

HDAC Downregulation of Xiaoqinglong Decoction in the Treatment of Allergic Rhinitis

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Keywords

Xiaoqinglong decoction · Allergic rhinitis · Histone deacetylase · Type 2 inflammation · Epithelial dysfunction

Abstract

Introduction: As one of the most common allergic diseases, allergic rhinitis (AR) has attracted wide attention all over the world. More appropriate treatment of AR should be explored thoroughly. In recent years, traditional Chinese medicine has attracted more attention in AR treatment. As a classical Chinese medicine prescription, Xiaoqinglong decoction (XQLD) has been commonly used in treating AR. Even though its therapeutic effect on AR has been clinically confirmed, more molecular mechanism remains to be further investigated. Our research aimed to investigate the therapeutic mechanism of XQLD for AR management. **Methods:** The study was evaluated in an ovalbumin sensitized mouse model and liquid chromatography-mass spectrometry was adopted to test the stability of XQLD's effective components. **Results:** The results confirmed the stability and safety of the effective components of XQLD. XQLD significantly downregulated the expression of HDACs (HDAC1, HDAC3, and HDAC4) and Th2 inflammatory factors (IL4, IL5, and IL13) in AR mice.

XQLD and the HDAC inhibitor JNJ-26481585 promoted the expression of epithelial tight junction proteins (claudin-1 and ZO-1) and decreased the expression of mucins (Muc5a and Muc5b) in the nasal mucosa of AR mice. **Conclusions:** In conclusion, our findings present the beneficial effects of XQLD on AR and recovery of the nasal epithelium. We also identify the decreased HDAC as a potential target of XQLD for AR treatment. This study provides an important experimental proof for elucidating the therapeutic mechanism of XQLD.

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Introduction

Allergic rhinitis (AR) is one of the most common allergic diseases mediated by immunoglobulin E (IgE), with a high prevalence of up to 40% around the world [1]. High recurrence and easy co-morbidity with chronic rhinosinusitis, asthma and/or atopic dermatitis make AR re-

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sult in huge burden for whole society [2]. Better strategies for AR management tend to be an urgent need.

In recent years, an increasing number of studies have shown that traditional Chinese medicine (TCM) might be helpful in the therapy of AR [3, 4]. Xiaoqinglong decoction (XQLD) is a typical TCM prescription for AR treatment [5] and has been proven to have a reliable clinical effect [6]. Although it has been used for patients, the lack of clarification on its therapeutic mechanism still restricts its further promotion. Therefore, further therapeutic research is needed to clarify the role of XQLD and provide more options for AR management.

After stimulation by allergens, the destruction of nasal mucosa and submucosal type 2 inflammatory responses are the main pathological changes of AR. Epithelial dysfunction can exacerbate the invasion of pathogens and promote mucosal immune responses in AR [7] which also results in the disorder of the mucociliary clearance system and destruction of epithelial apical tight junctions [8]. The immune reaction is the consequence of mucosal impairment after exposure to environmental allergens [9]. Activation of type 2 inflammatory response directly leads to IgE production, eosinophils infiltration, and mast cells degranulation et al. by release classic type 2 cytokines, such as IL4, IL5, and IL13, which eventually results in the occurrence of allergic symptoms. Therefore, repairing the function of the nasal epithelium and inhibiting the progression of type 2 inflammatory response are essential for AR control. Previous studies have confirmed that the ingredient of XQLD can help alleviate the inflammatory response [10], but the effect of XQLD on epithelial function and the related mechanism still need further exploration.

Epigenetic regulation is an important regulator for pathological changes, such as histone chromatin modification. Histone deacetylase (HDAC) has been reported to be vital in the pathogenesis of allergic diseases. Studies have shown that HDACs aggravate the airway allergic response by affecting Th1/Th2 differentiation and promoting the expression of inflammatory cytokines [11]. HDAC1 has been shown to disrupt nasal epithelial barrier integrity by significantly inhibiting *Trek1* expression in AR [12]. HDACs have also been found to be a driver of damage to the AR nasal epithelial barrier by impairing tight junctions [13]. Therefore, the HDACs are considered to be the promising target in AR treatment. In this study, we used OVA-sensitized mouse to explore the therapeutic mechanism of XQLD on AR and took advantage of HDAC as the entry point to clarify the role of XQLD in inhibiting type 2 inflammation and repairing the epithelial function of AR.

Materials and Methods

Mouse Preparation

This project was approved by the Ethics Committee of the Experimental Animal Center of the First Affiliated Hospital of Guangzhou University of Chinese Medicine with the approval number of TCMF1-2019048. C57BL/6 mice (female, SPF grade, aged 6–8 weeks) were provided by Changsha Tianqin Biotechnology Co., Ltd. (license: scxk (Xiang) 2019-0014). They were reared in an SPF level feeding room. All mice were randomly divided into 7 groups: the control group, AR model group, loratadine group, HDAC inhibitor group, and XQLD high/middle/low dose groups.

Pharmacological intervention: On the 1st to 15th day, except for the blank control group, the other 6 groups were sensitized by intraperitoneal injection of 300 μ L PBS + 25 μ g OVA + 1 mg aluminum hydroxide. From day 22–28, the mice in the control group were intranasally instilled with 10 μ L PBS/unilateral nose daily; the other 6 groups were sensitized by intranasal instilled with 50 mg/mL OVA solution (dissolved in PBS) daily, also 10 μ L/unilateral nose. From the 15th to 28th day, the control group was treated with 100 μ L PBS by gavage; XQLD low/middle/high dose groups received 1, 2, and 4 times the clinical equivalent dose (11.83, 23.66, and 47.31 g/kg) by gavage; the loratadine group received 1.547 mg/kg loratadine daily by gavage; and the HDAC inhibitor group was endonasally challenged with JNJ-26481585 12.5 μ g/days. On the 29th day, the mice were sacrificed after anesthesia for sampling. The whole sensitization process is also shown in online supplementary Figure 1 (see www.karger.com/doi/10.1159/000527429 for all online suppl. material).

XQLD Preparation

XQLD was prepared by the water refluxing method. First, 9 g of *Ephedra sinica* was placed in a round-bottom flask, and 10 times distilled water (780 mL) was added to the volatile oil collector to collect the volatile oil. After slightly boiling for 20 min, the other herbs were weighed according to the prescription dosage and added together. After boiling, the solution was simmered for 20 min, and 8 times distilled water (624 mL) was added to the residue. The mixture was simmered for 20 min and the solution was filtered while hot. The two filtrates were combined and concentrated by a rotary evaporator. Each dose of XQLD was concentrated to 32.5 mL (equivalent to 2.4 g/mL crude drug) and stored at -80°C . The information of all drug components of XQLD is provided in online supplementary Table 1 of supplementary materials. The plant name has been checked with data in <http://www.theplantlist.org>.

Liquid Chromatography-Mass Spectrometry Analysis

First, 100 μ L XQLD was placed in an EP tube. Then, 400 μ L pure methanol was added for protein precipitation. The tube then underwent vortex oscillation and was placed in an ice bath for 5 min. Next, the tube was centrifuged at 15,000 rpm and 4°C for 10 min. Afterward, the supernatant was removed and 1/2 volume of mass spectrometry grade water was added. The solution was diluted to 53% with methanol and centrifuged at 15,000 g, 4°C for 20 min. The supernatant was analyzed by a mass spectrometer. The blank sample was replaced by a 53% methanol water solution containing 0.1% formic acid, and the pretreatment process was the same as for the experimental sample.

Behavioral Evaluation of Mice

From the first experimental day, the behavior, state, physical signs, feeding, drinking, defecation, and other general conditions of the mice were observed. Nasal symptoms such as nasal itching, sneezing, and nasal secretions were also observed, and the numbers of sneezes and scratches at 10 min after the last stimulation were observed to evaluate the drug effect on nasal symptoms.

Detection of Serum OVA-IgE, IL4, IL5, IL13, IL2, and IgG in Mice

An enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Jianglai Biotechnology Co., Ltd.) was used to detect serum OVA-IgE, IL4, IL5, IL13, IL2, and IgG. The optical density value of each well was measured at a wavelength of 450 nm. In an Excel worksheet, the standard concentration was taken as the abscissa, and the corresponding optical density value was taken as the ordinate. The concentration of each sample was calculated according to the curve equation.

Liver and Kidney Function Test

Creatinine (CRE) in serum was detected by the creatine oxidase method, blood urea nitrogen (BUN) in serum was detected by diacetyl oxime colorimetry, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum were detected by Reitman-Frankel colorimetry. The test kit was purchased from Nanjing Jiancheng Bioengineering Research Institute, and all analytical procedures were performed according to the kit instructions.

Hematoxylin-Eosin Staining and Immunohistochemical Staining

The tissues of mice were soaked and fixed in 4% paraformaldehyde. The nasal mucosa of mice wrapped by nasal bone and skull was fixed in 4% paraformaldehyde for more than 1 day and then placed into ethylene diamine tetra-acetic acid (EDTA) decalcification solution. After alcohol dehydration and paraffin embedding, the paraffin sections were stained with hematoxylin-eosin and sealed with neutral gum. Immunohistochemical specimens were prepared after alcohol dehydration and paraffin embedding; DAB was used to develop color after incubating. The images were collected under an optical microscope.

Statistics: under an optical microscope, 6 high-power fields ($\times 400$) were selected for each section, and images of nasal mucosal tissues were taken. Gray values were measured by Image J; the average optical density method was adopted, with average optical density mean density = IOD (integrated optical density) cumulative optical density/area, and analysis and comparison between groups were conducted.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from the nasal mucosa tissues using an RNA Extraction Kit (Takara, Japan). The reverse transcription reaction system was configured with the reverse transcription kit (Takara, Japan), and the total RNA was transformed into cDNA for the experiment. The reverse transcription-polymerase chain reaction system was configured with the reverse transcription kit (Takara, Japan). All analytical procedures were performed according to the usage instructions. The sequences of the gene-specific primers are shown in the online supplementary Table 2 of supplementary materials.

Western Blot

The nasal mucosa of mice was taken and placed in 300 μ L lysate. A bicinchoninic acid assay (Shanghai Biyuntian Biotechnology Co., Ltd) was used to determine the protein concentration. The protein loading solution was incubated at 100°C for 10 min to denature the protein and stored at -80°C . An SDS-PAGE gel preparation kit (Beijing Solibao Technology Co., Ltd.) to configure the gel. The protein transmembrane electrophoresis system (BioRad, Germany) was used for electrophoresis and transmembrane and sealed with 5% skim milk. The primary and secondary antibodies used are shown in the online supplementary Table 3 of supplementary materials.

Statistical Methods

GraphPad Prism 8.0 was used to analyze the data and make charts. The histogram was used in the statistical chart. If it did not conform to the normal distribution, it was represented by M (p25–p75), and the statistical chart is a box chart. One-way ANOVA was used if the measurement data were normally distributed and the variance was homogeneous, and Dunnett's test was used for comparisons between groups. If the measurement data did not meet the normality and/or homogeneity of variance, the Kruskal-Wallis test was used, and Dunn's test was used for comparisons between groups. A *p* value of less than 0.05 was considered statistically significant.

Results

Effective Composition and Drug Stability Detection of XQLD

XQLD is composed of nine components (online suppl. Table 1). After all components are combined, the stability of the active ingredients needs to be determined. To identify the stability of the effective ingredients contained in XQLD, we analyzed the decoction by liquid chromatography-mass spectrometry (LC-MS). The XQLD mass spectrometry analysis data of five batches in our study is displayed in Figure 1a, b.

A total of 1,516 compounds were detected by LC-MS. Among them, 38 alkaloids, 3 catechins, 77 terpenes, 182 flavonoids, 38 cinnamic acids, 8 chalketes, 29 phenylpropanoid compounds, 25 benzenes, and their substituted derivatives were included. The correlation analysis of those components detected in five batches of XQLD showed that the correlation coefficients were greater than 0.965, indicating that different batches of XQLD components had high correlation (Fig. 1c). We also screened 27 bioactive components from those detected compounds (Table 1). Comparing the signal intensity of 27 components in different batches, we found that the content of effective bioactive components in different batches of XQLD was stable (Fig. 1d).

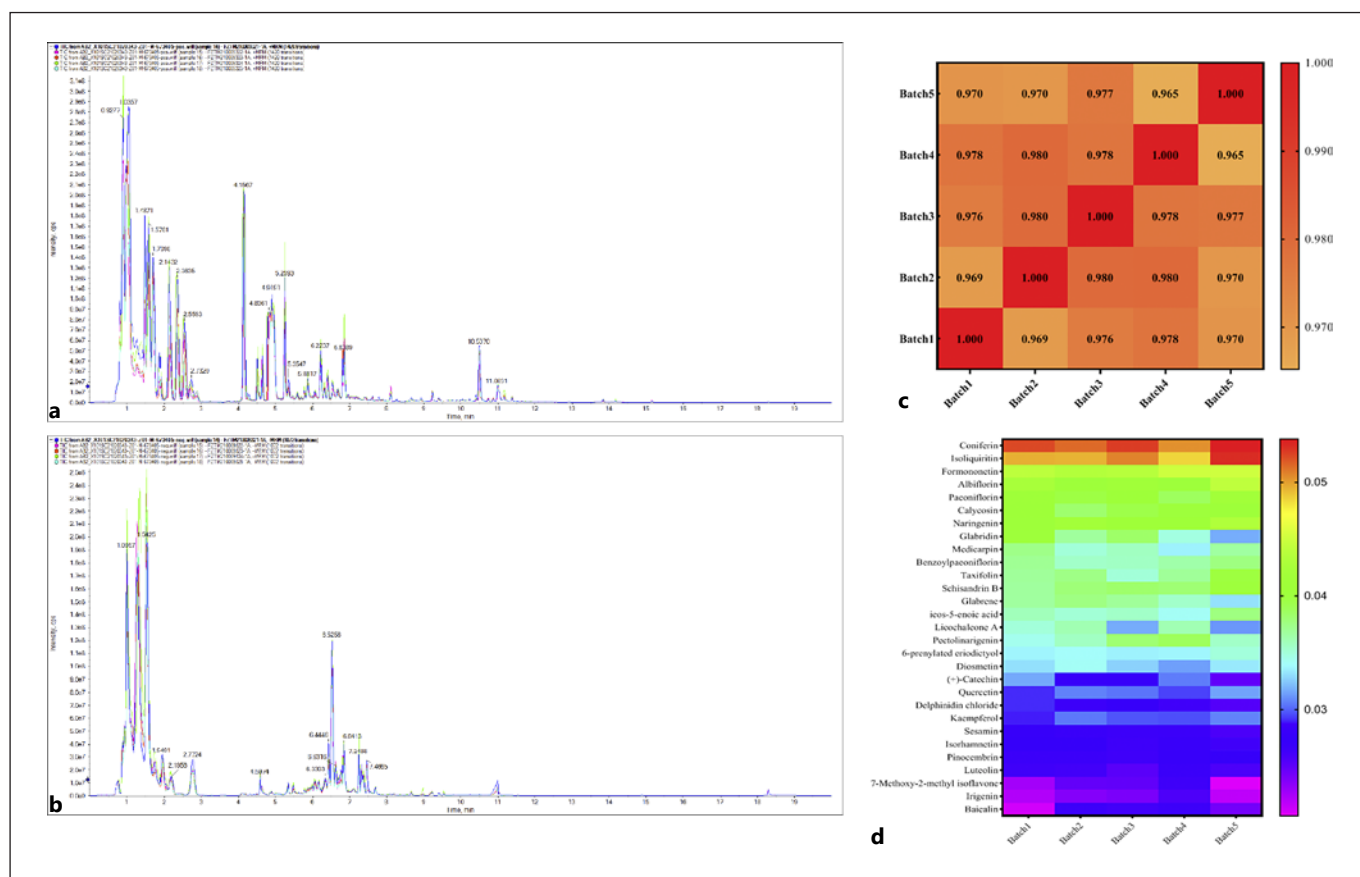


Fig. 1. LC-MS detection of 5 batches of XQLD. **a** HPLC-MS positive ionic TIC chromatograms of 5 batches of XQLD. **b** HPLC-MS negative ionic TIC chromatograms of 5 batches of XQLD. **c** Correlation analysis of total components. **d** Relative contents of 29 active biological components.

Safety Evaluation of XQLD in Treating AR Mice

XQLD is administered orally and metabolized mainly through the liver and kidney. Therefore, the liver and kidney function after XQLD intervention is the main index of its safety. CRE, BUN, ALT, and AST levels in blood were detected to evaluate the effects of all drugs (different doses of XQLD, loratadine, and the HDAC inhibitor JNJ-26481585) on mouse liver and kidney functions. The results showed that there was no significant change in the expression of CRE, BUN, ALT, and AST among all groups after drug intervention ($p > 0.05$), indicating that XQLD, loratadine, and HDAC inhibitor did not obviously affect the liver and kidney function of mice (Fig. 2a–d).

Hematoxylin-eosin sections of the liver and kidney in each group of mice also showed no obvious change in the structure. Liver slices of each group showed a clear lobular structure, orderly arrangement of cell cords, monolayer arrangement of hepatocytes in clusters or cords, and

polygonal shape of hepatocytes (Fig. 2e; online suppl. Fig. 2). The structure of the nephron was clear and complete, the glomerular mesangial cells and matrix did not proliferate abnormally, and the basement membrane did not thicken (Fig. 2e; online suppl. Fig. 2).

XQLD and HDAC Inhibitor Alleviates Allergic Symptoms in AR Mice

The symptoms of AR mice were observed and recorded within 10 min after the last nasal drip. Times of nasal sensitivity symptoms (sneezing and scratching the nose) in each group of mice were statistically analyzed to evaluate the alleviation of symptoms after drug intervention. It was observed that the nasal sensitivity symptoms of the AR mice were obvious. After XQLD or HDAC inhibitor intervention, the symptoms of sneezing and scratching the nose in the medication groups were significantly reduced ($p < 0.05$) compared with the AR group, indicating

Table 1. XQLD bioactive ingredients and chemical structure

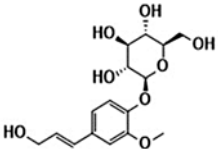
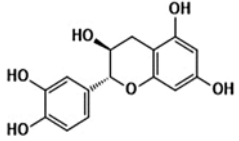
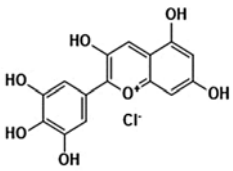
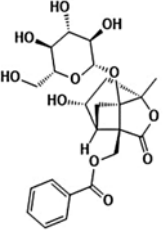
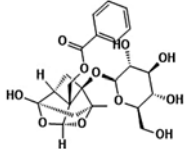
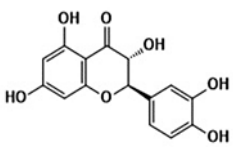
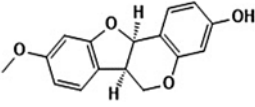
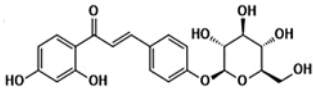
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Coniferin	0.97	C ₁₆ H ₂₂ O ₈	342.132	"531-29-3"	
(+)-Catechin	5.37	C ₁₅ H ₁₄ O ₆	290.27	"154-23-4"	
Delphinidin chloride	5.602	C ₁₅ H ₁₁ ClO ₇	338.7	"528-53-0"	
Albiflorin	6.176	C ₂₃ H ₂₈ O ₁₁	480.462	"39011-90-0"	
Paeoniflorin	6.333	C ₂₃ H ₂₈ O ₁₁	480.45	"23180-57-6"	
Taxifolin	6.885	C ₁₅ H ₁₂ O ₇	304.25	"480-18-2"	
Medicarpin	7.26	C ₁₆ H ₁₄ O ₄	270.285	"32383-76-9"	
Isoliquiritin	7.471	C ₂₁ H ₂₂ O ₉	418.394	"5041-81-6"	

Table 1 (continued)

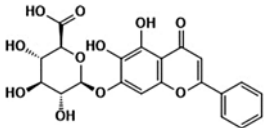
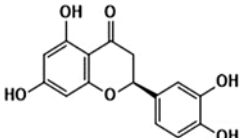
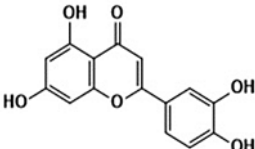
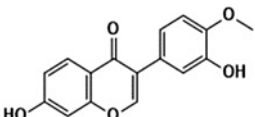
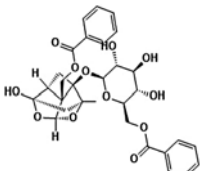
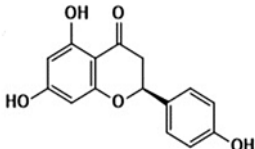
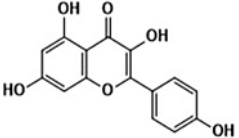
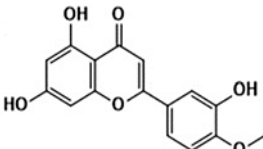
Ingredient name	Retention time	Chemical equation	Molecular weight	CAS	Chemical structure
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6-Prenylated eriodictyol	7.999	C ₁₅ H ₁₂ O ₆	288.252	"552-58-9"	
Luteolin	8.06	C ₁₅ H ₁₀ O ₆	286.24	"491-70-3"	
Calycosin	8.18	C ₁₆ H ₁₂ O ₅	284.263	"20575-57-9"	
Benzoylpaeoniflorin	8.53	C ₃₀ H ₃₂ O ₁₂	584.568	"38642-49-8"	
Naringenin	8.683	C ₁₅ H ₁₂ O ₅	272.25	"480-41-1"	
Kaempferol	8.811	C ₁₅ H ₁₀ O ₆	286.24	"520-18-3"	
Diosmetin	8.911	C ₁₆ H ₁₂ O ₆	300.26	"520-34-3"	

Table 1 (continued)

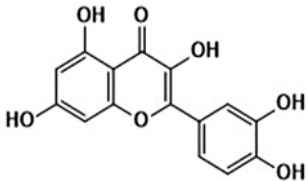
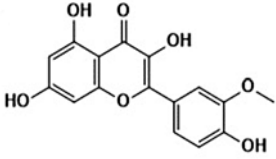
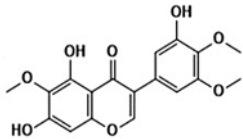
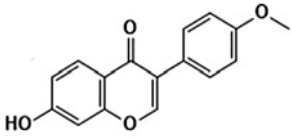
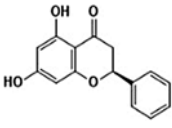
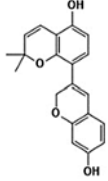
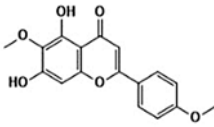
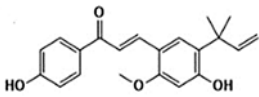
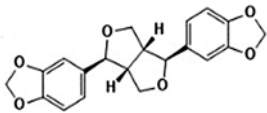
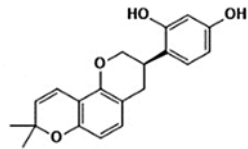
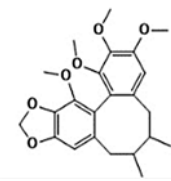
Ingredient name	Retention time	Chemical equation	Molecular weight	CAS	Chemical structure
Quercetin	8.96	C ₁₅ H ₁₀ O ₇	302.236	"117-39-5"	
Isorhamnetin	8.969	C ₁₆ H ₁₂ O ₇	316.262	"480-19-3"	
Irigenin	9.17	C ₁₈ H ₁₆ O ₈	360.32	"548-76-5"	
Formononetin	9.556	C ₁₆ H ₁₂ O ₄	268.27	"485-72-3"	
Pinocembrin	10.372	C ₁₅ H ₁₂ O ₄	256.25	"480-39-7"	
Glabrene	10.49	C ₂₀ H ₁₈ O ₄	322.361	"60008-03-9"	
Pectolarigenin	10.78	C ₁₇ H ₁₄ O ₆	314.295	"520-12-7"	
Licochalcone A	11.087	C ₂₁ H ₂₂ O ₄	338.4	"58749-22-7"	

Table 1 (continued)

Ingredient name	Retention time	Chemical equation	Molecular weight	CAS	Chemical structure
Sesamin	11.456	C ₂₀ H ₁₈ O ₆	354.35	"607-80-7"	
Glabridin	11.47	C ₂₀ H ₂₀ O ₄	324.37	"59870-68-7"	
Schisandrin B	14.09	C ₂₃ H ₂₈ O ₆	400.46	"61281-37-6"	

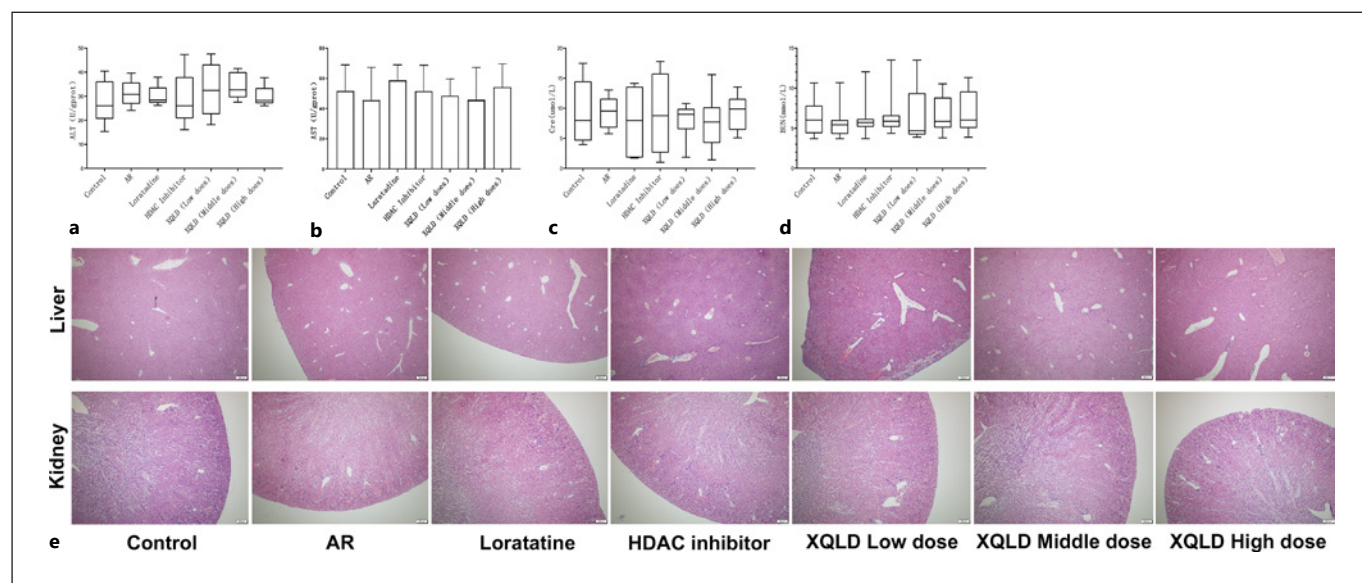


Fig. 2. Detection of liver and kidney function and structure after drug intervention in mice. **a–d** CRE, BUN, ALT, and AST levels in blood (CRE, ALT, AST $n = 6$, BUN $n = 17$; compared with the control group: $*p < 0.05$; compared with the AR group: $\#p < 0.05$). **e** HE images of mouse liver and kidney. The structure of mouse liv-

er and kidney, including liver lobules, interlobular bile ducts, interlobular arteries and veins, the renal cortex, medulla, and pelvis of the kidney, etc. were not damaged after drug intervention. Images were made at $\times 40$ magnification. HE, hematoxylin-eosin.

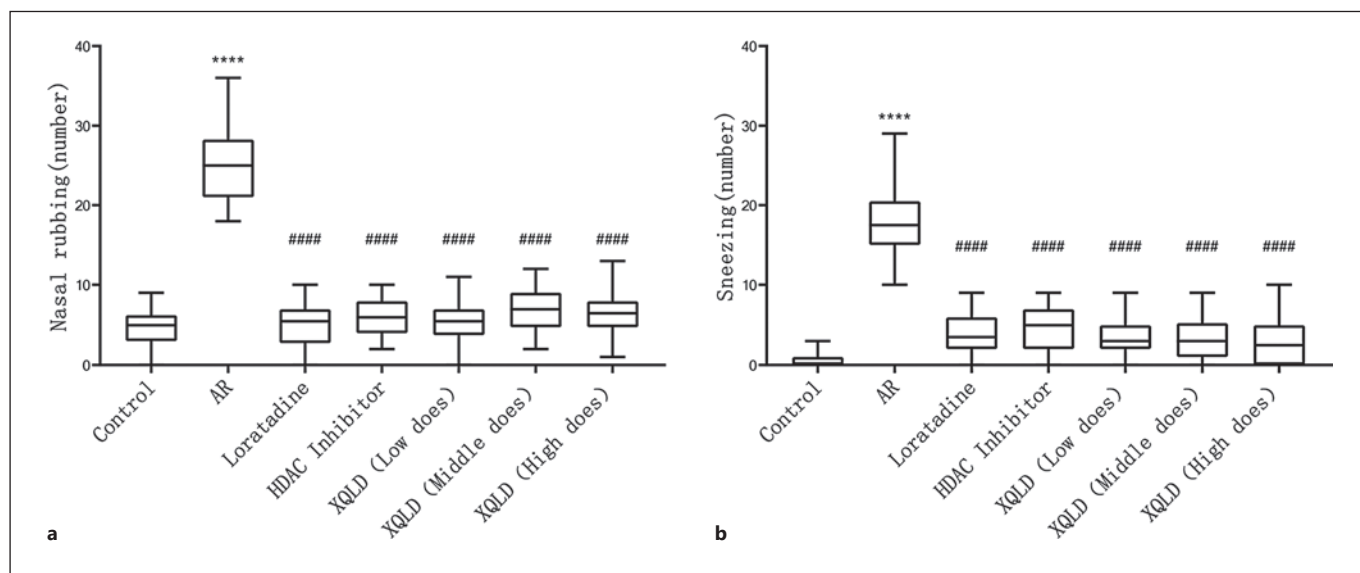


Fig. 3. Nasal allergy symptoms in mice. **a, b** Nasal rubbing and sneezing times within 10 min. Error bars are shown as the means of 6 SEMs ($n = 30$; compared with the control group: $*p < 0.05$ and $****p < 0.0001$; compared with the AR group: $#p < 0.05$ and $####p < 0.0001$).

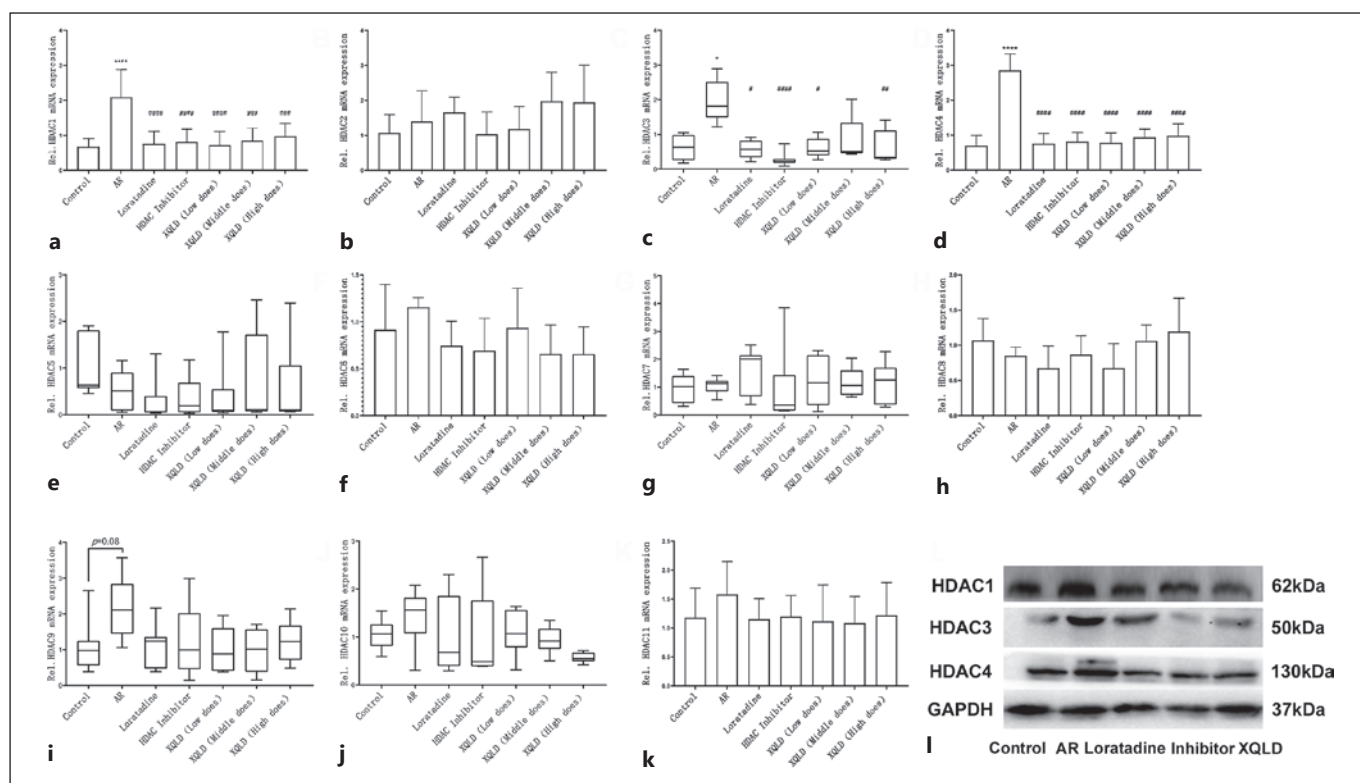


Fig. 4. Expression of HDAC family members in the nasal mucosa of mice and their variation after different doses of XQLD and non-selective HDAC inhibitor intervention. **a–k** mRNA levels of HDAC1–11. **l** The protein levels of HDAC1, HDAC3, and HDAC4. Inhibitor represents HDAC inhibitor, XQLD represents high dose

of XQLD (HDAC 1–2, HDAC 4–8, HDAC 10–11 $n = 6$; HDAC3 $n = 8$; HDAC9 $n = 7$; compared with the control group: $*p < 0.05$ and $****p < 0.0001$; compared with the AR group: $#p < 0.05$ and $####p < 0.0001$).

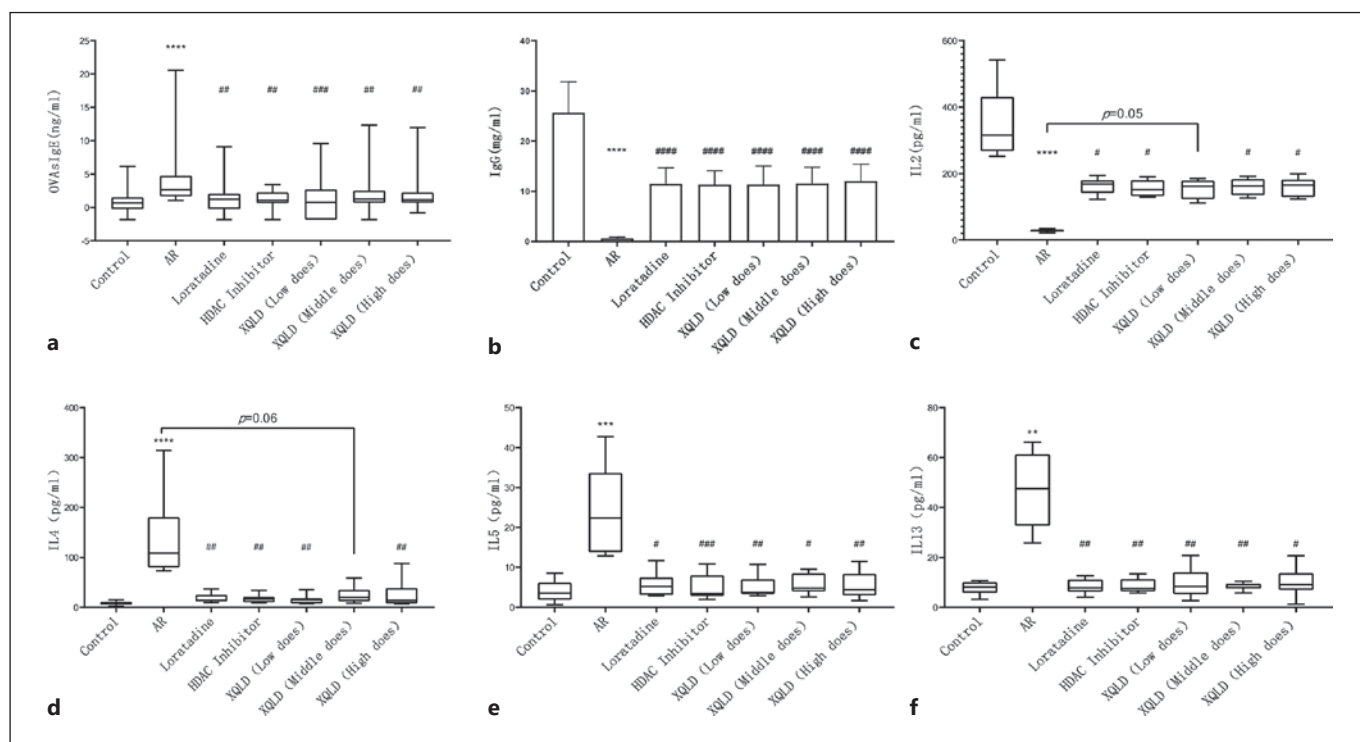


Fig. 5. Expression of OVA-sIgE, IL2, IgG, IL4, IL5, and IL13 in the serum of mouse. **a** OVA-sIgE in mouse serum. **b, c** type 1 inflammation-related factors IL2 and IgG in mouse serum. **d–f** type 2 inflammation-related factors IL4, IL5, and IL13 of serum (OVA-sIgE $n = 29$; IL4 $n = 9$; IgG $n = 6$; IL5, IL13, IL2 $n = 8$, compared with the control group: * $p < 0.05$ and **** $p < 0.0001$; compared with the AR group: # $p < 0.05$ and #### $p < 0.0001$).

that XQLD, loratadine and HDAC inhibitor could alleviate the nasal sensitivity symptoms of AR mice (Fig. 3).

XQLD Inhibits the Expression of HDACs in the Nasal Mucosa of AR Mice

Our results found that XQLD and HDAC inhibitors could both alleviate nasal allergy symptoms in mice. Then we detected the mRNA expression of HDAC1–11 (Fig. 4a–k) in nasal mucosa tissues to observe the changes of HDACs after XQLD and HDAC inhibitor intervention. The results showed that the mRNA expressions of HDAC1, HDAC3, and HDAC4 (Fig. 4a, c, d) in AR group were significantly higher than those in the control group ($p < 0.05$). However, after intervention with XQLD or HDAC inhibitors, HDAC1, HDAC3, and HDAC4 expression decreased significantly (Fig. 4a, c, d). We also verified Western blot results were consistent with the mRNA results (Fig. 4l).

XQLD and HDAC Inhibitors Reduce the Mucosal Immune Response in AR Mice

Effects of XQLD and HDAC Inhibitors on the Expression of OVA-sIgE and Type 1/Type 2 Cytokines in the Serum of AR Mice

To further confirm XQLD can inhibit AR inflammation by regulating expression of HDACs, we observed the changes of mucosal immune response after intervention with XQLD and HDAC inhibitors simultaneously in AR mice. We found that the content of serum OVA-sIgE in groups (XQLD, loratadine, and HDAC inhibitor group) significantly decreased compared with that in the AR group ($p < 0.05$). This result indicated that different doses of XQLD, loratadine, and HDAC inhibitors could inhibit the expression of serum-specific OVA-sIgE in AR mice (Fig. 5a). The levels of serum IL2 and IgG in the AR group were obviously lower than those in the blank control group ($p < 0.05$), while IL2 and IgG levels were significantly upregulated in the XQLD, loratadine, and HDAC inhibitor groups compared with the AR group (Fig. 5b, c) ($p < 0.05$). The high levels of IL4, IL5, and IL13

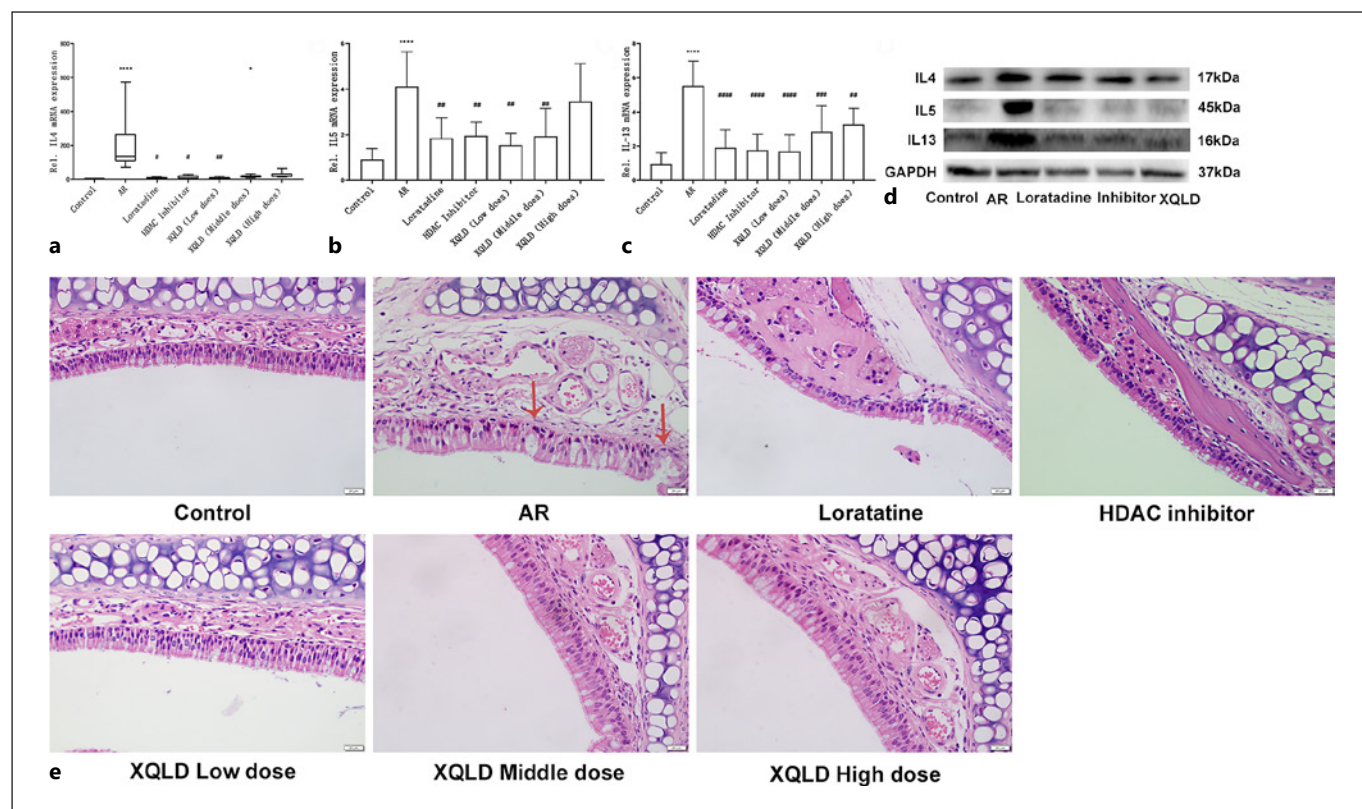


Fig. 6. Expression of IL4, IL5, and IL13 and pathological sections of nasal mucosa in mice. **a–c** mRNA levels of IL4, IL5 and IL13. **d** Protein levels of IL4, IL5, and IL13 in nasal mucosa. Inhibitors represent HDAC inhibitors, and XQLD represents a high dose of XQLD. **e** Pathological sections of nasal mucosa of mice in different groups. In the AR group, the integrity of the nasal mucosa was im-

paired, and goblet hyperplasia was observed, as shown by the red arrow. After XQLD and HDAC inhibitor intervention, the integrity of the nasal mucosa was restored. Images were made with $\times 400$ magnification (IL4, IL5, IL13 $n = 6$; compared with the control group: * $p < 0.05$ and **** $p < 0.0001$; compared with the AR group: # $p < 0.05$ and #### $p < 0.0001$).

in the AR groups were significantly inhibited after XQLD, loratadine, and HDAC inhibitor intervention ($p < 0.05$) (Fig. 5d–f).

XQLD and HDAC Inhibitors Downregulate the Expression of Type 2 Inflammatory Factors and Restore the Epithelial Integrity of Nasal Mucosa in AR Mice

We confirmed that XQLD at different concentrations was responsible for inhibiting the high levels of type 2 crucial inflammatory factors (IL4, IL5, IL13) in serum. To characterize the function of the XQLD on nasal mucosa, we detected the expression of IL4, IL5, and IL13 in nasal mucosa tissues. In this part, we also found that after interference with the XQLD or HDAC inhibitor, the expression levels of IL4, IL5, and IL13 in nasal mucosa tissues were obviously downregulated in mice (Fig. 6a–d). Pathological sections of nasal mucosa showed that in the

AR group, the integrity of the nasal mucosa was impaired, and goblet hyperplasia was observed. After XQLD intervention, the integrity of the nasal mucosa was restored and goblet cells reduced, which was also observed in the HDAC inhibitor group (Fig. 6e).

XQLD Promotes Nasal Mucosal Epithelial Function in AR Mice

It has been demonstrated that XQLD directly regulated the expression of HDACs in our previous results. HDACs have also been reported to negatively correlate with the nasal epithelial function [9]. We then explored the curable effects of XQLD or HDAC inhibitor on epithelial function. The results showed that the expression of Muc5ac and Muc5b (predominant gel-forming mucins of nasal mucosa) in AR mice was significantly higher than that in control mice. After XQLD or HDAC inhibitor intervention, Muc5ac and Muc5b expression decreased sig-

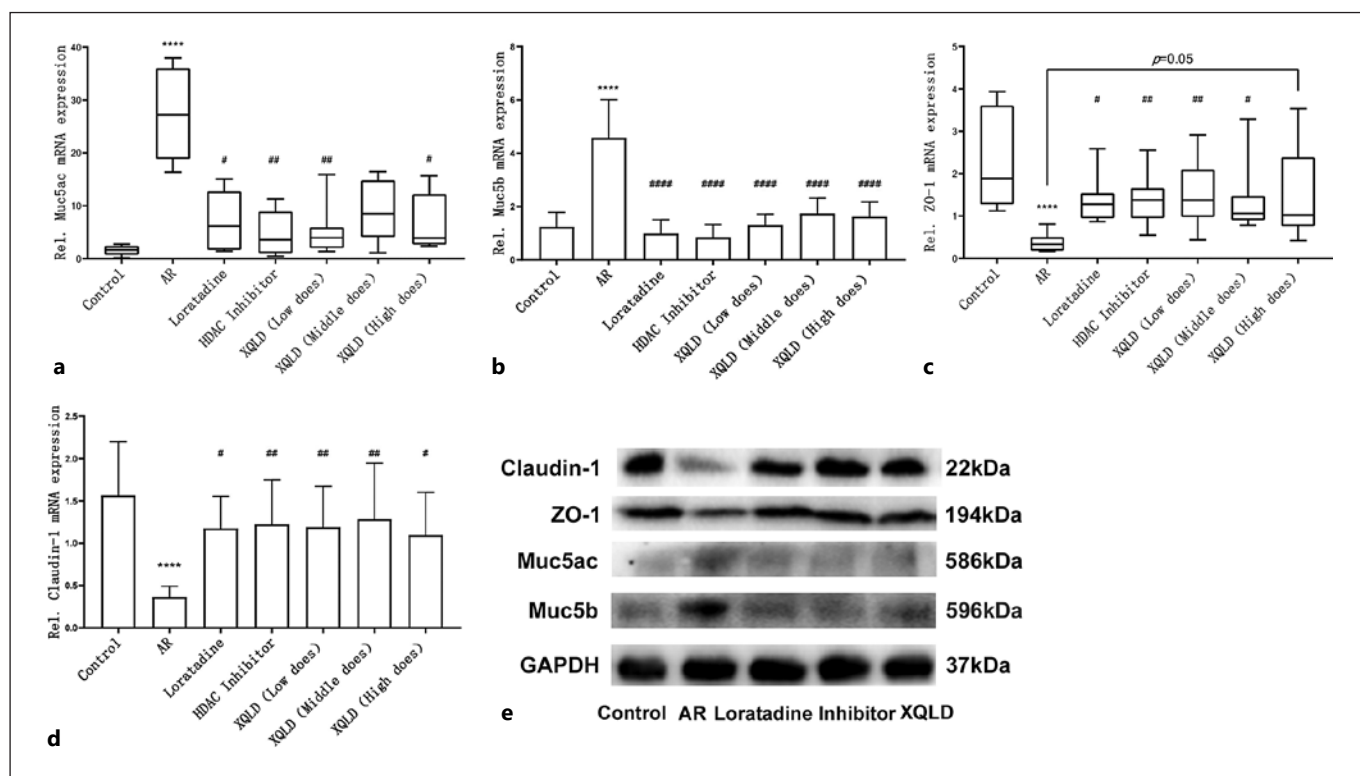


Fig. 7. The expression of epithelial barrier-related factors in nasal mucosa of mice. **a–d** The mRNA levels of Muc5ac, Muc5b, ZO-1, and claudin-1. **e** The levels of Muc5b, Muc5ac, claudin-1, and ZO-1 detected by WB. Inhibitors represent HDAC inhibitors, and XQLD represents a high dose of XQLD (Muc5b $n = 6$; Muc5ac, ZO-1, claudin-1 $n = 8$; compared with the control group: * $p < 0.05$ and **** $p < 0.0001$; compared with the AR group: # $p < 0.05$ and ### $p < 0.0001$). WB, Western blot.

nificantly (Fig. 7a, b). Moreover, the mRNA expression of ZO-1 and claudin-1 (main tight junction markers) in the nasal mucosa of AR mice was significantly lower than that of the control group ($p < 0.05$) but significantly increased with XQLD or HDAC inhibitor intervention (Fig. 7c, d). Western blot results also confirmed this conclusion (Fig. 7e).

Immunohistochemical staining was performed on the nasal mucosa tissues of mice in each group. The results showed that the expression of Muc5ac and Muc5b in the nasal mucosa of AR mice was higher than that of the control group, and the expression of Muc5ac and Muc5b in AR mice was inhibited after XQLD medication. The expression of claudin-1 and ZO-1 in the nasal mucosa of mice in the AR group was lower than that in the control group, while the expression of claudin-1 and ZO-1 was upregulated after XQLD treatment (Fig. 8).

Discussion

The combination drug therapy for patients is the key point to control symptoms and prevent recurrence of AR [14]. Therefore, exploring the better medication strategy and developing new pharmacological interventions are needed to improve AR management. In recent years, the effect of TCM in treating AR has been gradually recognized [15, 16], and the research on mechanism of TCM to treat AR has attracted a lot of attention.

As a classic Chinese medicine prescription, XQLD has been commonly used in the treatment of allergic diseases such as asthma and AR in clinical practice [6, 17]. However, because its therapeutic mechanism has not been fully described, XQLD still lacks a sufficient theoretical basis for its further clinical promotion and application. Therefore, it is of great significance to clarify the anti-inflammatory and therapeutic mechanisms of XQLD in treating

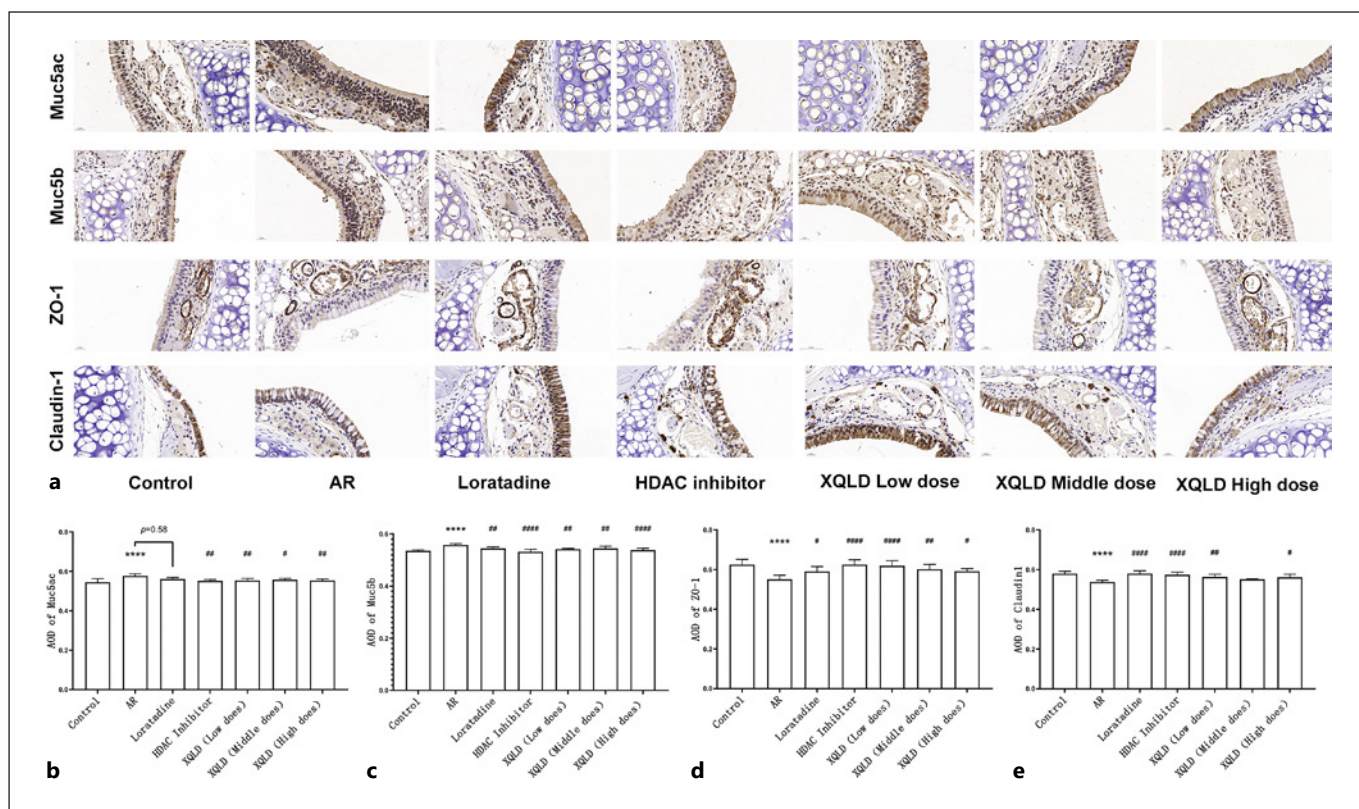


Fig. 8. Immunohistochemical images and AOD value of Muc5ac, Muc5b, ZO-1, and claudin-1 in the nasal mucosa of mice. **a** Immunohistochemical results. Compared with the control group, the positive expression of tight junction proteins ZO-1 and claudin-1 in nasal epithelial tissues of AR group were decreased, while the positive expression of mucins Muc5ac and Muc5b were increased. After loratadine, HDAC inhibitor and different doses of XQLD

intervention, the positive expression of ZO-1 and claudin-1 were significantly upregulated and expression of Muc5ac and Muc5b were downregulated. Images were made with $\times 400$ magnification. **b–e** AOD value of immunohistochemistry ($n = 6$; compared with the control group: * $p < 0.05$ and **** $p < 0.0001$; compared with the AR group: # $p < 0.05$ and #### $p < 0.0001$).

AR to provide more experimental basis for its effectiveness in treating AR.

Our results verified that XQLD could inhibit type 2 inflammatory response and restore the function of the nasal epithelium which might be mediated by inhibiting the expression of HDACs (HDAC1, HDAC3, and HDAC4). Type 2 inflammatory response and destruction of nasal epithelial function are key pathological changes in AR progression [18, 19]. Previous studies have reported the therapeutic effect of XQLD on immune response [17], but the regulation mechanism still needs more ascertained proof. In our study, XQLD has been demonstrated to directly inhibit the expression of type 2 inflammatory cytokines (IL4, IL5, and IL13). We also found that XQLD could downregulate the expression of HDAC1, HDAC3, and HDAC4. HDAC has been recognized it is closely related to immune regulation and can regulate the

innate and adaptive immune response [20, 21]. Recently, HDAC has been found it could also regulate the proliferation of type 2 innate lymphoid cells in airway inflammation [22] and modulate effector T cell phenotypes in severe COVID-19 patients [23]. All these demonstrated that HDAC is a promising immune regulator. Although our study was based on mouse models, we still demonstrated that XQLD could inhibit the type 2 inflammatory response and was responsible for inhibiting HDAC expression since the same trend was also observed in HDAC inhibitors group. More direct regulatory evidence needs to be further confirmed by HDAC gene knockdown in vitro or in vivo and hope to further demonstrate direct interaction between XQLD and HDAC.

The importance of epigenetics has been realized in the pathophysiology of AR [24]. HDACs are also closely related to the damage of nasal epithelial dysfunction in AR. Steelant

et al. [13] showed that HDAC inhibitors could restore epithelial integrity by upregulating the expression of tight junction proteins. In AR patients, HDACs member Sirtuin-1 disrupted the expression of e-cadherin in nasal epithelium [25]. From our study, we found the potential target of XQLD were HDACs. Additionally, we clarified that Muc5ac, Muc5b, ZO-1, and claudin-1 were regulated by XQLD and also regulated by HDAC inhibitor. It is reasonable to believe that XQLD achieving its regulatory effect may have relationship with regulating the HDAC expression. More importantly, the direct regulatory mechanism of XQLD on the nasal epithelial function also needs to be further clarified in subsequent experiment.

The role of XQLD on these two key pathological processes proves its effectiveness in AR treatment. In addition, protecting the nasal epithelial function is an important supplement to the therapeutic mechanism of XQLD which has not been reported before. Our findings also suggest that HDAC family members have close relationship with the acceleration of AR progression and may be a promising target of XQLD. Because of complex chemical composition of TCM, it is very important to detect the quality and stability of TCM composition which is crucial proof on the credibility of TCM effect. From our research, we first confirmed the pharmaceutical stability of XQLD by drug LC-MS profiles of different batches (Fig. 1). Drug safety of XQLD was also detected in ovalbumin sensitized mice (Fig. 2). The identification of drug stability and safety provides better evidence for the future clinical promotion of XQLD.

Chinese medicine is one optional and promising strategy in AR management [26, 27] because of its less drug resistance. It also can offer more choices to clinical specialists and patients, especially for those intolerance with glucocorticoid treatment. However, the therapeutic mechanism of TCM needs more experimental evidence to promote its further clinical promotion. Our study found the possible negative regulation of XQLD on HDACs in AR mice and also confirmed that XQLD and HDAC inhibitor effectively inhibited type 2 inflammation, restored epithelial function, and inhibited the expression of mucins in AR mice. These findings contribute new insight to the pathogenesis of AR and the treatment mechanism of XQLD.

Conclusions

Our data found that XQLD could inhibit the inflammatory progression of AR and promote the recovery of nasal epithelial dysfunction to help rebuild the patient's

own protection mechanism. It provides more experimental evidence for the AR treatment mechanism of TCM and provides new insights into the therapeutic mechanism of XQLD. These results also indicate that XQLD may inhibit Type 2 inflammatory progression and restore the function of nasal epithelium and is responsible for inhibiting the expression of HDACs in AR.

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Statement of Ethics

This project was approved by the Ethics Committee of Experimental Animal Center of the First Affiliated Hospital of Guangzhou University of Chinese Medicine with the approval number of TCMF1-2019048.

Conflict of Interest Statement

The authors declare that they have no competing interests.

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Author Contributions

Yan Ruan and Min Zhou conceived and designed the study. Hao-Lan Liu and Hui-Fang Chen performed experiments and analyzed data. Qin-Dong Liu and Wei-zhen Xu helped replenish the experiment data. Ya-jie Yan, Xue-Cheng He, and Jia-Jun Zhang helped analyze the experimental data.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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