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*Original Article*

# Liang Xue Qu Shi Zhi Yang soup ameliorates dinitrochlorobenzene-induced atopic dermatitis in mice

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**Abstract:** Aim: Atopic dermatitis (AD) is a chronic inflammatory skin disease with no curative treatment. Traditional Chinese medicine Liang Xue Qu Shi Zhi Yang (LXQSZY) soup with documented anti-pruritic property has long been employed to ameliorate the clinical symptoms of AD. The present study aimed to examine the therapeutic effect of LXQSZY soup on 2,4-dinitrochlorobenzene-induced AD in mice. Methods: To induce AD in male BALB/c mice, skin with hair removed was challenged with 2,4-dinitrochlorobenezne. Model mice were then given LXQSZY soup (therapeutic group, n=66) and Ebastine (control group, n=66) twice a day for two consecutive weeks. Before and after treatment, AD symptoms (erythema, edema, exfoliation, and lichenification) were scored. Leukotriene $B_4$ (LTB4) in AD lesions, and interleukin (IL)-2 and -4 in sera were measured using enzyme-linked immunosorbent assay. Gene and protein expression of aquaporin 3 (AQP3) in AD lesions was determined using real-time PCR and Western blot, respectively. Results: Treatment of AD mice with LXQSZY soup relieved erythema, edema, exfoliation, and lichenification of AD lesions, leading to an overall efficacy rate of 84.62%, which was significantly higher than that of Ebastine (64.62%, $P < 0.01$). Compared to Ebastine, treatment with LXQSZY soup significantly suppressed LTB4 in AD lesions and IL-4 in sera ($P < 0.01$). LXQSZY soup also up-regulated IL-2 in mice serum. In addition, LXQSZY soup but not Ebastine reduced gene and protein expression of AQP3 in AD lesions. Conclusion: LXQSZY soup ameliorated AD by modulating leukotriene $B_4$, IL-2, IL-4, and AQP3 in AD mice, of which the efficacy was higher than that of Ebastine. The soup would be further developed into new therapeutic agents for treatment of AD.

**Keywords:** Atopic dermatitis, 2,4-dinitrochlorobenzene, Liang Xue Qu Shi Zhi Yang (LXQSZY) soup, interleukin, leukotriene $B_4$, aquaporin 3

## Introduction

Atopic dermatitis (AD) a chronic inflammatory skin diseases affecting 11-30% of children and 2-10% of adults worldwide [1, 2]. The skin disease is characterized by pruritic and relapsing eczematous dermatitis. The underlying pathogenesis of AD is complex and multifactorial, involving interplays between genetic predisposition, environmental factors, skin barrier disorder, and dysfunction in immune system (i.e. excessive production of immunoglobulin E, hyperactivation of mast cells, and dysregulation of T helper cell) [3, 4]. These deregulated immune networks contribute to development of asthma, allergic rhinitis, and food allergies in patients with AD [5]. AD negatively affects the patients' quality of life, of which the prevalence could be unacceptably high, approximately 50% of children with AD [6]. Patients with severe AD are also at risk of depression, anxiety, and other mental disorders [7]. Disappointedly, the current mainstream treatment like anti-inflammatory medications can only achieve symptomatic relief. New curative therapeutic agents for AD treatment are urgently needed.

According to the pharmacopoeia of traditional Chinese medicine, and from our clinical experiences, AD is resulted from the undesirable accumulation of heat and humidity inside the body. Herbal regimens with blood-cooling (*Liang Xue*) and damp-clearing (*Qu Shi*) properties are therefore believed to hold promise in itch and AD control (*Zhi Yang*). In this study, Liang Xue Qu Shi Zhi Yang (LXQSZY) soup was used to

**Table 1.** Effects of different treatments on the symptoms of AD

| Group | Erythema | Edema | Exfoliation | Lichenification |
|---|---|---|---|---|
| Control (n=65) | | | | |
| Before Treatment | 2.77±0.23[a] | 2.58±0.23[a] | 1.48±0.20[a] | 1.46±0.12[a] |
| After Treatment | 2.41±0.19[b] | 2.19±0.15[b] | 1.44±0.38[b] | 1.33±0.10[b] |
| Therapeutic (n=65) | | | | |
| Before Treatment | 2.62±0.14 | 2.58±0.33 | 1.46±0.18 | 1.44±0.20 |
| After Treatment | 0.15±0.09[c,d] | 0.23±0.02[c,d] | 0.57±0.08[c,d] | 0.15±0.03[c,d] |

Remark: a, comparison between control and therapeutic groups before treatment ($P > 0.05$); b, comparison between before and after treatment within control group ($P > 0.05$); c, comparison between before and after treatment within therapeutic group ($P < 0.01$); d, comparison between control and therapeutic groups after treatment ($P < 0.01$).

**Table 2.** Overall treatment outcomes of therapeutic and control groups

| Group | *n* | Cured | Significantly improved | Improved | No Response | Overall efficacy (%) |
|---|---|---|---|---|---|---|
| Control | 65 | 24 (36.92) | 18 (27.69) | 18 (27.69) | 5 (7.69) | 42 (64.62) |
| Therapeutic | 65 | 44 (67.69) | 11 (16.92) | 10 (15.38) | 0 (0) | 55 (84.62) |

treat AD with satisfactory results. The underlying mechanism of LXQSZY soup however is far from clear. In this context, the present study aimed to examine the therapeutic effects of LXQSZY soup on a mouse model of chemically-induced AD. Few AD mediators including leukotriene $B_4$ (LTB4), interleukin (IL)-2 and 4, and aquaporin 3 (AQP3) were studied. The findings provide valuable insights into the novel use of TCM in AD treatment.

## Materials and methods

### *Animal*

SPF-graded male BALB/c mice aged 5 weeks with body weight of about 22±3 g were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). Mice were acclimated for at least 3 days before the experiment, and were kept in temperature (22±1°C) and humidity (40±10%) controlled animal facility in 12 light/12 dark cycles with free access to water and food.

### *Herbal regimen and drug*

LXQSZY soup consisted of 30 g water buffalo horn, 15 g Rehmannia glutinosa, 12 g Paeonia suffruticosa, 12 g Paeonia lactiflora, 10 g Sophora flavescens, 30 g raw coix seed, 15 g Poria cocos, 10 g Aconitum gymnandrum, 30 g Cogongras rhizome, 15 g Kochia scoparia, and 6 g Glycyrrhiza uralensis. All the medicinal herbs were purchased from Beijing Tcmages Pharmaceutical Co., LTD (Beijing, China). To prepare the decoction, herbs were boiled, filtered and concentrated (1 g crude drug per mL).

The control drug Ebastine (10 mg) was obtained from Lianhuan Pharmaceutical (Yangzhou, Jiangsu, China).

### *Dinitrochlorobenzene-induced atopic dermatitis*

The chemically-induced AD model was established in 134 mice as described [8]. In brief, at the dorsal and abdominal sides of mice, hair was removed from two areas designated as area A (1 × 2 cm) and B (2 × 2 cm), which were 1 cm apart from each other. On area A, 7% 2,4-dinitrochlorobenzene was applied, and two weeks later, 0.1% 2,4-dinitrochlorobenzene was applied on area B. The challenge was done once a week for four consecutive weeks.

### *Animal grouping and treatment*

Two mice died at the end of the model establishment, with the surviving 134 model mice randomly assigned into therapeutic (n=66) and control (n=66) groups. Mice of both groups showed no significant difference in body weight ($P > 0.05$). Mice of the therapeutic group were intragastrically administered twice a day a concentrated LXQSZY soup (1 g) dissolved in 2 mL physiological saline. Mice of the control group were given intragastric treatment of Ebastine (10 mg) dissolved in 2 mL physiological saline twice a day. Both treatment regimens lasted for 2 weeks. During treatment, in both treatment and control groups, there was a model mouse dying from asphyxiation due to accidental drug administration to mouse's trachea.

### *Assessment of atopic dermatitis and treatment outcome*

Before and after treatment, atopic dermatitis in model mice was assessed following the pub-

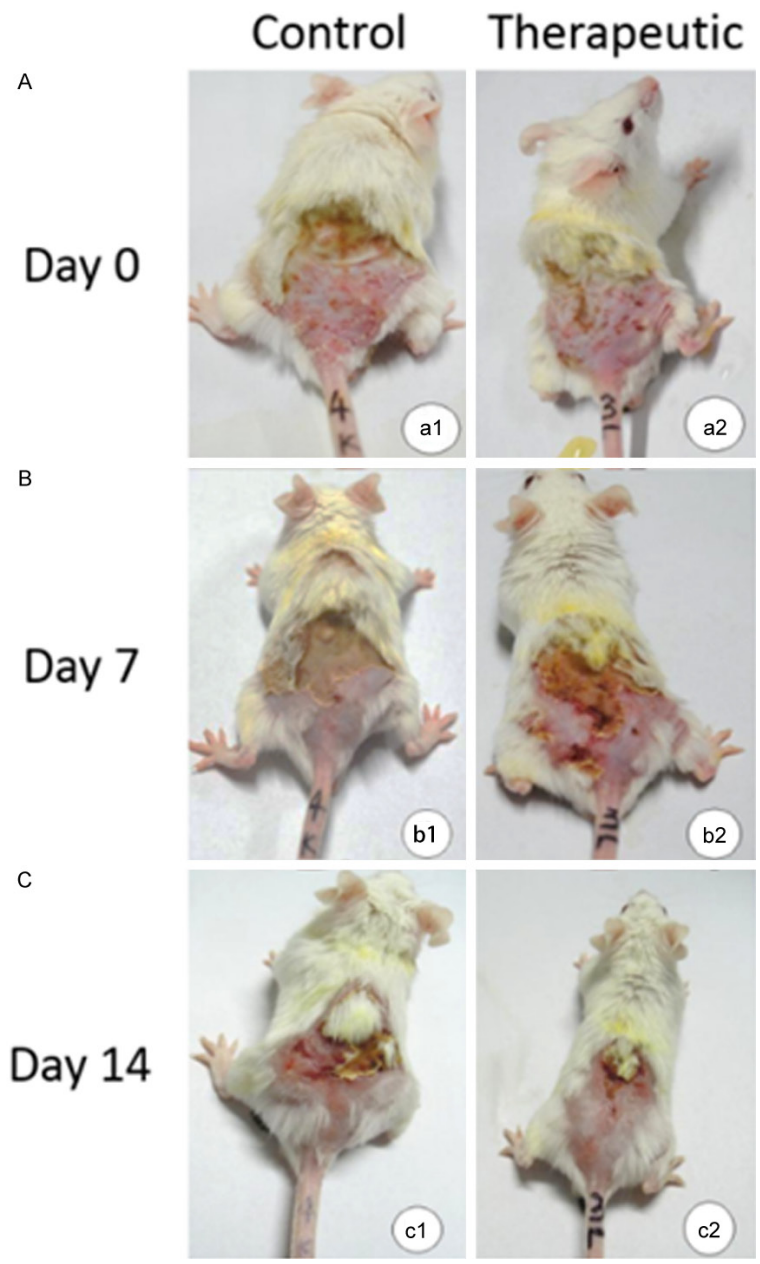


**Figure 1.** Effect of LXQSZY soup on 2,4-dinitrochlorobenzene-induced AD in mice. AD mice receiving Ebastine (control group) and LXQSZY soup (therapeutic group) were photographed at day 0, 7, and 14 of the treatment course. Significant amelioration of AD symptoms was noted in mice treated with LXQSZY soup.

**Table 3.** Effects of different treatments on the leukotriene $B_4$ level (ng/mL) in AD lesion

| Group | Control (n=65) | Therapeutic (n=65) |
|---|---|---|
| Before Treatment | 30.15±5.11 | 29.87±0.65 |
| After Treatment | 19.21±1.98 | 8.44±2.21 |

lished guideline of Eczema Area and Severity Index (EASI), with the four clinical symptoms of atopic dermatitis (i.e. erythema, edema, exfoliation, and lichenification) graded using Six Area, Six Sign Atopic Dermatitis (SASSAD) severity score, with a scale ranging from 0 to 3 (0, absent; 1, mild; 2, moderate; and 3, severe) [9]. The overall treatment outcomes were evaluated according to the Guiding Principles of Clinical Research on Traditional Chinese Medicine, and were classified as cured, significantly improved, improved, and no response. "Cured" represented complete resolution of skin erosion, with the severity of symptoms reduced by over 95%. "Significantly improved" referred to skin conditions with erosion largely resolved, with the severity of symptoms reduced by at least 70%. "Improved" referred to skin conditions with erosion partially repaired, with the severity of symptoms reduced by at least 50%. "No response" means no improvement in skin condition, with the severity of symptoms reduced by less than 50%.

*IL-2, IL-4 in serum and leukotriene $B_4$ in skin lesion*

The levels of IL-2, IL-4 in serum and LTB4 level in skin lesion were determined using enzyme-linked immunosorbent assay (ELISA). In brief, primary antibody against the analyte was coated on 96-well plates, following by the addition of diluted serum or skin tissue. After washing the bound analyte was detected using specific horseradish peroxidase (HRP)-conjugated antibody. Signal generated from TMB was then measured as optical density at 450 nm ($OD_{450}$). Concentrations of IL-2, IL-4, and

**Table 4.** Effects of different treatments on the IL-2 and IL-4 levels (pg/mL) in serum

| Group | Control (n=65) | | Therapeutic (n=65) | |
|---|---|---|---|---|
| | IL-2 | IL-4 | IL-2 | IL-4 |
| Before Treatment | 2.87±0.13 | 55.14±3.21 | 2.79±0.28 | 56.17±1.85 |
| After Treatment | 2.56±0.19 | 50.44±4.17 | 4.83±0.24 | 11.46±0.74 |

**Table 5.** Effects of different treatments on AQP3 protein level in AD lesion

| Group | Control (n=65) | Therapeutic (n=65) |
|---|---|---|
| Before Treatment | 1.46±0.19 | 1.49±0.18 |
| After Treatment | 1.29±0.24 | 0.58±0.03 |

leukotriene $B_4$ were subsequently calculated from the $OD_{450}$ values against the corresponding standard curve.

*Protein level of AQP3 in skin lesion*

AD mouse skin lesion was homogenized in lysis buffer. The resulting lysate was quantified using bicinchoninic acid assay, resolved in SDS-PAGE, and subsequently transferred onto PVDF membrane [10]. AQP3 protein was detected using goat anti-mouse AQP3 antibody (1:1000 dilution, Oubei Biotechnology, Beijing, China). Signal density of the detected protein was quantified using ImageJ software.

*Gene expression of AQP3 in skin lesion*

Total RNA was extracted from skin tissues, and was transcribed into first-strand cDNA using RevertAid RT Reverse Transcription kit (ThermoFisher Scientific, Waltham, MA, USA). AQP3 was then amplified from the resulting cDNA by Applied Biosystems 7500 Fast Real-Time PCR System (ThermoFisher Scientific) using specific oligonucleotides recognizing AQP3 (forward, 5'-CATAGGCACAGCAGCCCTTA-3'; reverse, 5'-CCCAATGACCAGGACCACAA-3') [11]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal control (forward, 5'-ACTCTGTGTGGATTGGTGGC-3'; reverse, 5'-AGAAAGGGTGTAAAACGCAGC-3'). The level of AQP3 was determined as relative expression by $2^{-\Delta\Delta CT}$ algorithm.

*Statistical analysis*

Data were analyzed using statistical software SPSS version 15.0. All measurement data are presented as mean ± standard derivation ($\bar{X}$ ± S), with the difference between treatment and control groups evaluated using paired *t* test. The overall treatment outcomes between groups were compared using Wilcoxon rank-sum test. Significant difference was indicated by *P* value < 0.05.

## Results

*Effect of LXQSZY soup on the severity of AD symptoms*

The severity of AD in model mice before and after drug treatment was assessed by scoring erythema, edema, exfoliation and lichenification (**Table 1**). In the control group, the severity score of each symptom decreased very slightly after Ebastine treatment, with the differences not statistically significant. In the therapeutic group, there was substantial reduction in the severity scores of all the symptoms after LXQSZY soup treatment ($P < 0.01$). Before any treatment, the severity of AD in both control and therapeutic groups was comparable to each other with no significant difference between the two ($P > 0.05$). Notably, the symptoms of AD in mice treated with LXQSZY soup were less severe than those treated with Ebastine ($P < 0.01$).

*Effect of LXQSZY soup on overall treatment outcome*

The overall treatment outcomes of the control and therapeutic groups were evaluated (**Table 2**; **Figure 1**). In the therapeutic group, 44 (67.69%) AD mice were completely cured, with 11 (16.92%) mice showing significant improvement in severity, leading to an efficacy rate of 84.62%. In the control group, there were only 24 (36.92%) AD mice were cured, with 18 (27.69%) mice with their skin condition significantly improved, resulting in an efficacy rate of 64.62%. Our analysis using Wilcoxon rank-sum test further suggested the overall treatment outcome of the therapeutic group was significantly improved compared to the control group ($x^2$=-3.624, $P < 0.01$).

*Effect of LXQSZY soup on LTB4 in AD lesions*

LTB4 in the AD lesions was quantified using ELISA (**Table 3**). Before treatment, LTB4 levels in therapeutic and control groups were compa-

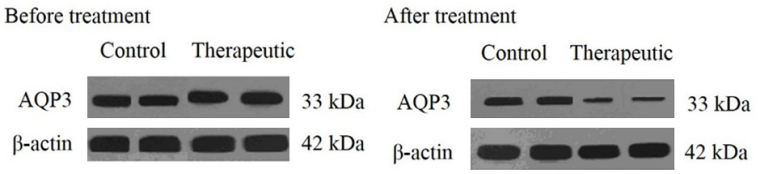


**Figure 2.** Protein levels of AQP3 in AD lesions of the control and therapeutic groups were examined using western blot. The intensities of protein bands were quantified using ImageJ.

**Table 6.** Effects of different treatments on AQP3 gene expression in AD lesion

| Group | Control (n=65) | Therapeutic (n=65) |
|---|---|---|
| Before Treatment | 2.10±0.29 | 2.11±0.37 |
| After Treatment | 2.04±0.18 | 1.15±0.16 |

rable, showing no significant difference between each other. In the control group, Ebastine treatment reduced LTB4 level by about 1.5 folds ($P < 0.05$), while in the therapeutic group, treatment with LXQSZY soup suppressed LTB4 level by nearly 3.6 fold ($P < 0.01$). Strikingly, the posttreatment level of LTB4 in the therapeutic group was substantially lower than that in the control group ($P < 0.01$).

*Effect of LXQSZY soup on serum IL-2 and IL-4*

IL-2 and IL-4 in the sera of AD mice was examined using ELISA (**Table 4**). Before treatment, IL-2 and IL-4 levels of therapeutic and control groups were comparable, showing no significant difference between each other. In control group, Ebastine treatment altered neither IL-2 nor IL-4. In the therapeutic group, treatment with LXQSZY soup significantly decreased serum IL-4 by nearly five-fold ($P < 0.01$), and oppositely increased serum IL-2 by about 1.7-fold ($P < 0.01$). Differences in the post-treatment levels of IL-2 and IL-4 between control and therapeutic groups were significant ($P < 0.01$).

*Effect of LXQSZY soup on AQP3 expression in AD lesions*

The modulating effect of LXQSZY soup on the protein and gene expression of AQP3 in skin lesions of AD mice was examined using Western blot and real-time PCR, respectively. Like leukotriene B4, IL-2, and IL-4, AQP3 protein levels of therapeutic and control groups before treatment were comparable, showing no significant difference between each other (**Table 5**; **Figure 2**). Treatment with Ebastine did not suppress AQP3 in skin lesion, however, administration of LXQSZY soup reduced AQP3 protein expression by about 2.5 folds ($P < 0.01$). Furthermore, the post-treatment AQP3 protein expression was significantly lower in the therapeutic group than in the control group ($P < 0.01$).

To determine whether LXQSZY soup would also module gene expression of AQP3 in skin lesions of AD mice, AQP3 gene expression was quantified (**Table 6**). Like the protein expression, the pretreatment levels of therapeutic and control groups showed no significant difference between each other. Treatment with Ebastine did not reduce AQP3 gene expression in AD lesions, however, administration of LXQSZY soup reduced AQP3 protein expression by about 1.8 folds ($P < 0.01$). In addition, the post-treatment AQP3 gene expression was significantly lower in therapeutic group than in control group ($P < 0.01$).

## Discussion

The present work demonstrates for the first time that TCM Liang Xue Qu Shi Zhi Yang soup can effectively suppress dinitrochlorobenzene-induced AD in mice. The overall efficacy of LXQSZY soup in reducing the severity of AD was higher than that of antihistamine Ebastine, implicating the potential use of LXQSZY soup in the management of patients with AD.

According to the pharmacopoeia of TCM, AD is developed from the accumulation of heat and humidity inside the body, which is resulted from the impaired pancreatic function. LXQSZY soup is one of the blood-cooling and damp-clearing regimen that has been used by TCM practitioners to relieve itch in patients with AD. In this study, we further demonstrate that a 2-week treatment of AD mice with LXQSZY soup could effectively ameliorate the symptoms of AD including erythema, edema, exfoliation, and lichenification. Notably, the efficacy rate of LXQSZY soup was higher than Ebastine, an anti-histaminic agent that is useful for symptomatic relief of AD (LXQSZY soup, 84.62%; Ebastine, 64.62%).

The anti-AD function of LXQSZY soup can be at least partly attributed to the suppression of LTB4 and IL-4 level in skin lesion and blood, respectively. LTB4 is a potent itch mediator [12], and is also a stimulant to the recruitment of inflammatory neutrophil into the site of AD [13]. Pharmacologic blockade of LTB4 was shown to inhibit AD-associated allergic skin inflammation [14]. For IL-4, it is a T helper 2 cytokine that plays key role in the pathogenesis of AD [15]. IL-4 was demonstrated to exacerbate the T cell-mediated inflammatory reaction of AD by suppressing the anti-inflammatory cytokine IL-10 and potentiating toll-like receptor 2 activation [16]. Indeed, a human anti-IL4 receptor monoclonal antibody could significantly improve clinical signs and symptoms in adults with AD in a phase 2 clinical trial [17].

LXQSZY soup was also found to significantly upregulate IL-2 level in AD mice. Although known to be essential to the differentiation of regulatory T cell [18], the role of IL-2 in the pathophysiology of AD is not well-documented as leukotriene B4 and IL-4. It has been suggested that production of IL-2 from immune cells like mast cells could contribute to the suppression of dermatitis [19]. An early study demonstrated that in patients with severe AD that were refractory to conventional therapy IL-2 treatment could relieve clinical symptoms and signs of AD. However, the symptoms were recurred 2-6 weeks after discontinuation of therapy [20]. Given LXQSZY soup was shown capable of elevating IL-2 level, it would be an alternative to IL-2 in AD therapy.

Our work further shows that LXQSZY soup can reduce expression of AQP3 in skin lesion. AQP3 is a water/glycerol-transporting protein predominantly found in keratinocytes of the epidermis. In patients with AD, increased expression and altered cellular distribution of AQP3 can contribute to the increased water loss in AD lesions [21]. AQP3 was also implicated in the epidermal hyperplasia in AD [22]. Suppressing AQP3 expression by nicotinamide decreased permeability and water loss in cultured human skin keratinocytes [23]. As such, by its ability to suppress AQP3, treatment with LXQSZY soup would improve the dry skin of AD.

In summary, the present study provides evidence supporting LXQSZY soup as a promising therapeutic agent for the treatment of AD. LXQSZY soup modulated LTB4, IL-2, IL-4, and AQP3 in AD mice, resulting in significant ameliorations in erythema, edema, exfoliation, and lichenification. AD, which is one of the most common skin disease affecting millions of populations worldwide and which lacks effective treatment. LXQSZY soup can be a potential alternative to anti-histamine and corticosteroid therapies. The safety, anti-pruritic property, and promoting effect of LXQSZY soup on skin integrity will further investigated.

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## Disclosure of conflict of interest

None.

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