Acupoint catgut-embedding therapy ameliorates DNCB-induced atopic dermatitis in BALB/c mice by regulating Th2 type immune response and reducing infiltration of CD4\(^+\) and CD8\(^+\) cells

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**Author contributions**

Qin C and Kong XY both made equal contributions to this effort, which included data analysis and manuscript drafting. The work was conceived, and supervised, and the manuscript was revised by Yang XS and Ye J. The data was gathered and the animal trials were carried out by Wang F, Xu J, and Zhang Z. The final draft that was accepted for publication was approved by all writers.

**Competing interests**

The authors declare no conflicts of interest.

**Acknowledgements**

This work was supported by grants from the National Natural Science Foundation of China (Grant No. 82269040), the Yunnan Provincial (Traditional Chinese Medicine) Clinical Dermatology Center, 12th Five-year Key Construction Discipline of State Administration of Traditional Chinese Medicine “Dai Pharmacy”; Open Project of Yunnan Key Laboratory of Dai and Yi Medicines (No. 3097101100) and Key Laboratory of Chemistry in Ethnic Medicinal Resources, State Ethnic Affairs Commission & Ministry of Education, Yunnan Minzu University.

**Peer review information**

Traditional Medicine Research thanks all anonymous reviewers for their contribution to the peer review of this paper.

**Abbreviations**

AD, atopic dermatitis; ACE, acupoint catgut-embedding therapy; DNCB, 2,4-dinitrochlorobenzene; NC, normal control; ADA, AD + acupoint catgut-embedding; ADS, AD + sham-acupoint catgut-embedding; ns, no significance.

**Citation**


**Executive editor:** Jing-Yi Wang.

**Received:** 07 December 2023; **Accepted:** 25 March 2024; **Available online:** 27 March 2024. © 2024 By Author(s). Published by TMR Publishing Group Limited. This is an open access article under the CC-BY license. (https://creativecommons.org/licenses/by/4.0/)

**Abstract**

**Background:** This study aimed to assess how acupoint catgut-embedding therapy influences Th2-type immune response and the infiltration of CD4\(^+\) and CD8\(^+\) cells in DNCB-induced atopic dermatitis in BALB/c mice. It also conducted an initial examination of the underlying molecular mechanisms. **Methods:** Seventy-two mice were randomly divided into four groups: normal control, DNCB-induced atopic dermatitis model (AD), AD with acupoint catgut-embedding treatment (ADA), and AD with sham-acupoint catgut-embedding treatment. After DNCB challenge to induce AD, the ADA group received acupoint catgut-embedding therapy treatment at Zusanli (ST 36) and Quchi (LI 11) acupoints every other week from day 8. Mice in the AD with sham-acupoint catgut-embedding treatment group underwent the same procedure as the ADA group but without catgut implantation. Severity was assessed using SCORAD on treatment days 1, 10, and 20. On day 18, nine mice per group were euthanized, and the remaining on day 28. Histopathological changes were observed using hematoxylin-eosin and immunohistochemistry staining. TNF-\(\alpha\), IL-4, IL-6, and IL-13 levels were analyzed by ELISA, and GATA3 and STAT6 protein levels by western blot. **Results:** After 20 days of acupoint catgut-embedding therapy treatment, mice showed reduced dermatitis scores compared to DNCB-induced AD-like mice. Significant decreases occurred in serum IL-4, IL-6, IL-13, and TNF-\(\alpha\) levels. Skin analysis revealed marked reductions in CD4\(^+\) and CD8\(^+\) cell infiltration, as well as GATA3 and STAT6 protein levels. **Conclusion:** Acupoint catgut-embedding therapy may effectively alleviate atopic dermatitis by suppressing Th2 immune responses via the STAT6-GATA3 pathway and reducing CD4\(^+\) and CD8\(^+\) T cell infiltration in skin lesions.

**Keywords:** atopic dermatitis; acupoint catgut-embedding therapy; Th2 type immune response

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**Background**

Atopic dermatitis, or AD, is a common chronic inflammatory skin disease that typically appears in early childhood and can last into adulthood for certain individuals. It may also occur before related atopic conditions, including asthma, food allergy, and allergic rhinitis [1, 2]. AD affects people all over the world and is present in as many as 20% of adults and children [3]. With its high prevalence and significant impact on patients, AD is regarded as one of the most burdensome skin diseases worldwide [4]. A multitude of intricate elements interact to cause AD, such as a strong genetic susceptibility, compromised epidermal function, anomalies in the skin microbiota, immune system dysregulation, and involvement of the neuroimmune system [5]. It is now generally acknowledged that the main feature of AD patients is a high activation of the type 2 immune response. Topical moisturizers, corticosteroids, calcium inhibitors, antihistamines, and systemic immunosuppressants are widely used in the treatment of AD [5]. Nonetheless, patient compliance may be impacted by the long-term usage of these therapeutic approaches and any potential side effects. The satisfaction of AD patients with treatment still needs to be improved, and it is necessary to seek better alternative treatments for AD [6].

Acupuncture, as a popular form of alternative and complementary medicine worldwide, has a history of over 3,000 years in China. Acupuncture is widely used in skin conditions and its efficacy has been proven in atopic dermatitis [7]. Acupoint catgut-embedding therapy (ACE) is a new treatment developed from acupuncture. ACE is based on the meridian system, in which absorbable threads, such as catgut, are placed in the acupuncture point area to achieve the purpose of treating diseases through long-term acupoint stimulation. Compared to traditional acupuncture therapy, ACE reduces the number of doctor visits while increasing the treatment duration, thus improving patient compliance. A recent study demonstrated that ACE significantly improved respiratory function in a rat model of LPS-induced ARDS by reducing inflammation and oxidative stress, leading to notable amelioration of the condition [8]. Furthermore, ACE therapy has the ability to suppress the inflammatory activity of allergic asthma by preventing the secretion of Th2 cytokines, such as IL-4 and IL-13, and ILC2 cell proliferation. The inhibition of the NF-κB/COX-2 pathway could be associated with its mechanism [9]. Moreover, ACE treatment was found to enhance spatial learning and memory, mitigate hippocampal pathological damage, and inhibit rats’ vascular dementia-related inflammatory response [10]. To summarize, the data above suggests that ACE therapy exerts an effect on reducing inflammation and modulating the immune response. Numerous positive effects of acupuncture at ST36 have been shown, such as immune system stimulation and anti-inflammatory effects [11, 12]. According to traditional Chinese medicine theory, stimulating multiple different acupoints instead of just one can enhance the therapeutic effect and minimize potential side effects [13]. Zhang et al. conducted a literature analysis on the use of acupuncture to treat atopic dermatitis and discovered that the combination of ST36 and LI11 is frequently employed in this regard [14]. However, the underlying mechanism by which ACE exerts its therapeutic effect on AD is uncertain. The primary objective of the current study was to evaluate the impact of ACE on Th2 inflammatory responses, aiming to investigate a potential mechanism by which it could be employed for the treatment of AD.

**Materials and method**

**Animals**

The SPF male BALB/c mice, eight weeks old and weighing 22 ± 2 g, were obtained from Specific-pathogen-free (Beijing) Biotechnology Co., Ltd. (Beijing, China, SCXX (Jing) 2019-0010) and kept in a pathogen-free environment with unlimited access to food and water. They followed a 12-h light/dark cycle at 22 °C ± 1 °C with 55%–65% humidity. Following a week-long acclimation period, mice were assigned at random to four groups, each consisting of eighteen animals: normal control (NC), 2,4-dinitrochlorobenzene (DNCB)-induced AD model, AD + acupoint catgut-embedding (ADA), and AD + sham-acupoint catgut-embedding (ADS). Ethical approval was granted by the Experimental Animal Ethics Committee of Yunnan University of Traditional Chinese Medicine (license number: R-062023037). Each animal experiment carried out for this study complies with the “Guidelines for the Care and Use of Laboratory Animals”.

**Atopic dermatitis model establishment**

The establishment process of the mouse AD model is shown in Figure 1. The mouse AD model was induced with DNCB (Sigma-Aldrich, St. Louis, MO, USA) solution (DNCB crystals dissolved in acetone-olive oil mixture, 4:1 v/v). The dorsal hair of the mice was removed with a clipper in advance. The mice were applied with a 200 μL 1% DNCB solution for three days in a row during the first sensitization. Following four days, the mice were applied 150 μL of 0.2% DNCB solution on their backs every other day for three weeks in a row. The sensitized skin (secondary sensitization) was repeatedly stimulated for an AD-like inflammatory reaction. Edema, erythema, scab, and scratching appeared after the early 1% DNCB stimulation of mouse dorsal skin. The appearance of dark erythema and scales in the later stage of 0.2% DNCB stimulation was considered successful.
Treatment
As shown in Figure 1, mice in the ADA group have received a one-time ACE treatment every other week since the 8th day of the experiment. Following anesthesia, an ACE needle was placed into the mice's skin at a 15° angle and a depth of 1 cm. Subsequently, catgut was implanted into bilateral Zusanli (ST 36) and Quchi (LI 11) acupoints of each mouse in the ADA group. Mice in the ADS group were operated in the same method as mice in the ADA group, but without the implantation of catgut [15]. On day 18, nine mice were chosen at random from each group and euthanized using cervical dislocation with anesthesia by the approved protocol. On day 28, the remaining mice were put to death in the same way.

Dermatitis severity evaluation
Each mouse's dorsal skin dermatitis severity was assessed using the EFTAD-proposed SCORAD scoring criteria [16]. The specified scoring criteria are as follows: 0 for none, 1 for mild, 2 for moderate, 3 for severe, and 4 for extremely severe. Desquamation (dryness), edema (papule), erythema (bleeding), and epidermal exfoliation (scratch) were scored on the aforementioned 0–4 scale, and the integral change trend chart of mice was drawn to observe the changes in skin lesions in mice. The dermatitis score was determined by a researcher who was not aware of the treatment or control groups.

Histopathological evaluation
The back skin lesions of mice from each group were fixed in a 4% polyformaldehyde solution for 24 h, followed by a 5-min gradient dehydration in an ethanol solution, transparency achieved using xylene, paraffin embedding, and subsequent sectioning into 4-μm-thick slices. Following hematoxylin and eosin staining and sealing with resin, the prepared sections were examined under a light microscope to assess pathomorphological changes, specifically focusing on the degree of epidermal and dermal hyperplasia as well as inflammation.

ELISA analysis
Blood samples were obtained from the veins of mice immediately after euthanasia. The serum samples were obtained from blood using a centrifuge (1,200 g, 4 °C, 5 min) and stored at 80 °C until use. The serum levels of IL-4, IL-6, IL-13, and TNF-α were measured using mouse ELISA kits following the manufacturer's protocols (IL-4, IL-6, IL-13, and TNF-α; Mei Biao Biotechnology, Yancheng, China). The absorbance was measured at 450–540 nm using an automated microplate reader (Bio Tek Co., Ltd., Winooski, VT, USA).

Immunohistochemistry analysis
The dehydration, clearing, paraffin embedding, sectioning, dewaxing, hydration, antigen retrieval, peroxidase inactivation, and goat serum blocking of mouse back tissue were performed. A primary antibody (CD4 IgG and CD8 IgG, Affinity Biosciences, Changzhou, China; dilution 1:100) was added and blocked overnight at 4 °C. After removing the primary antibody, a secondary antibody was applied, followed by DAB color development, hematoxylin counterstaining, and slide covering. The infiltration of inflammatory cells in each group was observed under a fluorescence microscope.

Western blot analysis
The protein of skin lesions from each group's mice was routinely extracted, and the total protein of the extracted cells was quantified by BCA protein quantification kit (Beyotime Biotechnology, Changsha, China). 20 μg of sample protein from each group was subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane, and blocked with blocking solution for 1 h at room temperature. Add specific recognition antibody and block overnight at 4 °C. After washing the membrane by shaking with TBS, ECL color developing solution was added to develop color.

Statistical analysis
Statistical analyses were conducted using GraphPad Prism 9.5 software (GraphPad Software, San Diego, CA, USA) and SPSS 25.0 software (IBM SPSS Statistics, Chicago, IL, USA). Data is presented as mean ± standard deviations. ANOVA was used to do multiple comparisons, and the least-significant difference post hoc test was used to determine which difference was not significant. A statistically significant P-value was defined as one that was less than 0.05.

Results
ACE therapy's impact on skin symptoms in mice with DNBC-induced AD
Photographs were obtained of each mouse group's dorsal skin lesions on days 8, 18, and 28 of the experiment. Figure 2B shows representative photos of the dorsal areas of the mice in each group. Compared to the NC group, mice in AD group exhibited symptoms on...
the dorsal skin, including erythema, dryness, desquamation, scab formation, bleeding, and epidermal detachment, with a higher SCORAD index. The symptoms of the ACE group mice’s dorsal skin showed significant improvement, particularly in terms of scab formation and bleeding on the skin. By the 10th day of treatment (10.50 ± 1.93 vs. 7.38 ± 1.69), a noticeable improvement in dermatitis manifestations was evident, with the most prominent improvement observed on the 20th day (10.13 ± 1.96 vs. 5.75 ± 1.98). Although mice in the ADS group exhibited symptoms similar to those in the AD group, the severity of skin lesions was less pronounced in the ADS group.

ACE therapy’s impact on DNCB-induced AD-related histological changes in mice.

The hematoxylin and eosin staining results are shown in Figure 2. In the NC group, the skin structure is well-defined at all levels, with a thin epidermis and uniform distribution of collagen in the dermis. There is no evident infiltration of inflammatory cells observed. Compared to the NC group, the skin of mice in the AD group exhibits epidermal hyperplasia, excessive keratinization, evident spongiosis on experimental days 18 and 28. Additionally, there is noticeable dermal thickening and prominent inflammatory cell infiltration. The ADA group shows a significant reduction in epidermal thickness, alleviation of spongiosis, and a decrease in inflammatory cell infiltration within the dermal layer. These changes are more pronounced after 20 days of treatment. However, there is no apparent alteration in excessive epidermal keratinization following the treatment.

The immunohistochemistry staining is illustrated in Figure 3. We picked three fields at random at 200× magnification, counting the number of positive cells and calculating the average, in accordance with the approach described in earlier studies [17]. There isn’t any obvious CD4⁺ or CD8⁻ T cell infiltration in the NC group. Compared

![Figure 3](https://example.com/figure3.png)

Figure 3 Effect of ACE on histopathology and the infiltration counts of CD4⁺ and CD8⁻ cells in mice with DNCB-induced AD. (A) Representative histological images of the mice skin stained by HE. Scale bar = 100 μm. (B, C) CD4⁺ (B) and CD8⁻ (C) cells were examined by IHC. Scale bar = 50 μm. (D) The counting of CD4⁺ cells and CD8⁻ cells. N = 9, *P < 0.05, **P < 0.01, ***P < 0.0001. NC, normal control; AD, atopic dermatitis; ADA, AD + acupoint catgut-embedding; ADS, AD + sham-acupoint catgut-embedding; ns, no significance.
to the NC group, the infiltration of these two cell types in the skin of mice in the AD group significantly increases on experimental days 18 and 28, with the majority of infiltration occurring in the dermis. Compared to the AD group, on day 10 of treatment, the ADA and ADS groups show varying degrees of reduction in infiltrated CD4+ (94.50 ± 15.97 vs. 71.00 ± 8.83 vs. 71.50 ± 12.87) and CD8+ (91.00 ± 38.10 vs. 75.25 ± 15.63 vs. 68.00 ± 13.49) cells in the skin. On day 20 of treatment, there is a significant decrease in the infiltration of CD4+ (83.50 ± 10.85 vs. 57.00 ± 7.87 vs. 62.25 ± 10.72) and CD8+ (91.00 ± 38.10 vs. 68.00 ± 13.49 vs. 75.25 ± 15.63) cells.

ACE therapy lowers the levels of TNF-α and Th2-type cytokines, including IL-4, IL-6, and IL-13, in the serum

The results of the ELISA analyses are shown in Figure 4. Compared to the NC group, the levels of TNF-α, IL-4, IL-6, and IL-13 in the serum of mice in the AD group are significantly elevated on experimental days 18 and 28. After 10 days of ACE treatment, mice in the ADA group show a significant reduction in serum IL-4 (145.54 ± 14.44 vs. 130.14 ± 4.92) and IL-13 (45.21 ± 5.43 vs. 38.10 ± 1.99) compared to the AD group. After 20 days of ACE treatment, mice in the ADA group exhibit a significant decrease in serum IL-4 (148.43 ± 11.69 vs. 133.27 ± 13.02), IL-6 (101.40 ± 11.58 vs. 86.06 ± 2.88), IL-13 (43.77 ± 3.20 vs. 38.02 ± 1.97), and TNF-α (575.82 ± 64.18 vs. 508.01 ± 39.38) compared to the AD group. On days 10 and 20 of treatment, the ADS group exhibits a lowering trend in blood levels of TNF-α, IL-4, IL-6, and IL-13 in mice as compared to the AD group.

ACE therapy inhibits the expression of transcription factors GATA3 and STAT6 in DNCB-induced AD mice dorsal skin lesions

As indicated in Figure 5, on experimental days 18 and 28, mice in the AD group have higher levels of GATA3 and STAT6 expression in their dorsal skin compared to the mice in the NC group. After 10 days of ACE treatment, compared to the AD group, the expression of GATA3 is already suppressed in the ADS and ADA groups in the dorsal skin of mice. On day 20 of treatment, the expression of STAT6 is also significantly inhibited.
Discussions

AD is the initial stage of the “atopic march” (natural history of allergic diseases). With the progression of age, certain patients develop food allergies, allergic rhinitis, and allergic asthma [18]. In our current investigation, we observed edema, erythema, scabbing, and increased scratching or the initial 1% DNBC stimulation on mouse dorsal skin. As the stimulation continued with 0.2% DNBC, we observed the emergence of dark erythema and scales, indicative of AD-like changes in the mice. ACE treatment reduces these symptoms and alleviates histopathologic inflammatory cell infiltration and marked thickening of the epidermis and dermis suggests that ACE has a therapeutic effect in the treatment of AD. IL-4 and IL-13 play crucial roles in AD development and progression, as they are key cytokines associated with the Th2 immune response [19]. Upon administering ACE treatment on day 10, we observed a pronounced decrease in the serum levels of IL-4 and IL-13 in mice. On day 20 of treatment, the decline in both cytokines remains significant compared to the AD group.

Interestingly, on the 10th day of ACE treatment, serum levels of IL-6 and TNF-α were lower in the ADS and ADA groups of mice than in the AD group, but this difference was not statistically significant. Nevertheless, the ADA group’s levels of both cytokines significantly dropped on day 20 of treatment. Catgut, as a biomaterial, initiates a tissue response continuum upon implantation into acupoints. This spectrum encompasses several processes like damage, blood-material interactions, the start of the inflammatory response, the construction of a provisional matrix, acute and chronic inflammation, fibrosis, and fibrous encapsulation [20]. In this process, macrophages play a crucial role, as activated macrophages adhere to the cutag can secrete various cytokines, including TNF-α and IL-6, mediating the inflammatory and wound healing responses [21]. Therefore, we speculate that the tissue response continuum initiated by ACE therapy may explain the lack of statistically significant differences in serum TNF-α and IL-6 levels in mice during the early stages of treatment (day 10) compared to the control group. However, on day 20 of treatment, as ACE therapy alleviates the condition of AD, there is a significant decrease in both cytokines. A Chinese study partially supports our view. Following catgut implantation at the T36 acupoint, the ACE group rats showed a significant TNF-α increase near ST36 compared to the control group. The peak occurred on the third day, followed by a gradual decline starting on the seventh day [22].

STAT6 plays a crucial role in modulating immune responses and influencing Th2 cell differentiation through its regulation of the GATA3 gene expression [23]. Anticipatingly, the skin tissues of AD mice exhibited a considerable upregulation of STAT6 and GATA3 expression following DNCB induction. Notably, the ACE therapy effectively suppressed them. STAT6 is predominantly activated by IL-4 and IL-13, leading to the upregulation of GATA3. Apart from being induced by STAT6 signaling, GATA3 expression can also be triggered through T cell receptor stimulation with low doses of antigen and co-stimulatory molecules, or via the interaction of Notch receptors on T cells with Jagged ligands presented by antigen-presenting cells [24]. Therefore, ACE treatment may potentially alter the cytokine milieu during CD4+ T cell differentiation or regulate the expression of STAT6 and GATA3 through other pathways. The exact mechanism of ACE’s inhibitory effect on the STAT6-GATA3 pathway in the skin tissues of AD mice remains unclear, and determining if this influence is direct or indirect is difficult. There is still a need for research.

CD4+ T cells can produce a mixed pattern of Th1 and Th2 cytokines, including IL-4, IL-13, and IL-5, which are considered crucial for the development of atopic dermatitis. However, this hypothesis was modified as research on CD8+ T cells deepened. Seneviratne et al. established a strong correlation between CD8+ T cell levels and disease severity [25]. In a Der f-induced AD mouse model, Hennino et al. demonstrated that IFN-γ-producing CD8+ T cells infiltrated affected skin, leading to typical AD-related epidermal changes [26]. Subsequently, they confirmed the early and crucial role of CD8+ T cells in human AD lesions, possibly associated with keratinocyte apoptosis and epidermal spongiosis [27]. Moreover, Hijnen et al.’s research revealed the abundance of CD8+ T cells in AD patient skin, capable of producing a range of cytokines, such as IFN-γ, IL-13, and IL-22, which could play a pivotal role in epidermal thickening and skin barrier disruption [28]. Following that, our study was specifically aimed at investigating how ACE affects CD4+ cells and CD8+ cells present in the skin. The dorsal skin of mice in the ADS and ADA group had less CD4+ and CD8+ cells on day 10 of ACE treatment than did the mice in the model group, however this difference was not of statistical significance. We speculate that the cause of this situation is also related to the inflammatory response mediated by catgut implantation at acupoints. As ACE therapy continued, leading to the alleviation of the condition, the significant difference in the reduction of both cell types became apparent.

Reflecting on the entire experiment, some observed indicators only exhibited significant differences compared to the model group on the 20th day of ACE treatment. Given the characteristics of ACE therapy itself, where the use of catgut as a biological material implanted into the body can elicit inflammatory responses, and the relatively short duration of this experiment, along with the chronic and relapsing nature of AD in clinical settings, investigating the relationship between a more extended course of ACE treatment and its efficacy would be meaningful. This is also a limitation of our current study, and our next step involves researching the therapeutic effects of a longer course of ACE therapy.

During the experimental process, certain observed indicators also changed in the ADS group. We speculate that the procedure in the ADS group may have exerted effects similar to acupuncture treatment. However, due to the short duration and limited frequency of treatments, although many indicators showed a trend towards relief, we did not observe significant differences in statistical analysis.

Conclusion

The outcomes of this study suggest that the inhibition of the STAT 6-GATA3 pathway may be the explanation why the ACE treatment reduced AD-like skin inflammation and suppressed Th2 responses in the DNCB-induced AD animals. Furthermore, our research indicated that ACE treatment decreases CD4 and CD8 cell infiltration in the skin, a finding that may be partially explained by ACE’s suppression of the Th2-type immune response. However, more research is needed to determine the precise mechanism. When taken together, the ACE treatment may be used as a successful means of treating atopic dermatitis.

References


