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Luteolin Ameliorates Allergic Rhinitis in Mice through Modulating T Cell Subset Imbalance, Endoplasmic Reticulum Stress, and NLRP3 Inflammasome Axes

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ABSTRACT

Luteolin (LO) possesses pharmacological benefits like anti-inflammatory, antioxidant, and immune-boosting properties. This study aims to clarify the effect of LO on allergic rhinitis (AR) and its mechanisms and provide new insights for the clinical application of LO.

A mouse model for AR was developed through ovalbumin (OVA) stimulation. AR mice were gavaged with saline, low, medium, and high concentrations of LO, and montelukast. Nasal symptoms and scores were evaluated. The levels of OVA-specific immunoglobulins (OVA-sIgs), T helper cells (Th1, Th2, Th17), regulatory T cells (Tregs) cytokines, along with proinflammatory cytokines were measured using enzyme-linked immunosorbent assay (ELISA). Histopathological alterations were observed utilizing hematoxylin-eosin staining. Interleukin (IL)-1β and IL-18 levels were assessed through immunohistochemistry. Flow cytometry measured the percentage of T lymphocytes. The levels of endoplasmic reticulum stress (ERS)-related and NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome-related mRNAs and proteins were analyzed through reverse transcription-polymerase chain reaction (RT-PCR) and Western blot.

LO reduced nasal symptom scores in AR mice, upregulated OVE-sIgG2a levels, and downregulated OVE-sIgE, OVE-sIgG1, and histamine levels. After the administration of LO, AR mice showed an increase in Th1 and Treg cytokines levels, while Th2 and Th17 cytokines levels were reduced. LO ameliorated the splenic T cell subset imbalance and attenuated inflammatory cell infiltration. LO reduced the levels of ERS-related and NLRP3 inflammasome activation-related mRNAs and proteins in the nasal mucosa.

LO ameliorated AR symptoms by regulating T cell subset imbalance, hindering ERS and NLRP3 inflammasome activation.

Keywords: Allergic rhinitis, Endoplasmic reticulum stress, Luteolin, NOD-like receptor family pyrin domain containing 3 inflammasome

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INTRODUCTION

Allergic rhinitis (AR) is a persistent, noncommunicable condition affecting the upper respiratory system, currently afflicting 10% to 40% of the world's population, and its occurrence is on the rise due to industrialization.[1,2] While AR does not pose a direct risk to patients' lives, its complications significantly impact life quality and create a substantial social burden, making it a global health concern. Montelukast (MO) is an inexpensive and well-tolerated oral medication used to treat AR and chronic asthma.[3,4] However, some studies have shown that patients are at increased risk of adverse neuropsychiatric outcomes after MO sodium treatment, leading to potential mental health problems.[5,6] Therefore, it is imperative to search for powerful yet harmless drugs for managing patients with AR.

The involvement of helper T cells is essential in the pathophysiology of AR.[7] Allergen exposure induces the infiltration and activation of eosinophils by inducing the polarization of type 2 helper T cells (Th2), which triggers immunoglobulin E (IgE)-mediated inflammation, followed by the activation of mast cells, eosinophils, and lymphocytes and releases inflammatory factors like interleukin (IL)-4, IL-5, and IL-13.[8] In contrast, cytokines such as interferon (IFN)-γ and IL-2, produced by Th1, have been shown to inhibit the development of AR.[9,10] Not only that, Li et al analyzed clinical cases, AR patients have different degrees of Th17/Treg cell imbalance, which suggests that Th17/Treg cell imbalance may also play an important role in AR.[11] Therefore, AR is considered to arise from T cell subset imbalance, and restoring the T cell subset balance with therapeutic agents could be beneficial for managing AR.

In eukaryotes, the endoplasmic reticulum plays an essential role in upholding the regular functioning and stability of the internal cellular environment. Endoplasmic reticulum stress (ERS) occurs when endoplasmic reticulum homeostasis is disrupted by excessive protein processing load, inadequate nutrient supply, viral infections, and imbalanced redox reactions. ERS is crucial in the progression of chronic illnesses like cardiovascular disease and cancer.[12,13] Meanwhile, it has been shown that ERS is connected to the production and regulation of localized IgE in AR patients, suggesting that the reduction of localized IgE production by inhibiting ERS may offer a novel avenue for the treatment of AR.[14] Resano et al[15] proved that elovanoids N-34 (a lipid mediator and bioactivation messenger) can effectively counteract the effects of ERS and inflammatory factors on human nasal mucosal epithelial cells, and promote the homeostasis of the internal environment of human nasal mucosal epithelial cells, presenting a promising avenue for AR treatment.

The NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is a key player in the innate immune system, it is a multi-protein complex consisting of NLRP3, caspase-1, and apoptosis-associated-like protein containing a caspase recruitment domain (ASC).[16] NLRP3 is responsible for triggering the caspase-1 activation, and releasing cytokines like IL-1β and IL-18 when cells are damaged.[17] Mounting evidence indicates that NLRP3 inflammasome activation is critical in AR progression.[18] Studies have shown that the selective NLRP3 inhibitor improves symptoms and pathological changes in AR mice, indicating that focusing on NLRP3 may be a promising therapeutic strategy for AR.[19]

In recent years, numerous plants have shown promising potential in preventing and treating AR.[20] Various plants contain luteolin (LO), a flavonoid compound that displays pharmacological benefits including anti-inflammatory, immunomodulatory, and antioxidant effects.[21] LO has been shown to ameliorate palmitic acid-induced hepatocyte lipotoxicity by attenuating ERS.[22] Meanwhile, LO hindered the NF-κB signaling pathway, thereby suppressing mucus overproduction in airway epithelial cells.[23] However, whether LO can play an active role in AR treatment by modulating ERS and inflammasomes remains to be further investigated.

In this research, we established an ovalbumin (OVA)-induced AR model in mice and applied different doses of LO for intervention. By detecting T cell subset cytokines, ERS and NLRP3 inflammasome and other related indexes, we sought to examine the role of LO in AR and its mechanism.

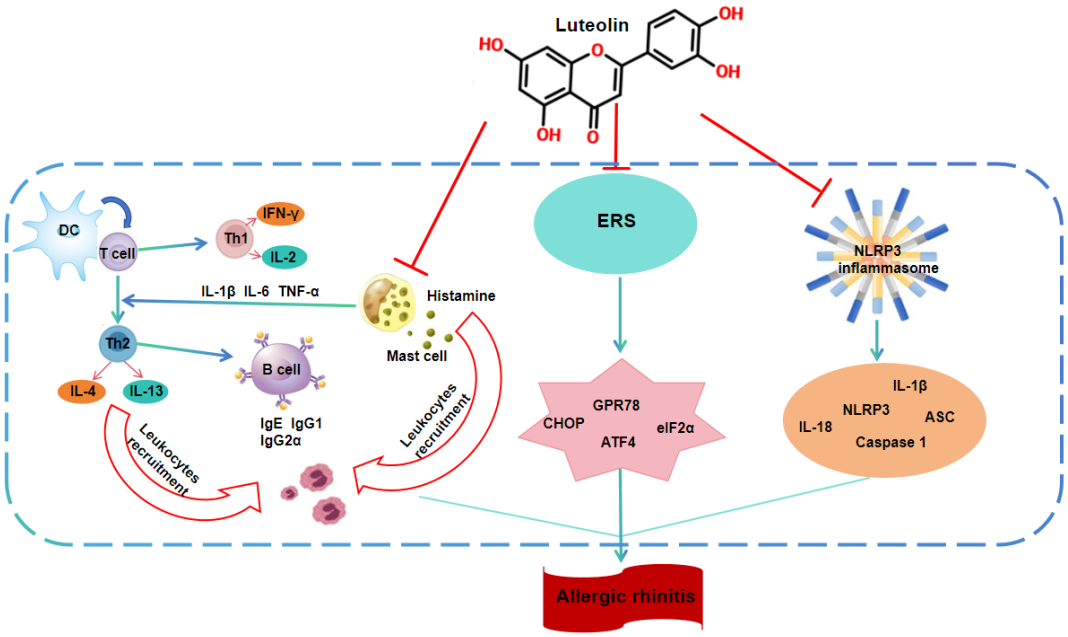

Figure 1. Luteolin (LO) regulated type 1 T helper (Th1)/ type 2 T helper (Th2) imbalance, reduced ovalbumin-specific immunoglobulin E (OVA-sIgE), ovalbumin-specific immunoglobulin G1 (OVA-sIgG1), and histamine production, and hindered endoplasmic reticulum stress (ERS) and NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome activation in nasal mucosal tissues, thereby improved allergic rhinitis inflammatory responses.

MATERIALS AND METHODS

Animals

5- to 6-week male SPF-grade Balb/c mice (n=48) with body mass of 18±2 g, were selected and supplied by Sipeifu Biotechnology Co., Ltd (Beijing, China). (Production License No. SCXK(Yu)2019-0002; Certificate of Conformity No. 410981201100011477). The mice were situated at the Laboratory Animal Center of Henan University of Traditional Chinese Medicine (Use Permit No. SYXK(Yu)2017-0001), with the indoor temperature controlled at 24±3°C, free diet and water intake, and acclimatized for 1 week before the experiment.

Establishment of Allergic rhinitis Mouse Model

The AR mouse model was established in 3 phases, consisting of basal sensitization, nasal excitation, and maintenance phase. In the basal sensitization phase, on days 1, 3, 5, 7, 9, 11, and 13 of the experiment, 40 μg of OVA (HY-W250978, MedChemExpress, Monmouth Junction, NJ, USA) and 1.5 mg of $Al(OH)_3$ (239186, Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 0.2 mL saline and injected into the right abdominal cavity of mice.[24,25] During the nasal excitation phase, mice were administered OVA (50 μL, 3 mg/100 μL) into each side of their nasal cavities once daily for a week, beginning on the 15th day of the experiment. The maintenance phase began on the 22nd day of the trial, with OVA (50 μL, 1 mg/100 μL) administered into the nasal cavities of mice every other day for 14 days in a row.

Experimental Grouping and Treatment

A total of 48 mice were distributed into 6 groups (n=8) in a random manner as follows: Sham, Model, LO low-dose (LO-L), LO medium-dose (LO-M), LO high-dose (LO-H), and MO. All groups, with the exception of the Sham group, had the AR mouse model established. The LO-L, LO-M, LO-H and MO group were gavaged with 2.5, 12.5, 25 mg/kg LO (1 mg/100 μL, HY-N0162, MedChemExpress) and 1.35 mg/kg MO (HY-13315A, MedChemExpress) on the basis of the models, respectively. Equal quantities of saline were administered to the mice in the Sham and Model groups. The administration of gavage continued for 14 days starting on the 22nd day of the experiment. Following the treatment period, mice were assessed for nasal

symptoms. Following an injection of sodium pentobarbital (50 mg/kg) (B005, Jian cheng, Nanjing, China), blood was taken from the eyeballs of the mice while they were under anesthesia. Subsequently, they were euthanized and the osseous nasal tissues were collected for future use.

Rhinitis Symptom Score

At the end of the experimental mice treatment, based on common clinical signs, with reference to literature reports,[26] the assessment of AR mice was conducted through 3 observers who were kept unaware of the treatment groups of the mice. Each symptom was numerically scored (0–3 points): (1) Rubbing: 0 point indicates that mice did not scratch their noses for 10 minutes; 1 indicates that mice rubbed their noses 1 to 3 times in 1 minute; a score of 2 means that mice rubbed their noses 4 to 6 times in 1 minute; while a score of 3 means that they rubbed their noses >6 times in 1 minute. (2) Sneezing: a score of 0 means the mouse did not sneeze for 10 minutes; a score of 1 means the mouse sneezed 1 to 3 times in 10 minutes; a score of 2 means the mouse sneezed 4 to 9 times in 10 min; a score of 3 points suggests that the mouse sneezed >10 times within a 10-minute period. (3) Nasal secretion: a score of 0 denotes no nasal secretion in the mouse; a score of 1 indicates the presence of nasal secretion in the nostrils; a score of 2 denotes nasal secretion outside the nostrils; and 3 points indicates that the mouse has overflowing nasal secretion in the nostrils. Achieving a total score exceeding 5 was deemed for AR model construction. The last score for rhinitis symptoms was calculated by taking the average of 3 observers' scores.

Enzyme-Linked Immunosorbent Assay (ELISA)

The blood collected was left at room temperature for 1 h. When the whole blood coagulated naturally and serum separated, the supernatant was taken for use after centrifuging. Nasal mucosa tissue was homogenized in phosphate buffer saline (PBS), centrifuged and the resulting liquid was gathered. The secretion levels of OVA-specific immunoglobulins (OVA-sIgs) like OVA-specific IgE (OVA-sIgE), OVA-specific IgG1 (OVA-sIgG1), OVA-specific IgG2a (OVA-sIgG2a), and histamine, IFN-γ, IL-2, IL-4, IL-17, IL-10, and IL-13 in mouse serum, as well as the levels of IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) in nasal mucous membrane tissues were quantified by ELISA. Mouse OVA-sIgE (ml063583), OVA-sIgG1 (ml037615), histamine (ml001877), IFN-γ (ml058350), IL-2 (ml063136), IL-4 (ml063156), IL-13 (ml063123), IL-17 (ml037866), IL-1β (ml098416), IL-6 (ml098430) and TNF-α (ml037211) kits were sourced from Enzyme-Link Biotechnology (Shanghai, China). Mouse IL-10 (ZC-37962) and OVA-sIgG2a (ZC-55530) kits were sourced from ZCIBIO Technology Co., Ltd. (Shanghai, China). 25 μL of serum (or tissue supernatant) and standard samples (50 μL) were placed in designated wells of a 96-well plate containing ELISA reagents. Following that, biotin (25 μL) and HRP Diluent (50 μL) were introduced into the sample wells and incubated at 37°C for 1 hour without light. Following 3 washes with wash buffer, substrate A and substrate B (50 μL each) were added and incubated for 20 minutes. After adding and thoroughly mixing termination solution, the OD450 value was measured and used to calculate the concentration.

Hematoxylin and Eosin (HE) Staining

Bony nasal tissues from mice were exposed to 4% paraformaldehyde (P0099, Beyotime, Shanghai, China) for 24 hours. The fixative was discarded, and 10 times the volume of decalcifying agent with ethylenediaminetetraacetic acid (ST1303, Beyotime) was added, and the decalcifying solution was renewed every other day for 18 days. The bony nasal tissue can be removed when there is no resistance to needling, and it was routinely dehydrated, paraffin embedded, sectioned (4–5 μm), deparaffinized with xylene (534056, Sigma-Aldrich), and hydrated with gradient ethanol. Following that, the tissue sections were dyed with hematoxylin (C0107, Beyotime) for 15 minutes. Subsequently, treated with ethanol hydrochloride differentiation solution (C0161M, Beyotime) for 5 minutes. The sample was treated with 85% ethanol for 3 minutes followed by incubation with eosin (C0109, Beyotime) for 1 minute. Afterward, the sample was dehydrated using a gradient of ethanol, clarified with xylene, and then sealed with neutral adhesive. Ultimately, the sections were viewed with a fully automated microscope (DMLA, Leica, Heidelberg, Germany).

Immunohistochemistry

The nasal tissue sections were treated by deparaffinizing with xylene, hydrating, and then dehydrating using a gradient of ethanol, antigen repair by microwave oven, and endogenous peroxidase

inactivation by 3% H_2O_2. Afterward, tissue sections were exposed to IL-1β antibody (ab9722, 1:50, Abcam, Cambridge, CA) or IL-18 antibody (ab191860, 1:50, Abcam) and left to incubate overnight at 4°C. On the following day, incubated with goat anti-mouse IgG (ab205719, 1:2000, Abcam) for 1 h before the color was developed in DAB solution (DA1010, Solarbio, Beijing, China). Mayer hematoxylin (C0107, Beyotime) was used for re-staining, and the film was sealed with neutral balsam. Observed under a DMLA fully automated microscope and photographed.

Preparation and Flow Cytometric Analysis of Splenic Lymphocyte Suspensions

Spleens were placed in Petri dishes, rinsed with PBS solution, and then repeatedly cut with ophthalmic scissors to make the tissue into little pieces which could be performed gently using a syringe needle core on the 200 mesh stainless steel filters to get cells. The suspension was transferred to a centrifuge tube and centrifuged, and the precipitate was resuspended with PBS solution, then added 2 times the volume of erythrocyte lysate, mixed thoroughly, leaved for 5 minutes, centrifuged and discard the supernatant. The cells were rinsed 2 times with PBS solution, and the supernatant was discarded; a splenocyte suspension (with a final density of 1×10^7 cells/mL) was created by mixing with PBS solution and then resuspended for later use. The APC-tagged $CD25^+$ (17-0251-82, Invitrogen, Carlsbad, CA, USA) and PE-tagged $Foxp3^+$ (12-5773-82, Invitrogen) were utilized to specifically label Treg cells, PE-tagged IL-$17A^+$ (12-7177-81, Invitrogen) was utilized to identify Th17 cells. APC-tagged IFN-γ+ (17-7311-82, Invitrogen) was used to label Th1 cells, and APC-tagged IL-4^+ (ab95715, Abcam) to label Th2 cells. Fluorescein isothiocyanate (FITC)-tagged $CD4^+$ (11-0041-82, Invitrogen) was used to label T cell subset. Ultimately, the labeled cells were examined utilizing flow cytometry.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Extraction of total nasal mucosal RNA was carried out with the RNeasy Plus Mini Kit (QIAGEN 74,104). Subsequently, reverse transcription was performed by adding AMV reverse transcriptase (TAKARA, Tokyo, Japan) to obtain cDNA. Then, the target gene was amplified using TB Green Premix Ex Taq II FAST qPCR kit (CN830S, TAKARA) in a PCR reaction with the cDNA as a template. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was designated as the internal reference. The $2^{-\Delta\Delta Ct}$ method was utilized to calculate and quantify the relative mRNA expression. The complete sequence of primers is shown in Suupplementary Table 1.

Western Blot

RIPA lysate (P0013B, Beyotime) was added to mouse nasal mucosa tissues to obtain the total proteins after high-speed centrifugation, and the BCA kit (P0012, Beyotime) was used for assessing protein concentrations. After separation using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12%, Invitrogen), the samples were shifted to a polyvinylidene fluoride (PVDF) membrane (Invitrogen), followed by blocking for 3 hours. After rinsing the membranes, placed them at 4℃ for an overnight incubation with associated primary antibodies NLRP3 (ab4207, 1:1000, Abcam), ASC (HY-P80548, 1:1000, MedChemExpress, Monmouth Junction, NJ, USA), caspase 1 (ab138483, 1:1000, Abcam), IL-1β (1:2000), IL-18 (1:400), C/EBP homologous protein (CHOP, MA1-250, 1:2000, Invitrogen), glucose-regulated protein 78 (GRP78, PA1-014A, 1:100, Invitrogen), eukaryotic initiation factor 2alpha (eIF2α, ab169528, 1:2000, Abcam), activating transcription factor 4 (ATF4, ab85049, 1:1000, Abcam), and GAPDH (MA5-15738, 1:1000, Invitrogen). On the following day, after being rinsed thrice, the membranes were cultured with goat anti-mouse IgG (ab205719, 1:20 000, Abcam) for 2 hours. Eventually, the developer solution (P0019, Beyotime) was used to cover each membrane surface for 2 minutes. The gray value of each protein band was obtained after processing the image using Image Lab 3.0 software (Bio-Rad, Hercules, CA).

Statistical Analysis

This study used SPSS 23.0 software (SPSS, Chicago, IL, USA) and GraphPad Prism 8.0.1 (GraphPad Software, San Diego, CA, USA) to analyze and construct graphs. The data is shown as the average value with the standard deviation. The Shapiro-Wilk test was employed to check the normality and homogeneity of the data. The experimental results were assessed through one-way analysis of variance (ANOVA), two-way ANOVA, and Tukey's multiple comparison test. $p<0.05$ signifying that there was a significant distinction.

RESULTS

Luteolin Ameliorates Nasal Symptoms in Allergic rhinitis Mice

Nasal symptoms commonly associated with AR include sneezing and nose rubbing, so we scored these symptoms to assess the success of AR modeling. Before the drug intervention, allergic behavior scores exhibited a significant increase in the Model group, as well as in the different concentrations of LO groups, and the MO group. Compared with the control group, the AR model was successfully created as indicated by the allergic behavioral scores exceeding 5 in the Model group, MO group, and different concentrations of LO group (p<0.001) (Figure 2A). The pharmacological intervention produced significant changes in allergic behavioral scores in all groups. There was an overall trend of allergic behavioral scores including rubbing, sneezing, and nasal secretion decreased in all groups administered the drug, and although allergic behaviors still occurred, they all decreased significantly compared to the Model group. The higher the administered dose of LO, the lower the allergic behavioral scores, indicating that LO can notably improve allergic symptoms (p<0.001) (Figures 2B–2D).

LuteolinInhibits OVA-specific Immunoglobulins and Histamine Release and Ameliorates Nasal Mucosal Injury in AR Mice

AR is marked by a heightened generation of serum OVA-sIgE and OVA-sIgG1, elevated histamine levels, and declined levels of IgG2a.[27,28] The Model group of mice showed markedly elevated serum levels of OVA-sIgE, OVA-sIgG1, and histamine, while IgG2a levels notably declined. In contrast, when compared with the Model group, the LO-L, LO-M, and LO-H groups, as well as the MO group, had markedly lower serum OVA-sIgE, OVA-sIgG1, and histamine levels, and higher OVA-sIgG2a level (p<0.001), which suggested that LO had the effect of attenuating OVA-specific allergic reactions in AR mice peripheral blood in a dose-dependent manner (Figures 3A–3D). Additionally, inflammatory factors like IL-1β, IL-6, and TNF-α levels showed a notable rise in the Model group, while a notable decline in their levels was observed in the LO-L, LO-M, LO-H, and MO groups (p<0.001) (Figures 3E–3G). In order to clarify the impact of LO on the inflammatory infiltration in AR mice nasal mucosal tissues, HE staining of the mice nasal mucosal tissues of each group was carried out in this study, and structural changes in the nasal mucosa and inflammatory infiltration were noted and recorded. Only sporadic inflammatory cells were observed in the Sham group, whereas the Model group displayed a higher level of inflammatory cell infiltration in the nasal submucosal connective tissue, vasodilatation, and congestion. The inflammatory injury of the nasal mucosal tissues was alleviated in the LO-L, LO-M, and LO-H groups, as well as the MO group (Figure 3H).

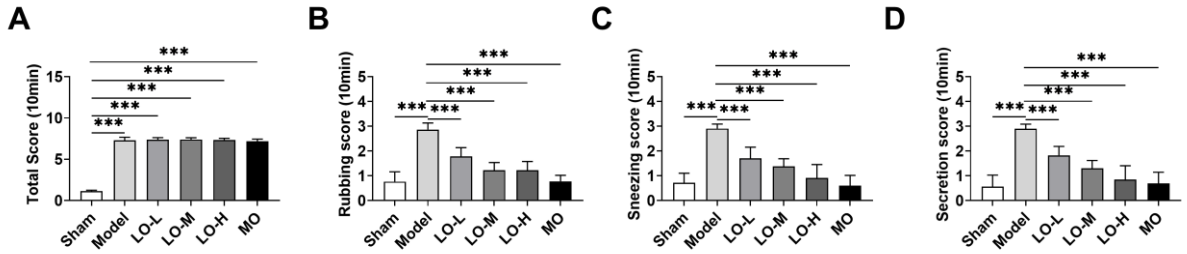

Figure 2. Scores of nasal symptoms in allergic rhinitis (AR) mice in each group. (A) Total score of nasal symptoms. In the AR model, a score above 5 was deemed as a successful sign. After constructing the AR mouse model, the luteolin (LO)-L, LO-M, and LO-H groups were gavaged with 2.5, 12.5, and 25 mg/kg LO (1 mg/100 µL), respectively, on the basis of the Model group. The montelukast (MO) group was gavaged with 1.35 mg/kg MO on the basis of the Model group. Saline solution was administered in equal amounts to both the Sham and Model groups. The mice were gavaged continuously for 14 days and scored for rubbing (B), sneezing (C), and secretion (D) on the last day. (*p<0.001)**

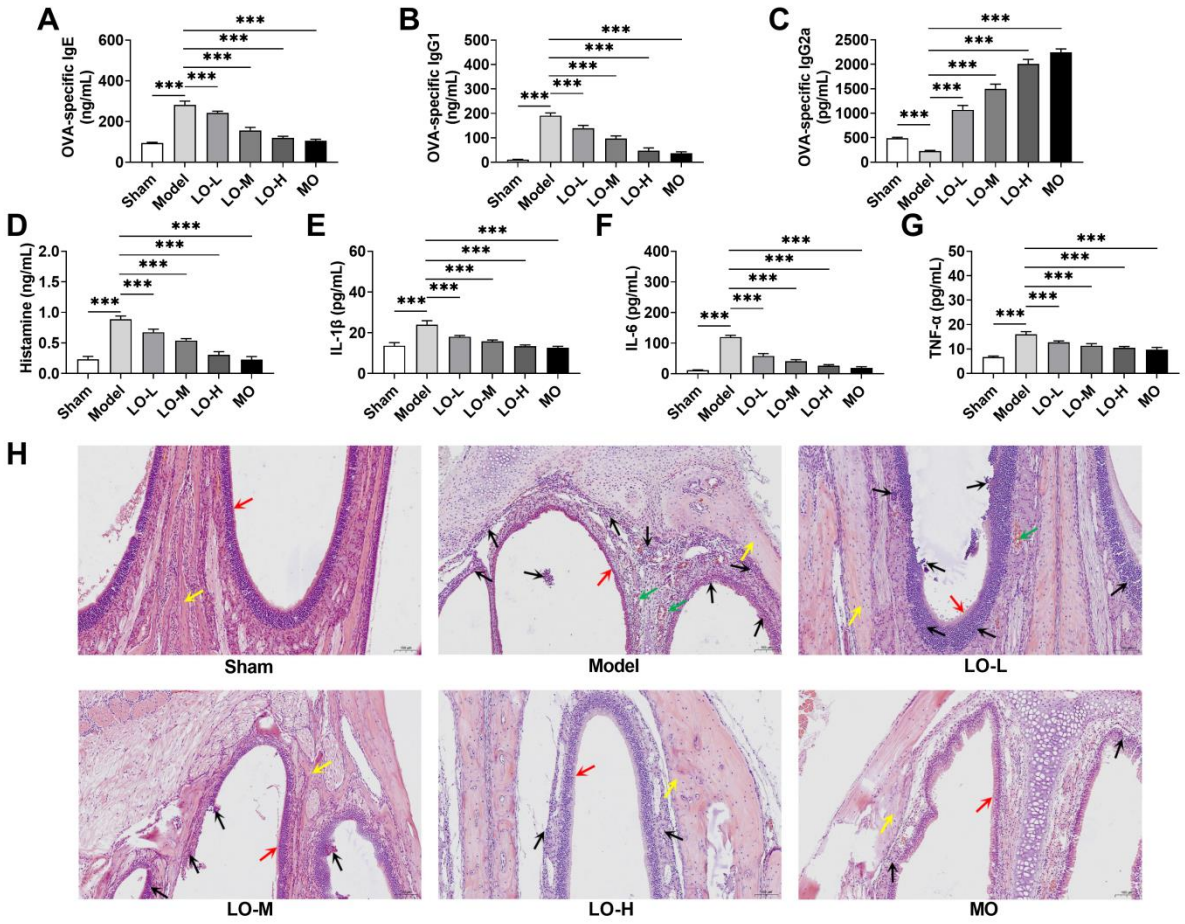

Figure 3. Luteolin (LO) inhibits ovalbumin-specific immunoglobulins (OVA-sIgs) and histamine release and ameliorates nasal mucosal injury in allergic rhinitis (AR) mice. (A-D) Serum samples from various treatment groups of mice were gathered, and the secretion of IgE, IgG1, IgG2a, and histamine were analyzed using enzyme-linked immunosorbent assay (ELISA). (E-G) Nasal mucosa tissue supernatant samples from various treatment groups of mice were gathered, and tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 levels were assessed using ELISA. (H) Differences in the infiltration of inflammatory cells in mouse nasal mucosa in various groups were observed by hematoxylin and eosin (HE) staining (200×, 100 μm). The red arrow shows the epithelium of nasal mucosa; the black arrow shows inflammatory cell infiltration in submucosal connective tissue; the green arrow shows vasodilation and congestion in the connective tissue; the yellow arrow shows cartilage. (*p<0.001)**

LO Ameliorates Th1/Th2 Imbalance in AR Mice

AR is primarily driven by T-cell imbalances, so we determined the effect of LO on Th1 and Th2 cytokines. The Model group showed significantly lower serum levels of IFN-γ and IL-2 (Th1 cytokines), and notably elevated levels of IL-4 and IL-13 (Th2 cytokines). However, when compared to the Model group, the MO, LO-L, LO-M, and LO-H groups showed significantly elevated levels of cytokines IFN-γ and IL-2, while IL-4 and IL-13 levels were notably reduced (p<0.001) (Figure 4A-4D). Flow cytometry assay showed that the level of Th2 cells in lymphocytes was notably elevated, whereas the level of Th1 cells was notably declined in the Model group (p<0.001). In the MO group and different concentrations of LO groups, there was a marked decline in Th2 cells and a marked increase in Th1 cell levels in spleen tissues (Figure 4E-4I). These results suggested that LO could effectively improve AR-mediated Th1/Th2 imbalance.

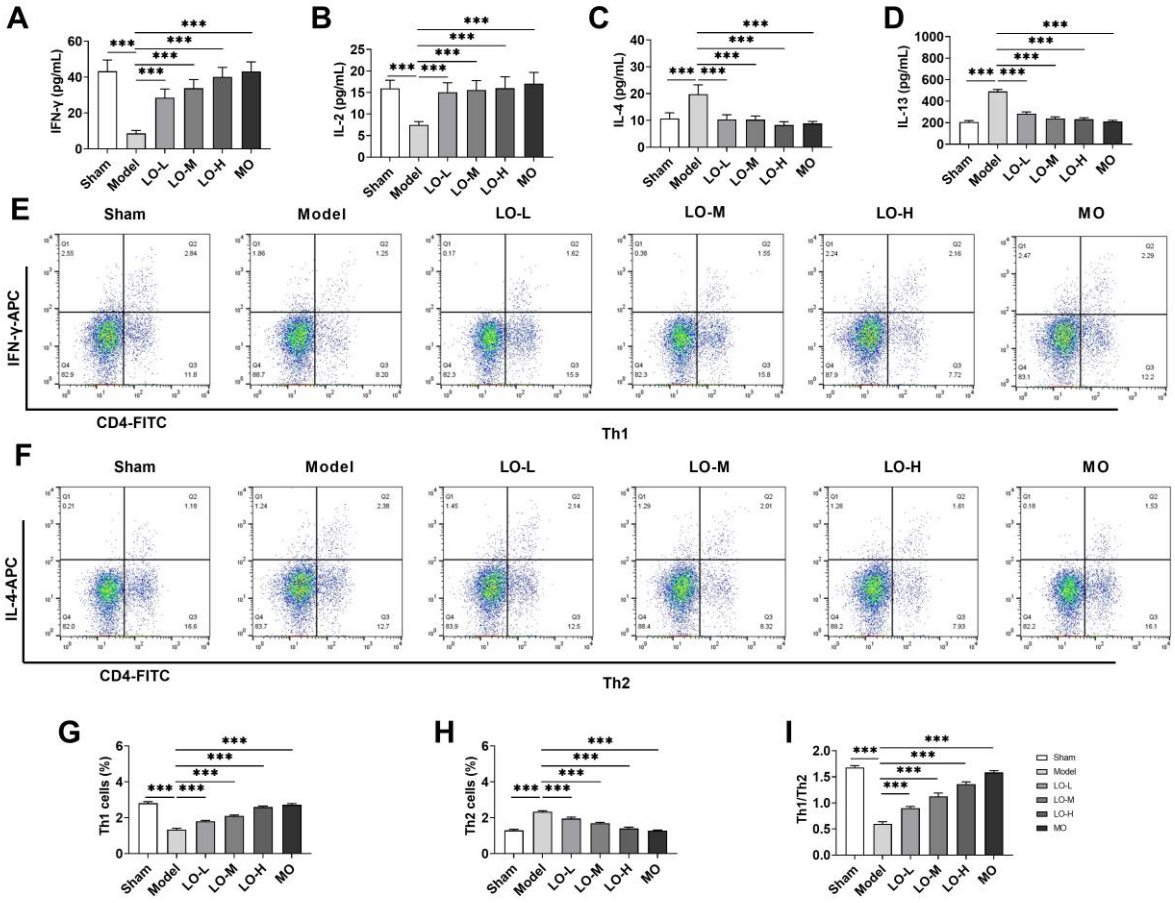

Figure 4. Luteolin (LO) ameliorates T helper cells (Th1 and Th2) imbalance in allergic rhinitis mice. A–D. The serum samples from mice in various treatment groups were obtained, and the levels of cytokines like interferon (IFN)-γ, interleukin (IL)-2, IL-4, and IL-13 were examined utilizing enzyme-linked immunosorbent assay (ELISA). E–I. Spleen tissues from different treatment groups were taken and lymphocyte suspensions were prepared, and the proportions of Th1 (IFN-γ^+ and $CD4^+$) and Th2 (IL-4^+ and $CD4^+$) cells were identified utilizing flow cytometry. (*p<0.001)**

Luteolin Ameliorates Th17/Treg Imbalance in Allergic Rhinitis Mice

Next, we determined the effect of LO on Th17 and Treg cytokines. The Model group showed a notably elevated level of IL-17 (Th17 cytokines), and a significantly lower serum level of IL-10 (Treg cytokines). However, when compared to the Model group, the MO, LO-L, LO-M, and LO-H groups showed a significantly elevated level of IL-10, while IL-17 level was notably reduced (p<0.001) (Figure 5A-5B). Flow cytometry assay showed that the level of Th17 cells in lymphocytes was notably elevated, whereas the level of Treg cells were notably declined in the Model group (p<0.001). In MO group and different concentrations of LO groups, there was a marked decline in Th17 cells and a marked increase in Treg cell levels in spleen tissues, suggesting that LO could effectively improve AR-mediated Th17/Treg imbalance (Figure 5C-5G). Combined with the previous results, LO may attenuate AR by regulating the T cell subset imbalance in the mice nasal mucosa.

LO Inhibits ERS of Nasal Mucosa in AR Mice

It has been shown that ERS is involved in the production and regulation of localized IgE in AR patients[14], so we determined the effect of LO on ERS in AR mice. RT-PCR findings indicated a notable increase in CHOP, GRP78, eIF2α, and ATF4 mRNA relative expression levels in the Model group. Treatment with LO or MO markedly decreased the levels of them in

mice nasal mucosa (p<0.001) (Figure 6A–6D). Additionally, Western blot analysis also revealed that CHOP, GRP78, eIF2α, and ATF4 levels were notably elevated after LO or MO treatment (p<0.001), and the influence of LO was dose-dependent, suggesting that LO reduces AR-mediated ERS (Figure 6E-6I).

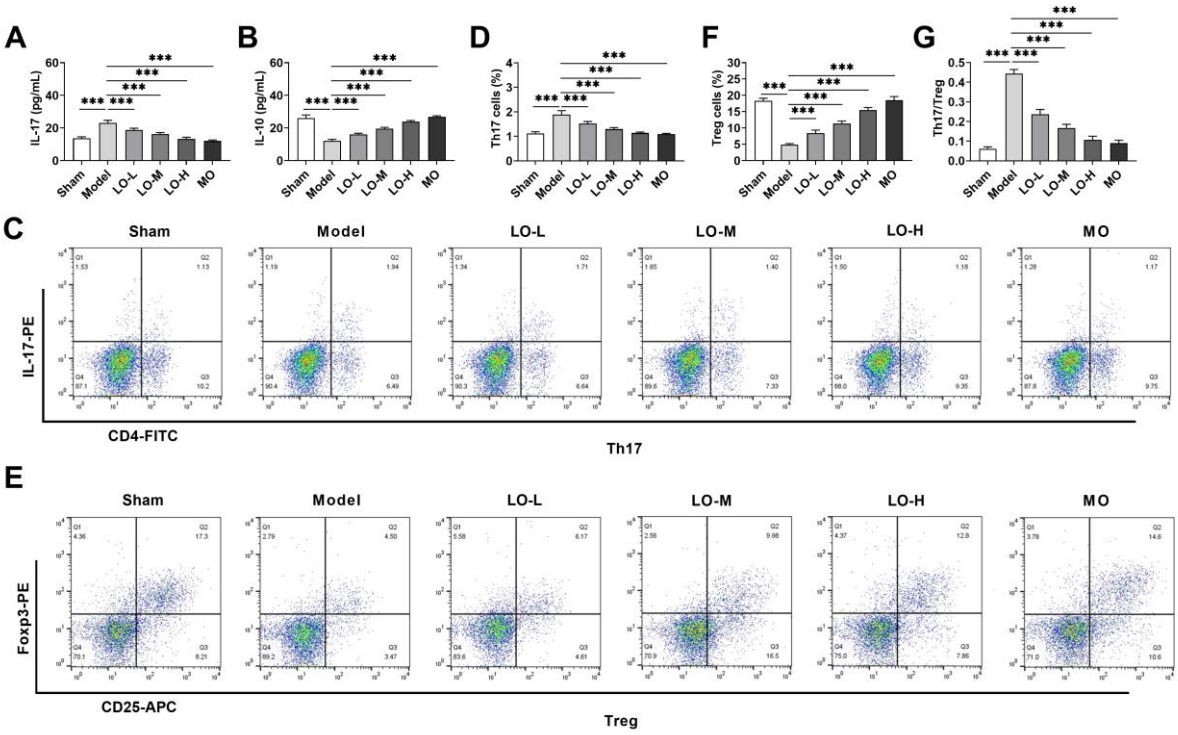

Figure 5. Luteolin (LO) ameliorates type 17 T helper (Th17)/ regulatory T (Treg) imbalance in allergic rhinitis (AR) mice. A–B. The serum samples from mice in various treatment groups were obtained, and the levels of interleukin (IL)-17 and IL-10 were examined utilizing enzyme-linked immunosorbent assay (ELISA). C–G. Spleen tissues from different treatment groups were taken and lymphocyte suspensions were prepared, and the proportions of Th17 (IL-17$^+$ and CD4$^+$) and Treg (CD25$^+$ and FoxP3$^+$) cells were identified utilizing flow cytometry. (*p<0.001)**

LO Inhibits NLRP3 Inflammasome Activation in the Nasal Mucosa of AR Mice

NLRP3 inflammasome is a key player in the innate immune system, it secretes proinflammatory cytokines IL-1β and IL-18 in response to cell injury.[17] The immunohistochemical analysis revealed the presence of IL-1β and IL-18 proteins in the ciliated epithelial cells of the nasal mucosa, which appeared brownish-yellow. The number of IL-1β- and IL-18-positive cells in mice nasal mucosa was low and their distribution was scattered in the Sham group. In contrast, the number of IL-1β and IL-18 positive cells was significantly increased in the Model group (p<0.001). Additionally, the amount of IL-18 and IL-1β-positive cells in the LO-L, LO-M, and LO-H groups, as well as the MO group were notably lower, suggesting that LO could inhibit the secretion of IL-18 and IL-1β (Figure 7A–7C). The levels of NLRP3, ASC, Caspase-1, IL-1β, and IL-18 were notably elevated in mice nasal mucosa tissues of the Model group. The LO-L group showed no significant alteration in IL-1β level in nasal mucosa tissues compared to the Model group, but there was a marked decrease in NLRP3, ASC, Caspase 1, and IL-18 levels (p<0.001). In addition, compared to the Model group, the LO-M, LO-H, and MO groups exhibited a notable reduction in NLRP3, ASC, Caspase 1, IL-1β, and IL-18 levels (Figure 7D–7I). These results suggested that LO hindered the NLRP3 inflammasome activation, which may be an important mechanism for its alleviation of AR.

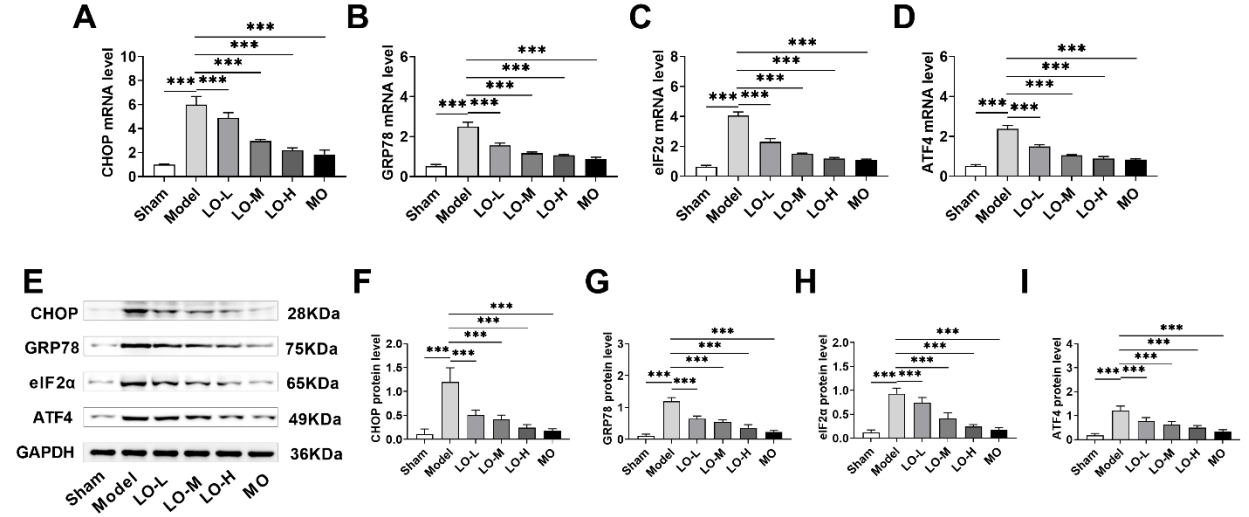

Figure 6. Luteolin (LO) inhibits endoplasmic reticulum stress (ERS) of nasal mucosa in allergic rhinitis (AR) mice. (A-D) In mice nasal mucosa tissues from various treatment groups, reverse transcription-polymerase chain reaction (RT-PCR) examined the levels of C/EBP homologous protein (CHOP), glucose-regulated protein 78 (GRP78), eukaryotic initiation factor 2alpha (eIF2*α*), and activating transcription factor 4 (ATF4) mRNA. (E-I) Examining CHOP, GRP78, eIF2*α*, and ATF4 protein levels after various treatments through Western blot. (*p<0.001)**

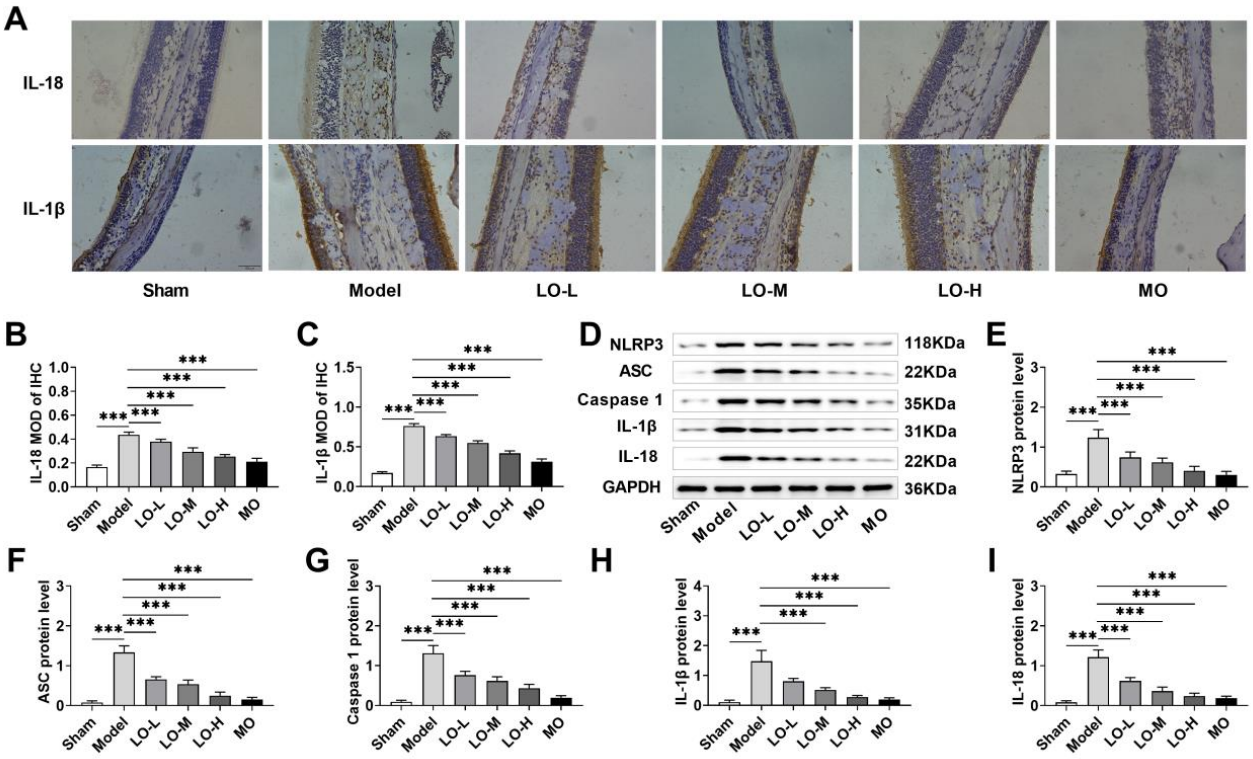

Figure 7. Luteolin (LO) inhibits NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome activation in the nasal mucosa of allergic rhinitis (AR) mice. A–C. Immunohistochemistry was used to detect the localization of interleukin (IL)-1β and IL-18 in mice nasal mucosa tissues in different treatment groups and to quantify their expression. (×400, 50 μm) D–I. Examining the levels of NLRP3, apoptosis-associated-like protein containing a caspase recruitment domain (ASC), Caspase 1, IL-1β, and IL-18 in mice nasal mucosa tissues through Western Blot, and quantified the level of each protein. (*p<0.001)**

DISCUSSION

AR is a common chronic respiratory disease that is highly prevalent worldwide, leading to significant social and economic challenges on a global level. The complexity of AR's pathogenesis is attributed to factors like immunity, infection, environment, and genetics.[29] However, its pathogenesis has not been fully elucidated up to now. Inhaled glucocorticoids are considered first-line anti-inflammatory agents in the management of AR.[30] Nevertheless, some patients have hormone dependence or hormone resistance, leading to unsatisfactory treatment results. Consequently, there is an urgent need to search for novel AR therapeutics.

LO has dual anti-inflammatory and anti-ERS activity.[31] Jang et al showed that LO decreased the cellular infiltration of lung parenchyma and nasal tissues.[32] Additionally, Qiao et al revealed that LO hindered IL-36γ secretion and modulated airway inflammation in an asthma rat model.[33] However, previous studies have not thoroughly investigated the potential mechanisms behind the immunomodulatory effects of LO. Therefore, our focus was on examining how LO actions and their effectiveness in treating AR. The crucial role of eosinophils is evident in the pathogenesis and regulation of AR. Activated eosinophils express FcεRI receptors that bind to high levels of IgE in the blood of AR patients, and release excessive inflammatory and allergic mediators, leading to recurrent nasal allergic and inflammatory reactions.[34] Furthermore, IgG1 and IgG2a are implicated in mediating the immune he immune reaction, with high expression of IgG1 and low expression of IgG2a during allergic reactions.[10,35] As seen in our experimental results, in AR mice, LO dose-dependently suppressed the serum levels of OVA-sIgE, OVA-sIgG1, and histamine levels, and increased OVA-sIgG2a level, suggesting that LO may exert an inhibitory effect on allergic responses such as eosinophil recruitment in AR mice.

Studies of late have suggested a link between the progression of AR and an imbalance in Th1/Th2 levels and Th17/Treg levels.[36,37] Zhu et al found that improving the balance of Th1/Th2 cells effectively attenuated allergic reactions in AR mice through immunotherapy.[38] You et al showed that D-Pinitol treatment increased Th1 cytokine levels and decreased Th2 cytokine levels and had a positive effect on OVA-induced AR.[39] In addition, a study showed that Baicalin can reduce the level of inflammatory factors in the nasal lavage fluid of OVA-induced AR mice by regulating the imbalance of Treg/Th17 cells, thereby alleviating AR symptoms[11]. Our experimental results showed that after administration of LO, AR mice showed a marked increase in Th1 cytokines (IFN-γ and IL-2) and Treg cytokines (IL-10) levels in their nasal mucosa tissues, while Th2 cytokines (IL-4 and IL-13) and Th17 cytokines (IL-17) levels were notably reduced. The ratio of splenic lymphocytes detected by flow cytometry further confirmed that a notable rise in Th1 and Treg cells and a marked decline in Th2 and Th17 cells in the mouse spleen in the different concentrations of LO groups. Not only that, we found that the inflammatory damage of mouse nasal mucosal tissue was improved in all dose groups of LO, and the inflammatory cell infiltration was significantly reduced. These findings suggest that LO can inhibit the inflammatory reaction in AR by regulating the T cell subset imbalance in the mice's nasal mucosa.

ERS leads to exacerbation of bronchospasm and inflammatory response in airway allergic inflammatory diseases, further worsening the condition.[40] It has been documented that herbal monomers, including LO, can block ERS and have potential therapeutic effects in a variety of diseases.[41] LO has been shown to lower GRP78 levels in astrocytes in a mouse model of Alzheimer's disease, providing more evidence of LO's anti-ERS effects.[42] In our experiments, after administration of LO treatment to AR mice, it was observed that the indicators of ERS, CHOP, GRP78, eIF2α, and ATF4, were improved to different degrees in the nasal mucosal tissues. This suggests that LO can provide anti-endoplasmic reticulum protection to AR mice, which in turn attenuates their nasal mucosal damage.

The activation of the NLRP3 inflammasome is strongly linked with respiratory disorders. Research has revealed that AR mice exhibit higher levels of NLRP3 and caspase-1 protein expression along with elevated cytokine IL-1β levels in comparison to normal Shams.[43] According to Yang et al the levels of NLRP3 and caspase-1 were increased in the nasal lavage fluid of individuals with AR.[44] NLRP3 inflammasome can trigger macrophage pyroptosis, activate caspase-1, and enhance IL-1β and IL-18 production. This process amplifies the inflammatory response, ultimately

contributing to the development of AR.[45] Therefore, suppressing NLRP3 inflammasome-triggered pyroptosis emerges as a potential therapeutic method to reduce the inflammatory reaction in AR. Notably, LO has been shown to inhibit NLRP3 activation, which in turn attenuates inflammatory damage in cardiomyocytes and has the potential to ameliorate myocarditis.[46] Our results showed that the application of LO to AR model mice resulted in a decline in the amount of IL-1β and IL-18-positive cells, as well as a marked decrease in the level of NLRP3, ASC, Caspase-1, IL-1β, and IL-18 proteins. This suggests that LO can inhibit NLRP3 activation in the AR model and has a favorable ameliorative effect on AR.

However, our current study has some limitations. First, although we found that LO was able to attenuate nasal allergy symptoms by modulating T cell subset imbalance and decreasing ERS and inflammatory responses, other pharmacological effects, including anti-oxidative stress properties, may also play a role in reducing AR symptoms. Second, we have not investigated the signaling pathway through which LO mediates ERS and inflammatory vesicles to exert its mechanism of action in treating AR. Finally, the route of administration of LO in our study was by gavage, and its intranasal efficacy needs to be further determined in future experiments and clinical efficacy demonstrated through clinical trials.

In conclusion, this study demonstrated that LO can effectively improve allergy symptoms by regulating T cell subset imbalance and inhibiting ERS and NLRP3 inflammatory vesicle activation in nasal mucosal tissues. This research elucidated the influence of LO on AR and its related action mechanism, offering new insights for using LO in clinical practice and treating AR.

STATEMENT OF ETHICS

The Laboratory Animal Ethics Committee of Henan University of Traditional Chinese Medicine approved the study protocol (Ethics Approval No. DWLL202203009), and the experimental process followed the rules of the laboratory, and the experimental animals were given humane care and protection following the 3R principle.

FUNDING

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

Not applicable.

DATA AVAILABILITY

The data supporting the findings of this study can be obtained from the corresponding author, Xiaoning Chen, upon request.

AI ASSISTANCE DISCLOSURE

Not applicable.

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