

# Effects of Xingbi gel on leukotriene E4 and immunoglobulin E production and nasal eosinophilia in a guinea pig model for allergic rhinitis

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## Summary

**Background:** Allergic rhinitis (AR) is a chronic inflammatory disease of the nasal airways. Many therapies do not have immediate effects, even which have side-effects. However, the effects of Xingbi gel for the treatment of AR was investigated.

**Objective:** We investigated the effects of Xingbi gel on serum levels of leukotriene E4 (LTE4) and immunoglobulin E (IgE), as well as eosinophil counts in the nasal mucosa using a guinea pig model of allergic rhinitis (AR).

**Methods:** In addition to a healthy control group without AR, guinea pigs with AR were randomly divided into untreated AR control group, low-dose Xingbi gel (0.2483 g/mL) group, high-dose Xingbi gel (0.4966 g/mL) group, and budesonide group.

**Results:** Compared to the healthy controls, untreated AR guinea pigs had significantly higher ethology scores, serum LTE4 and IgE levels, and nasal mucosa eosinophil counts ( $p < 0.01$ ). Treatments with low-dose Xingbi gel, high-dose Xingbi gel, and budesonide significantly reduced the ethology scores, serum LTE4 and IgE levels, and nasal mucosa eosinophil counts as compared to untreated AR model guinea pigs ( $p < 0.01$ ).

**Conclusion:** Xingbi gel alleviates AR in part through inhibiting LTE4 and IgE production and reducing eosinophilia in the nasal mucosa. (*Asian Pac J Allergy Immunol* 2015;33:99-106)

**Keywords:** allergic rhinitis, leukotriene E4, immunoglobulin E, eosinophilia, inflammation

## Introduction

Allergic rhinitis (AR) is a chronic inflammatory disease of the nasal airways,<sup>1</sup> resulting from the inhalation of substances to which patients are sensitized, such as pollens, dust, or animal danders. The resulting production of immunoglobulin E (IgE) leads to the release of histamine, leukotrienes, and cytokines (e.g., IL-4) from basophils and mast cells, thereby producing localized inflammation. Although often considered a “nuisance condition,” AR affects the quality of life of numerous individuals on a global scale as its incidence is increasing worldwide with recent estimates of 14% in the U.S., 7% in Latin America, and 9% in the Asia-Pacific region.<sup>2</sup>

The primary goal of AR therapy is to alleviate its symptoms, including rhinorrhea (excess nasal secretion), itching, and nasal congestion and obstruction.<sup>3</sup> Current first-line treatment options include inhaled corticosteroids (e.g., budesonide) and systemic steroids (e.g., prednisone). However, steroids do not always have immediate, long-lasting effects, and prolonged steroid use invariably involves significant side effects, including skin angioedema, hives, rash, dermatitis, and pruritus.<sup>4</sup> Second-line treatment options include antihistamines as well as leukotriene (LT) receptor antagonists;<sup>5,6</sup> however, their effects are also not long-lasting and have been associated with significant side effects, including drowsiness, dizziness, and headache. Identification of a therapeutic agent that acts rapidly and without inducing significant side effects would thus benefit AR patients.

AR is a Type I allergy due to the IgE-mediated release of histamine, bradykinin and LTs. Studies have confirmed that serum IgE increases

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significantly in AR patients.<sup>7</sup> Therefore, inhibition of IgE production and interference of Th2-dependent allergic inflammation via synthetic compounds are a key AR therapeutic approaches as IgE is the primary inducer of allergic inflammation.<sup>8</sup> In addition to IgE, leukotrienes (LTs), including LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>, possess vasoactive and chemotactic effects and can promote mucus secretion and increased nasal airway resistance in AR.<sup>9</sup> LTE<sub>4</sub> is a prime AR target as it increases vascular permeability and mucus production; it is also a chemotactic factor for various inflammatory cells, particularly eosinophils, which contribute to chronic inflammation.<sup>10</sup> Thus, analyzing the effects of AR therapies on LTs may help determine the mechanism of action of specific compounds given the roles of LTs in the pathogenesis of AR.

Previous studies have investigated alternative therapeutic options using traditional Chinese herbal medicines (CHM) that target IgE production<sup>11</sup> and the release of inflammatory mediators by cultured cells.<sup>12</sup> While these results showed promising, albeit limited, effectiveness, many of these effects were assessed in cultured cells, which may not translate to clinical studies. Our group has worked with another traditional Chinese medicine, Xingbi gel, that has been used for the clinical treatment of AR for more than 8 years. Xingbi gel is comprised of a formulation of four traditional herbs, including *Radix cynanchi Paniculati* (*Pycnostelma paniculatum* K.Schum), *Periostracum Cicadae* (cicada slough), *Calculus bovis* (bezoar), and *Borneolum Syntheticum* (borneol). Its viscous nature permits direct application to the nasal mucosa that may provide long lasting effects and may minimize any systemic effects of the drugs, which is ideal for AR treatment. Foreign body sensation has been only observed in a few patients; however, the majority of patients were tolerant to it. In a small clinical study of AR patients, Xingbi gel application for 4 weeks significantly improved symptoms and decreased LTE<sub>4</sub> levels as compared to budesonide nasal

spray.<sup>13</sup> To better characterize the effects of Xingbi gel on AR, an AR model was generated in guinea pigs, and symptom relief, serum levels of LTE<sub>4</sub> and IgE, and nasal mucosa eosinophilia were assessed after intranasal application of either Xingbi gel or budesonide in the present study. These results may form the basis of further clinical studies of Xingbi gel for AR.

## Methods

### Experimental animals

Three-month-old Hartley male guinea pigs (210–250 g; License No.SCXK 0042965) were obtained from Slack Shanghai Laboratory Animals (Shanghai, China). Guinea pigs were maintained in our specific pathogen-free (SPF) Laboratory Animal Center at a constant temperature and humidity and with free access to food and water. All experimental protocols were approved by our institution's ethics committee for the experimental use of animals.

### Herbal and commercial medicines

Xingbi gel was obtained from the Peoples Hospital affiliated with the Fujian University of Traditional Chinese Medicine and consisted of a mixed formula composed of four major Chinese traditional herbs (Table 1). Briefly, 100 g of glycerin and 2 g of potassium sorbate were dissolved in 800 mL of Xuchangqing extract (TongChun Drug Co., Fujian, China) followed by the addition of 9 g of Carbomer 940 and 10 g of bletilla gum. The solution was incubated overnight after which 2 g of triethanolamine was added to create the gel matrix. Next, 22 g of Paeonol, the active ingredient of Xuchangqing (China National Institutes for Food and Drug Control; Batch# 110708-200506; purity >99%), 45 g of cicada slough, 12 g of bezoar, and 2 g of a  $\beta$ -cyclodextrin inclusion complex of natural borneol were mixed using the equivalent incremental mixing method and added into the gel matrix. After thoroughly mixing, distilled water was added with stirring to bring the volume to 1000 mL.

**Table 1.** Herbal components of Xingbi gel (% by weight).

Scientific name	Chinese name	%
<i>Radix cynanchi Paniculati</i> ( <i>Pycnostelma paniculatum</i> K.Schum)	Xuchangqing	10
<i>Periostracum Cicadae</i> (cicada slough)	Chantui	4.5
<i>Calculus bovis</i> (bezoar)	Niuhuang	1.2
<i>Borneolum Syntheticum</i> (borneol)	Bingpian	0.2



**Figure 1.** Protocol to establish an *in vivo* AR model via OVA sensitization. Guinea pigs were sensitized on alternating days. After 12 days, AR guinea pigs were randomly divided into various treatment groups. During the treatment period, AR animals received OVA 1 h for five times.

### AR model establishment and drug treatment

Figure 1 outlines the protocol used to establish the AR model in guinea pigs as well as the treatment schedules.<sup>14</sup> Briefly, guinea pigs were intraperitoneally injected with 0.5 mg of ovalbumin (OVA; Sigma, St. Louis, MO, USA; lot number: 076K7045) plus 30 mg of aluminum hydroxide powder (Fuchen Chemical Reagent Factory, Tianjin, China; lot number: 20060112) in 1 mL of sterile normal saline on alternating days for 12 days to establish the AR model ( $n = 52$ ). Healthy control guinea pigs ( $n = 13$ ) were injected with an equal volume of saline solution. After seven injections, AR guinea pigs were randomly divided into the following four groups: untreated control AR group ( $n = 13$ ), low-dose Xingbi gel group ( $n = 13$ ; 0.2483 g/mL Xingbi gel), high-dose Xingbi gel group ( $n = 13$ ; 0.4966 g/mL Xingbi gel), and the budesonide group ( $n = 13$ ; 1.28 mg/mL, AstraZeneca, Lund, Sweden; lot number: HB527). After induction of AR, 50  $\mu$ L Xingbi gel or budesonide were administered in each nostril 3 times daily for 12 days. The healthy control and untreated control AR groups received intranasal normal saline solution in the same manner.

As shown in Figure 1, mice also received a total of five OVA challenges throughout the treatment period. Specifically, at day 4 after establishing the AR model, AR guinea pigs received 50  $\mu$ L of a 2% OVA suspension in each nostril 1 h after drug treatment every other day. The healthy control guinea pigs received an equal volume of saline solution.

### Ethology scores

In accordance with the 1997 revised Standards for Allergic Rhinitis Diagnosis and Efficacy Evaluation used in China,<sup>15</sup> guinea pigs were scored for sneezing, nose scratching, and rhinorrhea after each OVA challenge over a 30 min period as shown

**Table 2.** Ethology scoring system.

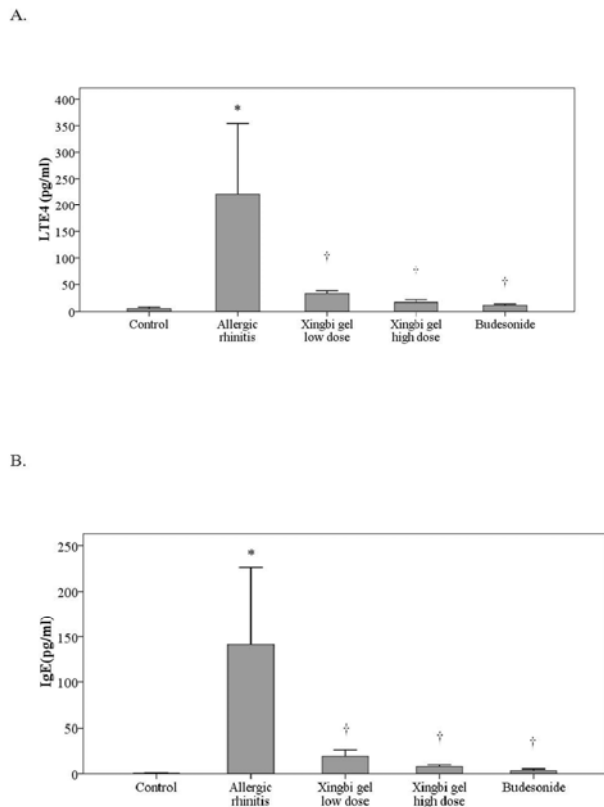
Scores for sneezing
0: no sneeze
1: 3-9 sneezes
2: 10-14 sneezes
3: > 15 sneezes
Scores for nose scratching
0: no nose scratching
1: nose scratching 2-3 times
2: nose scratching 3-5 times
3: nose scratching > 5 times
Scores for rhinorrhea
0: no rhinorrhea
1: presence of mucous in the nostrils
2: presence of mucous outside the nostrils
3: presence of mucous on the face

in Table 2. Total scores were calculated by the superposition method. A score of  $\geq 5$  was indicative of successful AR model establishment.<sup>14</sup>

### Serum LTE4 and IgE

At the end of the treatment period, guinea pigs were sacrificed by decapitation, and blood samples were immediately collected. After centrifugation of the samples, serum LTE4 and IgE levels were determined using commercially available enzyme-linked immunosorbent assay kits specific for guinea pig LTE4 and IgE, according to the manufacturers' instructions (Adlitteram Diagnostic Laboratories, Chicago, IL, USA; lot number: RT110371). Results were determined with a microplate reader (Model EL311, BioTek Instruments, Winooski, VT, USA) at a wavelength of 450 nm. Standard curves were prepared using standards provided with the kits.





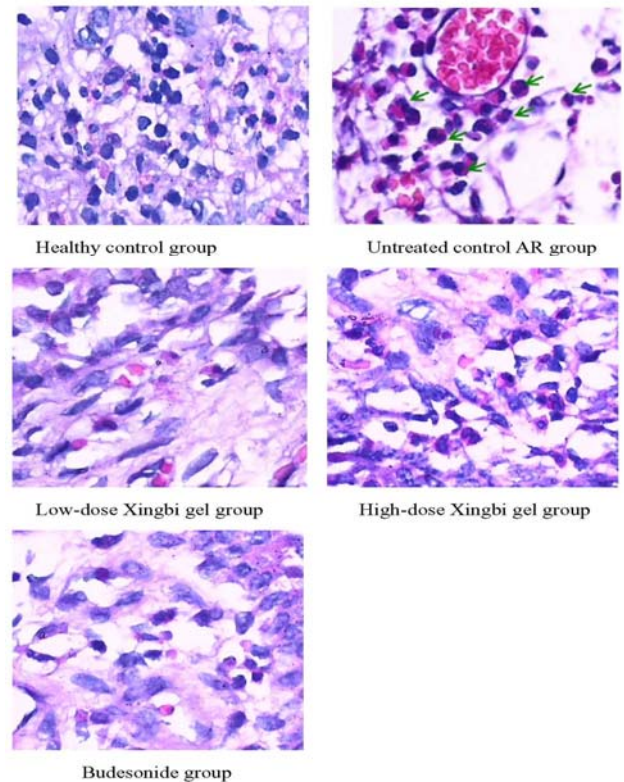
**Figure 2.** Xingbi gel reduces serum LTE4 and IgE levels. At the end of the experiment, serum was analyzed for (A) LTE4 and (B) IgE levels. Results shown are means  $\pm$  standard deviations. \*  $p < 0.01$  vs. the healthy controls group; †  $p < 0.01$  vs. the untreated AR group.

### Nasal mucosa pathology

At the end of the study period, nasal conchae and septal mucosa tissue samples were fixed in 10% formalin, paraffin-embedded, and sectioned at 5  $\mu$ m thickness. Tissue sections underwent H&E staining. Analysis of a total of five microscopic fields (10  $\times$  40  $\mu$ m) were observed in each tissue using a BH-2 binocular optical microscope (Olympus, Tokyo, Japan), and the average eosinophil counts were recorded for each group.

### Statistical analysis

Continuous variables are presented as means and standard deviations. Repeated measures analysis of variance (ANOVA) with Bonferroni post-hoc tests were used to assess OVA nasal challenge time and treatment effects for ethology scores for all five groups. One-way ANOVA with Bonferroni post-hoc tests were used to compare serum LTE4 and IgE



**Figure 3.** Xingbi gel reduces eosinophilia of the nasal mucosa associated with AR. At the end of the experiment, nasal conchae and septal mucosal tissues were stained with H&E, and eosinophils were counted. Representative photomicrographs are shown. Arrows indicate eosinophils. Magnification  $\times 400$ .

levels and eosinophil counts among the groups. Statistical analyses were undertaken using SPSS software version 17 (SPSS Inc, Chicago, IL, US). A two-tailed  $p$ -value of  $< 0.05$  was considered significant.

## Results

### Effect of Xingbi gel on AR symptom relief

The effectiveness of Xingbi gel and budesonide for alleviating AR symptoms induced after each OVA challenge was determined by ethology scoring using the variables shown in Table 2. As shown in Table 3, significantly greater ethology scores were observed in the untreated control AR group as compared to the healthy controls, indicating a successful induction of AR ( $p < 0.01$ ). Although all treatment results in a decreased ethology score as compared to the untreated control groups at the fourth and fifth OVA challenges ( $p < 0.01$ ), only



**Table 3.** Effects of Xingbi gel and budesonide on AR symptoms.

	Nasal symptom score after each OVA challenge				
	1st	2nd	3rd	4th	5th
Healthy controls (n=10) <sup>1</sup>	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Untreated controls (n=9) <sup>1</sup>	2.3±2.1*	2.8±2.0*	2.0±1.7*	4.9±2.4*	4.6±1.8*
Low-dose Xingbi gel (n=10) <sup>1</sup>	1.4±1.6*	1.8±1.9*	1.7±1.9*	2.6±1.4**†	2.2±1.9**†
High-dose Xingbi gel (n=8) <sup>1</sup>	1.0±1.2	1.4±1.5	1.4±1.4	1.9±2.1**†	2.5±1.7**†
Budesonide (n=8) <sup>1</sup>	1.6±1.7*	1.9±1.4*	1.9±1.9*	2.3±2.2**†	2.3±0.7**†

<sup>1</sup>Some guinea pigs died during the process of nasal administration of drug or saline, in part due to suffocation.

\* significantly different from the healthy control group.

† significantly different from the untreated control group.

the high-dose Xingbi group had scores that were comparable to the healthy control group after the first three OVA challenges. Thus, high-dose Xingbi gel was more effective than budesonide at alleviating AR symptoms in vivo in the early challenges; however, similar effectiveness was observed with later challenges.

#### ***Xingbi gel reduces serum LTE4 and IgE levels***

As shown in Figures 2A and 2B, respectively, serum LTE4 and IgE levels were significantly higher in the untreated control AR group as compared to the healthy control group at the end of the study ( $p < 0.01$ ). However, both serum LTE and IgE levels were significantly reduced in all of the treatment groups as compared to the untreated controls (all,  $p < 0.01$ ). There were no differences in either LTE4 or IgE levels between the low-dose and high-dose Xingbi gel groups.

#### ***Xingbi gel reduces eosinophils of the nasal mucosa in AR***

Eosinophil infiltration of the nasal mucosa was determined by histological analysis of H&E stained tissues. Figure 3A shows representative images for each group with eosinophils indicated by arrows. As shown in Figure 3B, eosinophil counts in the nasal mucosa were significantly greater in the untreated AR group compared to the healthy control group ( $p = 0.001$ ). However, treatment with either low- or high-dose Xingbi gel significantly reduced the eosinophil counts as compared to the untreated AR group (3.36 and 3.43 vs. 6.84,  $p < 0.01$ ). Although budesonide did not significantly reduce the eosinophil counts as compared to that observed for the untreated AR controls, the levels were comparable to those found in the healthy control group.

## **Discussion**

Inhaled corticosteroids or systemic steroids are considered current first-line treatments for AR; however, these therapies do not have immediate effects, which are of limited duration, and significant side-effects have been associated with prolonged use. In addition, side-effects have been associated with second-line treatment options, including antihistamines and leukotriene receptor antagonists. In this study, the effects of Xingbi gel for the treatment of AR was investigated. We successfully established a model of AR in guinea pigs, which exhibited the symptoms as well as the inflammation (e.g., increased serum IgE and LTE4 levels and nasal mucosal eosinophilia) characteristic of AR. Xingbi gel effectively reduced the AR symptoms, serum levels of IgE and LTE4, and the eosinophil counts in the nasal mucosa to levels similar to that observed for the inhaled corticosteroid, budesonide. These results indicate that Xingbi gel has both localized and systemic effects similar to budesonide.

Previous studies also investigated CHM for possible therapeutic effects associated with AR and included human clinical trials<sup>11,16,17</sup> as well as in vitro studies with mast cells,<sup>8</sup> monocytes and neutrophils,<sup>18</sup> basophils,<sup>19</sup> and an IgE-producing B cell line.<sup>20</sup> Although it has been used in the clinical treatment of AR for longer than 8 years, to our knowledge, this is the first report to show the therapeutic benefits of Xingbi gel for AR. Its direct administration into the nasal cavity may prolong the time of action as compared to nasal sprays. Furthermore, unlike the previous studies assessing CHM effects on AR, we established an in vivo model with which we could investigate both the

clinical affects of our preparation and explore the possible underlying mechanisms that account for these effects.

Because Xingbi gel is comprised of a formulation of four traditional herbs, including *Radix cynanchi Paniculati* (*Pycnostelma paniculatum* K.Schum), *Periostracum Cicadae* (cicada slough), *Calculus bovis* (bezoar), and *Borneolum Syntheticum* (borneol), further studies will need to elucidate the respective roles and active ingredients of these herbs in mediating the therapeutic effects in AR patients. Paeonol is the active ingredient of *Pycnostelma paniculatum* K.Schum; cicada slough contains high levels of chitin protein, L-valine,  $\gamma$ -aminobutyric acid, tyrosine, glutamic acid as well as adenosine triphosphatase.<sup>21</sup> Bezoar contains sodium taurocholate, bile acid, ursodeoxycholic acid, hyodeoxycholic acid, chenodeoxycholic acid, deoxycholic acid and cholesterol; the major component of borneol is D-borneol.<sup>21</sup> In addition, given that AR is a chronic inflammatory disease that involves a complex network of inflammatory cells and mediators further studies will also focus on identifying the specific biochemical pathways that mediate its reduction in serum IgE and LTE4 levels.

AR is a typical IgE-mediated type allergic reaction and IgE levels are significantly increased in serums of AR patients and in animal AR models.<sup>22</sup> As such, IgE tests are a valuable tool, which can improve clinical diagnostic and optimize interventions and economic benefits.<sup>23</sup> In the present study, IgE levels reached up to 150 pg/mL in the untreated AR control group, which was similar to the trend reported in children.<sup>24</sup> After sensitized nasal mucosa cells come in contact with the sensitizing allergen, an allergen-IgE reaction results in degranulation of mast cells and basophils, which release histamine<sup>1</sup> and thereby promote capillary permeability and increased mucus secretion. Previous studies also found reduced IgE production using different CHM formulations,<sup>16,20</sup> which suggests that CHM formulations can effectively alleviate one mechanism associated with AR symptoms. As with our formulation, the active ingredient(s) that lowered IgE production was unknown.

Because disruption of only the immediate aspects of inflammation (i.e., IgE-mediated histamine release) may have limited efficacy for AR, we also analyzed the effects of Xingbi gel on another downstream mediator, LTE4.<sup>25</sup> LTE4 is the

metabolic end product of cysteinyl leukotriene,<sup>26</sup> which also has pro-inflammatory effects; it mediates contraction of smooth muscle cells in the respiratory tract and acts as a chemoattractant for pro-inflammatory cells, particularly eosinophils, which contribute to increased endothelial permeability and mucus secretion.<sup>27</sup> Moreover, the accumulation of eosinophils in the nasal mucosa can lead to changes in the entire mucosal cellular network and is thought to result in the chronic inflammatory status associated with AR.<sup>1</sup> In the present study, Xingbi gel reduced LTE4 levels, which is similar to previous reports using CHM that showed beneficial effects in terms of mediator release and leukotrienes, in particular.<sup>12,19</sup> We also observed that Xingbi gel significantly reduced the eosinophil counts to levels comparable with the control group; however, similar effects were not observed in the budesonide group which was likely due to the large variance associated with the effects. Of note, one study also found that Niu Huang disrupted eosinophil chemotaxis towards LTE4.<sup>18</sup> As shown in Table 1, Niu Huang is one of the four CHM in Xingbi gel. Thus, this ingredient warrants further investigation as a potential active ingredient, mediating the effects of Xingbi gel on AR.

Similar to the present study, we previously showed that Xingbi gel application for 4 weeks significantly improved symptoms and decreased serum LTE4 levels as compared to budesonide nasal spray.<sup>13</sup> Thus, topical application of Xingbi gel induced systemic changes. This may be related to the inhibition of IgE synthesis and/or reduction in IgE levels, influencing the biological synthesis of LTs. In addition, it may act on LT receptors to inhibit LT release, thereby attenuating the chemotactic effect on neutrophils, eosinophils and monocytes. This would, in turn, reduce the production of inflammatory mediators and pro-inflammatory cytokines as well as attenuate the infiltration and aggregation of inflammatory cells, alleviating nasal inflammation. However, further studies are necessary to fully elucidate the underlying mechanisms related to the systemic changes observed.

Although this was only a preliminary investigation of the effects of Xingbi gel for use in treating AR, we should note several study limitations. First, we only investigated IgE, LTE4, and eosinophils in the nasal mucosa. Although these are major contributors, there are numerous other cell types and inflammatory mediators involved in AR

pathogenesis that we did not investigate, including the IL-5, IL-6, TNF- $\alpha$  and NF-KB signaling pathways.<sup>28,29</sup> Therefore, further investigations are required to understand the effects of Xingbi gel on other inflammatory cells of the nasal mucosa using in vitro studies as well as clinical samples. Additionally, while Xingbi gel reduced the levels of these inflammatory markers, the underlying biochemical pathways for these effects were not determined. Finally, as with much CHM, the Xingbi gel was not pharmaceutically formulated; therefore, its precise compositions remain unknown.

In summary, using a guinea pig model of AR, Xingbi gel alleviated AR symptoms and reduced the serum levels of IgE and LTE4 as well as eosinophilia of the nasal mucosa. Thus, once the active ingredient(s) in Xingbi gel are identified, this preparation may prove useful for disrupting both the immediate IgE-mediated reactions, but also the chronic inflammation in AR, which makes it and other atopic disorders so intractable.

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