNF- α and (E) IL-6 were measured in guinea pig sera using ELISA. Three independent experiments were performed, and the data are presented as the mean $\pm$ S.D. A difference was considered significant when $*P < 0.05$, $**P < 0.01$, or $***P < 0.001$.

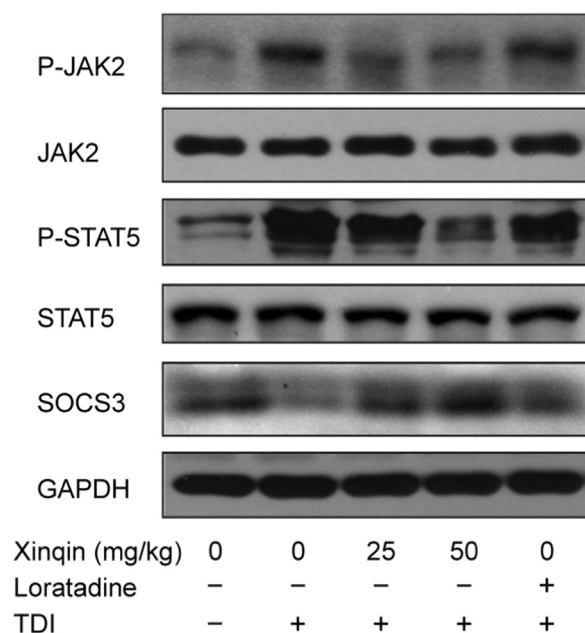


Fig. 6. Xinqin suppresses the phosphorylation of JAK2 and STAT5 and induces activation of SOCS3 in the TDI-induced AR guinea pig model. The TDI-induced AR guinea pigs were treated with Xinqin (25 mg/kg and 50 mg/kg) or loratadine (50 mg/kg) once a day for 10 days. The total protein of the nasal mucosa tissue was used for the detection of the phosphorylated or total forms of JAK2 and STAT5 as well as the negative regulator SOCS3. The protein bands are representative of three independent experiments.

Xinqin resulted in significant decreases in serum levels of histamine and LTD4 (Fig. 5B and C). Furthermore, treating TDI-challenged guinea pigs with Xinqin significantly decreased the serum levels of inflammatory cytokines TNF- α and IL-6 (Fig. 5D and E). Additionally, Xinqin (50 mg/kg) exhibited more potent inhibiting the expression of IgE, histamine, LTD4, TNF- α , and IL-6 than the loratadine (50 mg/kg). These results suggest that Xinqin exhibits anti-allergic activity via inhibiting the expression of allergic and inflammatory mediators in the AR guinea pig model.

3.6. Xinqin suppresses the phosphorylation of JAK2 and STAT5 and induces activation of SOCS3 in the TDI-induced AR guinea pig model

To further investigate whether the JAK2/STAT5 signal transduction pathway plays a critical role in Xinqin-mediated anti-allergic and anti-inflammatory activities, we analyzed the phosphorylation of JAK2 and STAT5, as well as the negative regulator SOCS3, in AR guinea pigs using western blot analysis. As shown in Fig. 6, the significant increases in levels of phospho-JAK2 and phospho-STAT5 and decrease in level of SOCS3 were observed in the nasal mucosa tissue of TDI-induced AR guinea pigs compared to that in untreated control guinea pigs. Pretreating with Xinqin attenuated the TDI-induced phosphorylation of JAK2 and STAT5 and downregulation of the negative regulator SOCS3. However, either Xinqin or the stimulation of TDI had no effect on the expression of total JAK2 and STAT5. In addition, Xinqin at high dose (50 mg/kg) exhibited more potent regulatory effect on the JAK2/STAT5 signaling pathway than loratadine (50 mg/kg). Thus, these results further confirmed that the JAK2/STAT5 signal pathway was involved in Xinqin-mediated anti-allergic and anti-inflammatory effects.

4. Discussion

In the present study, we demonstrate for the first time that Xinqin potentially exhibits anti-allergic and anti-inflammatory activities through inhibiting the mast cell degranulation in HMC-1 cells, and suppressing

allergic and inflammatory mediators, such as histamine, LTD4, IgE, TNF- α , and IL-6 both in vitro and in vivo. We also found that Xinqin exhibited anti-allergic efficacy by interrupting the JAK2/STAT5 signaling pathway. Notably, we found that Xinqin exhibited more potent anti-allergic effect and anti-inflammatory effect than that of loratadine.

Activated mast cells have a key role in AR through release of numerous mediators, such as histamine, leukotrienes and cytokines, via degranulation (Church and Levi-Schaffer, 1997). Thus, mast cell degranulation has been considered a major anti-allergic drug target. HMC-1 cells exhibit characteristics of human mast cells, including the synthesis of LT and the expression of histamine, trypsin, heparin, and a similar cell surface antigen-profile (Nilsson et al., 1994; Valent and Bettelheim, 1992). A23187 and PMA induced an increase in cytosolic calcium concentration, leading to mast cell degranulation (Baba et al., 2008). Thus, in this study, HMC-1 mast cells with A23187 and PMA stimulation were used to investigate how Xinqin exerts anti-allergic effect in vitro. Our data clearly showed that Xinqin attenuated the release of histamine and LTD4 induced by the combination of A23187 and PMA in HMC-1 mast cells by suppressing their degranulation.

AR nasal symptoms are mediated by histamine, LTs, prostaglandins, and trypsin secreted by sensitized mast cells, eosinophils and basophils (Shinmei et al., 2009). TDI, a highly reactive chemical, caused the mast cells activation and led to the development of nasal hypersensitivity in guinea pigs (Abe et al., 1993; Shill et al., 2016). In the present study, the TDI-induced guinea pig model presented the characteristic pathology of AR, including nasal discharge flow, sneezing, nasal rubbing, congestion, and edema. Furthermore, our study also revealed that Xinqin treatment strongly alleviated rhinitis symptoms, and the histamine and LTD4 release in TDI-challenged guinea pigs. Collectively, these results demonstrate that Xinqin alleviates rhinitis symptoms and protects mast cells against damage by suppressing histamine and LTD4 activity in the AR model in vivo.

AR is characterized by immediate type I allergic inflammatory reactions occurring due to interactions between IgE and certain antigens. Briefly, antigens stimulate the host thymic lymphocytes (T cells) to produce inflammatory cytokines, and promote specific IgE production in sensitized patients as well as the infiltration of immune cells, like mast cells (Benson et al., 2001). The allergic response in nasal mucosa challenged with TDI is characterized by the activation of mast cells and T cells to produce inflammatory cytokines (Abe et al., 1992; Benson et al., 2001). Specifically, TNF- α and IL-6 have been shown to be important in the pathogenesis of AR and are released via the IgE-dependent activation of mast cells (Grzelewska-Rzymowska and Pietrzakowicz, 2004; Oh et al., 2011). In turn, TNF- α upregulates the expression of endothelial cell adhesion molecules and activates eosinophils, mast cells and T cells (Bradding et al., 1993). IL-6 induces specific cellular allergic responses to inflammatory stimuli and activates T cells. Importantly, our findings also revealed that Xinqin treatment significantly reduced both IgE production and the expression of TNF- α and IL-6 in HMC-1 mast cells stimulated by the combination of A23187 and PMA and in the AR guinea pig model induced by TDI. Therefore, our results suggest that Xinqin exerts a strong anti-allergic effect via suppressing IgE production and reducing TNF- α and IL-6 expression.

The JAK/STAT signaling pathway has been reported to have a critical role in regulating the immune system, which regulates the balance of Th1/Th2 cells, as well as controlling mast cell homeostasis, proliferation, and secretion of inflammatory mediators (Rengarajan et al., 2000; Shuai and Liu, 2003). SOCS3 as a suppressor negatively regulates the JAK/STAT signal transduction pathway in the mast cells (Tamiya et al., 2011). In mast cells, JAK2 is the major JAK family member and STAT5 is essential for mast cell development (Shelburne et al., 2002; Shuai and Liu, 2003). It has been shown that STAT5 deficiency reduces IgE-mediated degranulation and prevents histamine, LT, TNF- α and IL-6 production in mast cells (Barnstein et al., 2006). Importantly, the results of our study indeed showed that Xinqin

treatment blocking the JAK2/STAT5 signal pathway both in vivo and in vitro models of AR.

In summary, our observations show that Xinqin exerts anti-allergic effects by inhibiting allergic and inflammatory mediator production both in vivo and in vitro. Furthermore, the JAK2/STAT5 signal pathway is involved in Xinqin-mediated anti-allergic activity and thereby resulting in inhibiting of allergic and inflammatory cytokine production, in activated mast cells. Overall, we present Xinqin exerts the anti-allergic activity by modulating mast cell-mediated allergic responses via inhibiting the JAK2/STAT5 signaling pathway. Our study could provide a mechanistic basis for the application of Xinqin in the treatment of AR.

5. Conclusion

The present results indicate that Xinqin alleviates TDI-induced rhinitis symptoms in a AR guinea pig model and decreases in levels of histamine, LTD4, IgE, TNF- α , and IL-6 in serum of guinea pig model of AR and in A23187/PMA-stimulated HMC-1 mast cells by down-regulating JAK2/STAT5 signaling pathway. It is suggested that the JAK2/STAT5 signaling pathway was involved in Xinqin-mediated anti-allergic effect. Thus, results from this study provide a mechanistic basis for the application of Xinqin in the treatment of AR.

Author contributions

Q.Z., N.G. and R.Z. conceived and designed the study and wrote the manuscript. Q.Z., Y.X., G.L., Q.T., M.C., J.H. and M.X. performed the experiments. C.H., Y.G. and Q.W. analyzed the data. N.G. and R.Z. integrated the data. Q.Z., C.H., Y.G. and Q.W. contributed reagents, materials and analysis tools. All authors reviewed and approved the manuscript.

Conflict of interest

The authors have declared that no competing interests exist.

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