Ind. J. Pharm. Edu. Res., 2027; 61:10.5530/ijper.20272027.
https://www.ijper.org

Original Article

Effects of Luteolin (LT) on the TLR4/NF-κB Pathway and Oxidative Stress in Ovalbumin-Induced Asthma Young Rat Model

Cui Changqiang, Zha Li, Ren Tailiang, Lin Xiaofei*

Department of Pediatrics, Huai'an Maternal and Children's Health Care Hospital, Huai'an, Jiangsu Province, CHINA.

ABSTRACT

Background: Ovalbumin (OVA)-induced asthma in young rats is a widely used model to investigate airway inflammation and related molecular pathways. This study evaluated the therapeutic effects of LT on OVA-induced asthma and explored the involvement of the TLR4/NF-κB(p65) signaling pathway. **Materials and** Methods: Young rats were divided into six groups: Normal, Model, LD-LT (25 mg/kg), MD-LT (50 mg/kg), HD-LT (100 mg/kg), and HD-LT + TLR4 inhibitor (100 mg/kg LT + TAK-242). Except for the Normal group, all groups were sensitized and challenged with OVA to induce asthma. LT and TAK-242 treatments were administered based on group allocation. Bronchoalveolar Lavage Fluid (BALF) was analyzed for total cell count and differential cell populations. Oxidative stress markers (MDA, NO, SOD, ROS), apoptosis levels, and TLR4/NF-κB(p65) gene and protein expression were evaluated. Results: Compared with the Model group, LT treatment significantly reduced total BALF cells and eosinophils, while neutrophils and macrophages significantly increased ($p < 0.05$). LT also down-regulated MDA and NO levels ($p < 0.05$), reduced apoptotic cell numbers ($p < 0.05$), increased SOD concentration ($p < 0.01$), and decreased ROS levels ($p < 0.05$). Additionally, LT markedly suppressed TLR4/NF-κB(p65) gene and protein expression. The effects observed in the HD-LT group were comparable to those of the HD-LT + TLR4 inhibitor group. Conclusion: LT ameliorates OVA-induced asthma in young rats, likely through inhibition of the TLR4/NF-κB(p65) signaling pathway. High-dose LT exhibits therapeutic effects similar to LT combined with a TLR4 inhibitor, suggesting that LT may act via TLR4-mediated mechanisms *in vivo*.

Keywords: Luteolin, Asthma, OVA, TLR 4, NF-κB(p65).

Correspondence:
Dr. Lin Xiaofei
Department of Pediatrics, Huai'an Maternal and Children's Health Care Hospital, Huai'an, Jiangsu Province, CHINA.
Email: linxiaofei1221@163.com

Received: 07-04-2026;
Revised: 29-06-2026;
Accepted: 18-08-2026.

IN PRESS

INTRODUCTION

Asthma is a common chronic inflammatory airway disease characterized by recurrent airway obstruction, excessive mucus secretion, and infiltration of inflammatory cells into the airway tissues, resulting in symptoms like wheezing, shortness of breath, chest tightness, and coughing.[1] Excessive secretion of mucus in the airways can impede air flow into the respiratory tract, leading to difficulty breathing, suffocation, and even death.[2] Asthma is an increasingly serious public health problem affecting over 300 million people worldwide, and its incidence is rapidly increasing, imposing a heavy economic burden on society.[3] Commonly used medications for asthma include β2-adrenergic agonists, inhaled corticosteroids, and methylxanthines. Among these, inhaled corticosteroids are currently considered the most effective treatment, but they can cause serious systemic side effects when used for long term. Therefore, it's urgently needed to find a new and effective medication.[4]

Asthma is often accompanied by infiltration of eosinophils, neutrophils, and macrophages in the airway tissue. These inflammatory cells can lead to airway inflammation and further enhance the release of inflammatory mediators and cytokines, which induce high levels of Reactive Oxygen Species (ROS) and tissue damage, resulting in a decrease in tissue antioxidant capacity.[5] ROS can also exacerbate airway tissue inflammation by activating the TLR4/NF-κB(p65) signaling pathway.[6] In recent years, clinical and experimental research has made significant progress in the treatment of asthma with traditional Chinese medicine, which has the advantages of minimal side effects and sustained relief of clinical symptoms in the treatment of asthma.[7]

Luteolin (LT) is a glycoside found in various plants, with higher concentrations in honeysuckle, chili peppers, wild chrysanthemum, honeysuckle, and perilla. It possesses antitussive and expectorant properties. According to recent research, respiratory symptoms are closely related to chronic

DOI: 10.5530/ijper.20272027

Copyright Information :
Copyright Author (s) 2027 Distributed under Creative Commons CC-BY 4.0

Publishing Partner : Manuscript Technomedia. [www.mstechnomedia.com

airway inflammation. Conditions like bronchial asthma, Chronic Obstructive Pulmonary Disease (COPD), chronic pharyngitis, allergic rhinitis, and others that cause cough, sputum production, and wheezing, are all considered to be associated with local inflammatory infiltration. Local inflammation disrupts the immune response in the airways, leading to increased airway reactivity in patients. The primary approach to treatment should aim at eliminating chronic inflammatory infiltration in the airways. Currently, glucocorticoids are commonly used to eliminate airway inflammation, but they come with numerous side effects and are not suitable for long-term use. Research indicates that LT inhibits macrophage phosphorylation, suppresses the activity of the transcription factor NF-kB, and can inhibit Lipopolysaccharide (LPS)-induced production of cytokines IL-6 and TNF-α, both of which play a crucial role in the inflammatory mechanism and serve as sensitive indicators of inflammation. LT also increases IFN-γ, reduces specific IgE, and decreases eosinophil infiltration. In addition to its anti-inflammatory and anti-allergic effects, LT also inhibits Phosphodiesterase (PDE), possesses anti-SARS and anti-HIV virus characteristics. For example, it inhibits the activity of SARS virus's precursor S protein, thereby preventing its entry into host cells. LT is also used to treat chronic cough caused by conditions such as COPD, bronchial asthma, chronic pharyngitis, and allergic rhinitis. LT is a natural flavonoid compound with anti-inflammatory and free radical scavenging properties.[8,9] Current research has found that LT can alleviate bronchoconstriction and airway hyper-reactivity in allergic rats, but there is no study on LT's role in asthma from the perspective of the TLR4-related pathway. This study aims to induce a rat's model of asthma with Ovalbumin (OVA) and explore the antioxidant effects of LT on asthmatic rats. LT's impact on the activation of the TLR4/NF-κB(p65) pathway induced by Reactive Oxygen Species (ROS) will also be investigated. This research aims to provide a theoretical basis for the role of LT as an antioxidant in the treatment of pediatric asthma and the inhibition of airway inflammation.

MATERIALS AND METHODS

Experimental Animals

A total of 54 neonatal specific pathogen-free male SD rat, aged 5 days, weighting 18 to 22 g, were obtained from the Experimental Animal Center of Anhui Medical University. All rat were housed in a controlled environment with temperature maintained between 22 and 26°C, humidity between 50% and 60%, 12 hr/12 hr light-dark cycle, and free access to food and water.

Experimental Animals and Reagents

OVA powder was purchased from Sigma-Aldrich; aluminum hydroxide and luteolin, were obtained from MCE (China); pentobarbital sodium salt was purchased from Merck Millipore Laboratory Equipment (Shanghai) Co., Ltd. Cell dilution solution, concentrated 2,2'-Diaminobenzidine (DAB) kit, proteinase K solution, and BCA protein concentration determination reagent kit were procured from Beijing Soleibao Technology Co., Ltd. Wright-Giemsa staining solution was obtained from Wuhan Servicebio Co., Ltd. Methanol was purchased from China National Pharmaceutical Group Corporation Chemical Reagent Co., Ltd. Terminal deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling (TUNEL) detection kit was purchased from Roche Pharmaceuticals (Shanghai) Co., Ltd. Superoxide Dismutase (SOD), Malondialdehyde (MDA), and Nitric Oxide (NO) detection kits were obtained from Nanjing Jiancheng Bioengineering Institute. RIPA lysis buffer, rabbit anti-NF-κB monoclonal antibody, rabbit anti-TLR4 monoclonal antibody, rabbit anti-GAPDH monoclonal antibody were purchased from Wuhan Bioswamp Biotechnology Co. Ltd., Rabbit anti-p-NF-κB monoclonal antibody was obtained from Abcam. Type II and Type IV collagenases were purchased from Gibco. The Reactive Oxygen Species (ROS) detection kit was procured from G-clone (Beijing) Biotechnology Co. Ltd., TAK-242 (TLR4 inhibitor) was purchased from MCE (USA).

Methods

Construction of Asthma Model

For the establishment of the asthma model, OVA and aluminum hydroxide were mixed in sterile PBS at 200 μg/mL and 10 mg/mL respectively, and after acclimating the rat for 3 days, 200 μL of the OVA solution were injected intraperitoneally on days 1 and 14. From day 28 to 31, the rat were exposed to a 5% OVA solution aerosol for 30 min and intranasally administered 20 μL of 40 mg/mL OVA solution once. Symptoms like increased respiration, cyanosis of the lips, nodding respiration, among others, indicated successful construction of the asthma model. Rat in the normal control group were treated with physiological saline instead at the same time.

Grouping and Treatment of Experimental Animals

The modeled rat were randomly divided into 5 groups of 9 rat: the asthma model group (Model group), Low-Dose Luteolin Group (LD-LT), Medium-Dose Luteolin Group (MD-LT), High-Dose Luteolin Group (HD-LT), and High-Dose Luteolin combined with TLR4 inhibitor group (HD-LT+TLR4 inhibitor group). After construction of the asthma model, the LD-LT, MD-LT, and HD-LT groups were orally administered LT at 25, 50, and 100 mg/kg, respectively.[10] The HD+TLR4 inhibitor group received an oral dose of 100 mg/kg of LT and 0.5 mg/kg of TAK-242. The rats were treated as above once a day, for a total of 7 days.[11] Two hours after the final administration, the rats were anesthetized with pentobarbital sodium, and their neck skin was cut open to expose the trachea. A small incision was made in the trachea, and a tracheal cannula was inserted. The lungs were lavaged with PBS through the Bronchoalveolar Lavage (BAL) method three times to collect the Bronchoalveolar Lavage Fluid (BALF). Blood was obtained by eye enucleation, and serum was separated and stored

IN PRESS

at -80°C. The right lung was dissected, and the middle lobe was fixed in 10% formalin. The remaining lung tissue samples were placed in DNase-free EP tubes, immersed in RNAlater reagent, and immediately placed in a refrigerator at 4°C for 24 hr before being transferred to a freezer at -80°C.

All procedures were conducted in accordance with the Guidelines for the Use and Care of Laboratory Animals (NOM-062-ZOO-1999, Ministry of Agriculture and Livestock, SAGARPA, Mexico), and the protocol was approved by the Institutional Committee for the Care and Use of Laboratory Animals. This study was approved by Ethics Committee of Huai'an Maternal and Children's Health Care Hospital.

Cell Counting in BALF of Experimental Animals

BALF was centrifugated at 4°C, 175 × g for 10 min, the supernatant was discarded, and cells were resuspended in 1 mL of PBS solution containing 1% BSA. 20 µL of the cell suspension was added to 0.38 mL of white blood cell diluent, thoroughly mixed, and total number of white blood cells in the BALF was counted with a counting chamber. Fresh cell suspension was centrifuged at 4°C, 490 × g for 5 min. After discarding the supernatant, 1.8 mL of methanol was added and vigorously mixed to fix the cells for 15 min. The fixed suspension was centrifuged at 490 × g for 5 min, the supernatant was discarded, and 0.5 mL of PBS buffer was added to resuspend the cells thoroughly. Then, 200 µL of the cell suspension was put on a glass slide and air-dried. The samples were stained with Wright-Giemsa staining solution A for 30 sec, followed by the addition of 2-3 volumes of Wright-Giemsa staining solution B for 1-3 min. After washing, samples were quickly air-dried, cleared with xylene for 5 min, and sealed with neutral gum. 200 cells were counted under a microscope, and cell classification was performed.

Histopathological Examination and TUNEL Staining of Lung Tissue

To observe the histopathological changes in lung tissue, lung tissue sections were prepared and stained with Hematoxylin and Eosin (HE). For TUNEL staining, after embedding the lung tissue in paraffin and sectioning, the lung tissue samples were immersed in xylene I and xylene II solutions for 15 min. The dewaxed sections were then treated with gradient ethanol and rinsed with running water for 10 min. Then, the lung tissue samples were incubated with proteinase K working solution at 37°C for 15 min. After that, 50 µL of the TUNEL reaction mixture was added to the samples, which were placed in a humid chamber and incubated at 37°C for 60 min. After washing three times with PBS, 50 µL of the substrate chromogen solution from the DAB concentration kit was added to the samples, and incubated at room temperature for 10 min. The tissue sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted on slides. Finally, the slides were observed and photographed under a microscope.

Detection of Oxidative Stress Levels in Serum and Lung Tissue

Following the instructions of the reagent kit, the levels of SOD, MDA, and NO were measured in both serum and lung tissue of the rat.

Detection of ROS Levels in Lung Tissue Using Flow Cytometry

The lung tissue was washed three times with PBS containing 5% BSA and then minced. The tissue was then resuspended in 10% type II collagenase and 10% type IV collagenase, and allowed digestion in a 37°C incubator for 30 min. Cells were gently dissociated into suspension using DMEM culture medium containing 10% FBS, passed through a 200-mesh filter, and centrifuged at 175 × g for 10 min. The cells were then washed three times with PBS, and resuspended in red blood cell lysis buffer. After neutralizing with an equal volume of PBS, the cells were centrifuged at 175 × g for 5 min. The supernatant was discarded, and the remaining cells were resuspended in PBS. Flow cytometry was performed to detect ROS levels in lung tissue as instructed by the manual of the kit, and analysis was conducted using the NovoCyte analysis software.

Detection of TLR4, NF-κB(p65), and p-NF-κB(p65) expression in lung tissue by Western blotting

The lung tissue was minced and put into a homogenizer, for every 20 mg of tissue, 200 µL of lysis buffer containing protease inhibitors and phosphatase inhibitors was added. The tissue fragments were homogenized until completely lysed. The homogenate was then centrifuged at 4°C for 10 min, and the supernatant was collected and stored at -80°C. PAGE gel was placed in an electrophoresis chamber, and the samples were loaded and subjected to electrophoresis. After electrophoresis, wet transfer was performed, and the PVDF membrane was blocked with 5% non-fat milk at 4°C overnight. As instructed by the manual, rabbit anti-TLR4, NF-κB(p65), p-NF-κB(p65), and GAPDH antibodies were diluted in PBS buffer and incubated at room temperature for 1 hour. The membrane was washed three times with PBS+Tween. Anti-rabbit IgG-HRP secondary antibody was then diluted at a 1:10000 ratio and incubated with the PVDF membrane at room temperature for 1 hr. After washing three times with PBS+Tween, the chemiluminescent substrate was added, and the membrane was placed in an automated chemiluminescence analyzer to detect the relevant protein bands and obtain their grayscale values.

Real-time fluorescence quantitative PCR

Rats' lung tissue was extracted, and after perfusion with distilled water pre-chilled to 4°C, total RNA was extracted using the RNA extraction kit. The RNA was then reverse transcribed into cDNA using the reverse transcription kit. The PCR kit was used to measure the mRNA levels of TLR4 and NF-κB(p65) in lung tissue,

IN PRESS

with GAPDH as the internal reference. The relative expression levels were calculated using the 2-ΔΔCt method. The primer sequences are provided in Table 1. The amplification program consisted of an initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 15 sec, and extension at 72°C for 30 sec.

Immunohistochemistry Staining

Rats' lung tissue was extracted, embedded in paraffin, and then cut into 5 μm sections. After dewaxing, hydration, and antigen retrieval, the sections were fixed at room temperature for 10 min in fixative, followed by three washes with washing buffer for 5 min each time. The sections were then blocked in blocking solution at room temperature for 1 hr. Rabbit monoclonal antibodies against TLR4 and p-NF-κB(p65) were added at a dilution of 1:1000 and incubated overnight at 4°C. After three additional washes with washing buffer, fluorescently labeled goat anti-rabbit IgG was added and incubated at room temperature for 1 hr. After three final washes with washing buffer, the sections were counterstained with hematoxylin, observed, and photographed under an optical microscope. The relative Integrated Optical Density (IOD) intensity was calculated using Image J pro plus 1.15.

Data analysis

All data were processed and analyzed using the statistical software SPSS 26.0. Continuous variables were presented as Mean ± Standard Deviation (Mean ± SD). Data passed the tests for normal distribution and homogeneity of variance. One-way Analysis of Variance (ANOVA) was used for comparisons among multiple groups, followed by the LSD test for pairwise comparisons between groups. Counting data was analyzed using the chi-square test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Construction of Asthma Model and Histopathological Observation

In the Normal group, young rats exhibited no symptoms like sneezing, runny nose, or nasal itching. After OVA stimulation, rats in the Model group showed frequent nose rubbing, increased respiratory rate, sneezing, and cyanosis around the mouth and nose, accompanied by irritability and agitation. Rats in the LD-LT, MD-LT, and HD-LT groups, as well as the HD-LT+TLR4 inhibitor group, exhibited relatively smooth breathing, lighter color around the mouth and nose, and almost no nose rubbing. Histopathological examination of lung tissues revealed clearly arranged alveoli in the Normal group, intact and clear bronchial structure with neatly arranged cells, and no abnormal tissue proliferation or significant infiltration of inflammatory cells. While in the Model group, lung tissue structure was disrupted, cell arrangement was irregular, bronchial epithelial cells were detached and swollen, with a significant influx of inflammatory cells, indicating successful construction of the asthma model in young rat induced by OVA. In the LD-LT, MD-LT, HD-LT, and HD-LT+TLR4 inhibitor groups, the bronchial epithelial tissue structure appeared relatively orderly and clear, with a reduction in inflammatory cell infiltration. The pulmonary pathological changes in the HD-LT+TLR4 inhibitor group were like those in the HD-LT group. See Figure 1 for details.

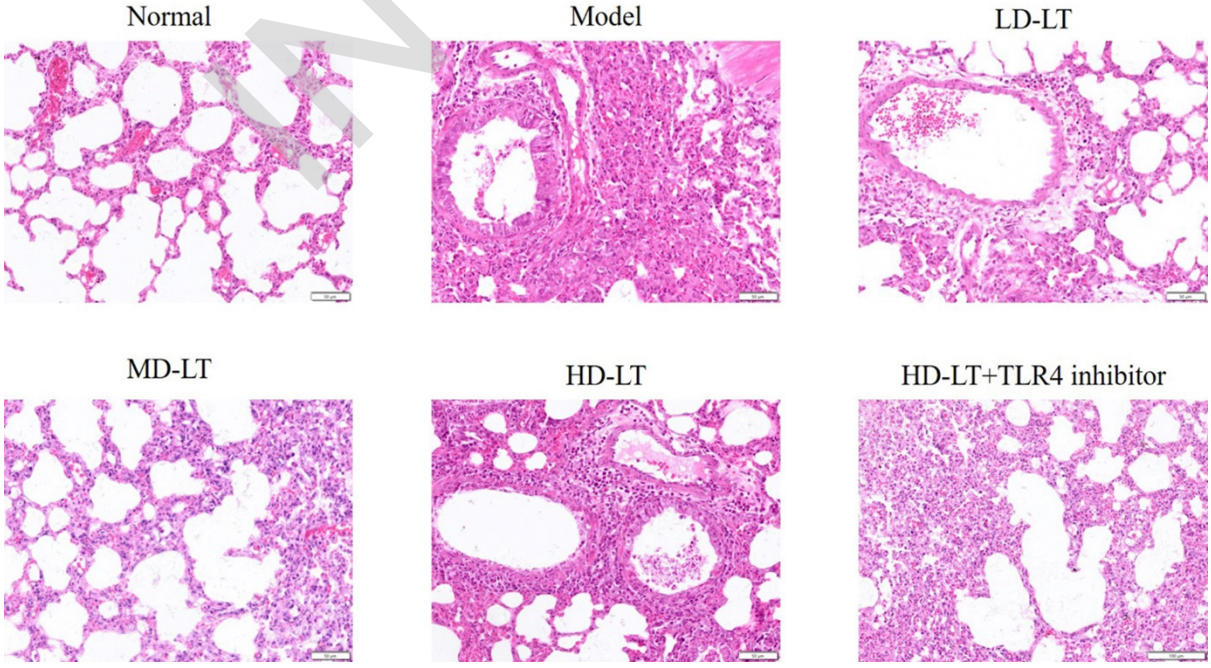


Figure 1: H-E staining of lung tissue in each experimental group (200×).

IN PRESS

Cell Count in BALF (Bronchoalveolar Lavage Fluid)

Compared to the Normal group, the Model group exhibited a significant increase in total cell count and eosinophil count in BALF (p<0.001, Figure 2). Neutrophil and macrophage counts, on the other hand, showed a significant decrease (p<0.001, Figure 2). All LT intervention groups (LD-LT, MD-LT, and HD-LT) and the HD-LT+TLR4 inhibitor group demonstrated a significant decrease in total cell count and eosinophil count in BALF compared to the Model group (p<0.05, Figure 2). Neutrophil and macrophage counts, conversely, showed a significant increase (p<0.05, Figure 2). Significant dose-response relationships were observed among the LT intervention groups in terms of total cell count, eosinophil count, neutrophil count, and macrophage count (p<0.05, Figure 2). However, there was no significant difference between the HD-LT group and the HD-LT+TLR4 inhibitor group.

Effect of LT on Expression of TLR4 and NF-κB(p65) mRNAs in Lung Tissues as Cell apoptosis of lung tissues by TUNEL assay

The TUNEL staining results showed that the nuclei of the Normal group were blue, while the apoptotic nuclei in the Model group were brown, yellow in color. The number of positive apoptotic cells significantly increased (p<0.001, Figure 3); Compared with the Model group, the number of positive apoptotic cells in the LD-LT, MD-LT, and HD-LT groups was significantly reduced (p<0.05, Figure 3); And showed a significant dose-response relationship (p<0.05, Figure 3); There was no significant difference in the number of positive apoptotic cells between the HD-LT group and the HD-LT+TLR4 inhibitor group.

Table 1: Primer sequence.

Gene Name	F:(5'-3')	R:(5'-3')
TLR4	TCCAGAGCCGTTGGTGTATCTT	ACAATTCGACCTGCTGCCTCA
NF-κB(p65)	TCATCTTCCCGGCAGAGCCAG	GTGGGTCTTGGTGGTAGCTGT
GAPDH	TCTGACTTCAACAGGGACACC	CTGTTGGTGTAGCCAAATTCGT

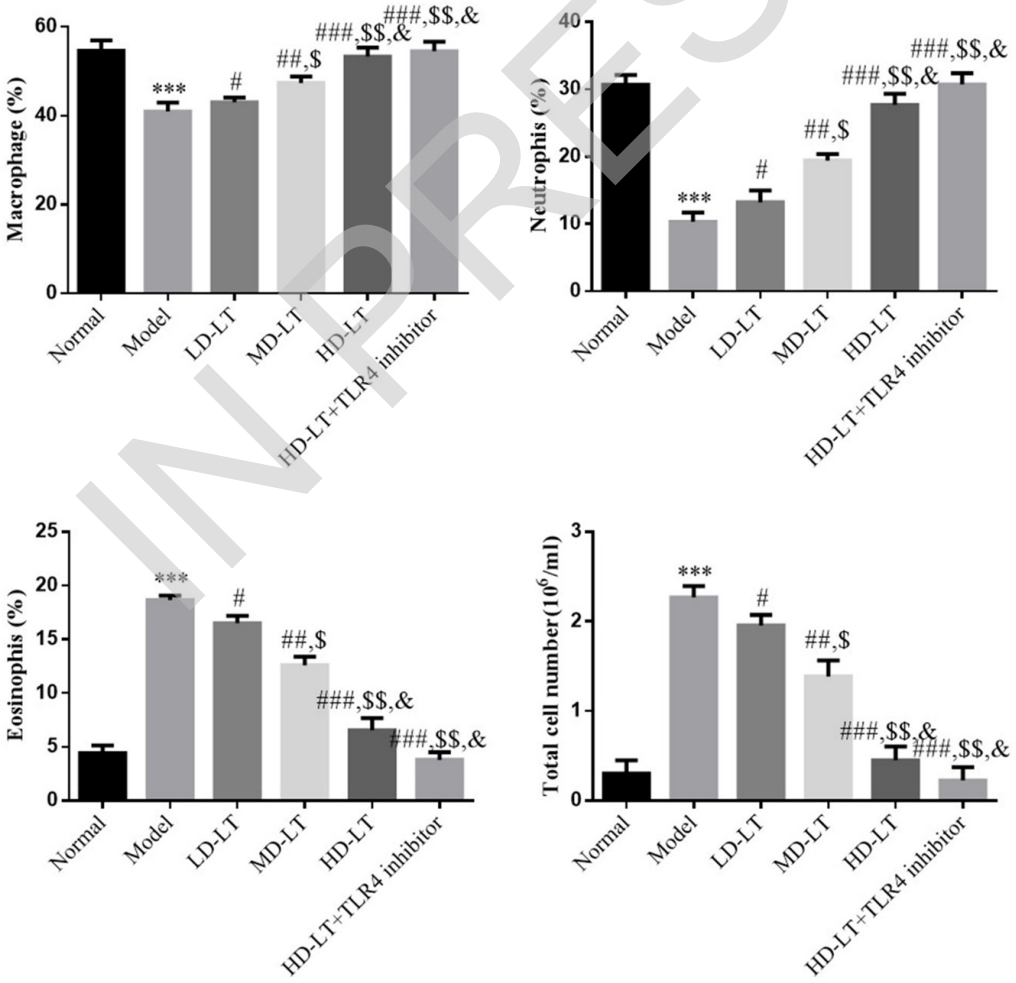


Figure 2: Statistical comparison of immune cell counts in BALF.

***: p<0.001, compared with Normal group; #: p<0.05, ##: p<0.01, ###: p<0.001, compared with Model group; $: p<0.05, $$: p<0.01, compared with LD-LT group; &: p<0.05, compared with MD-LT group.

IN PRESS

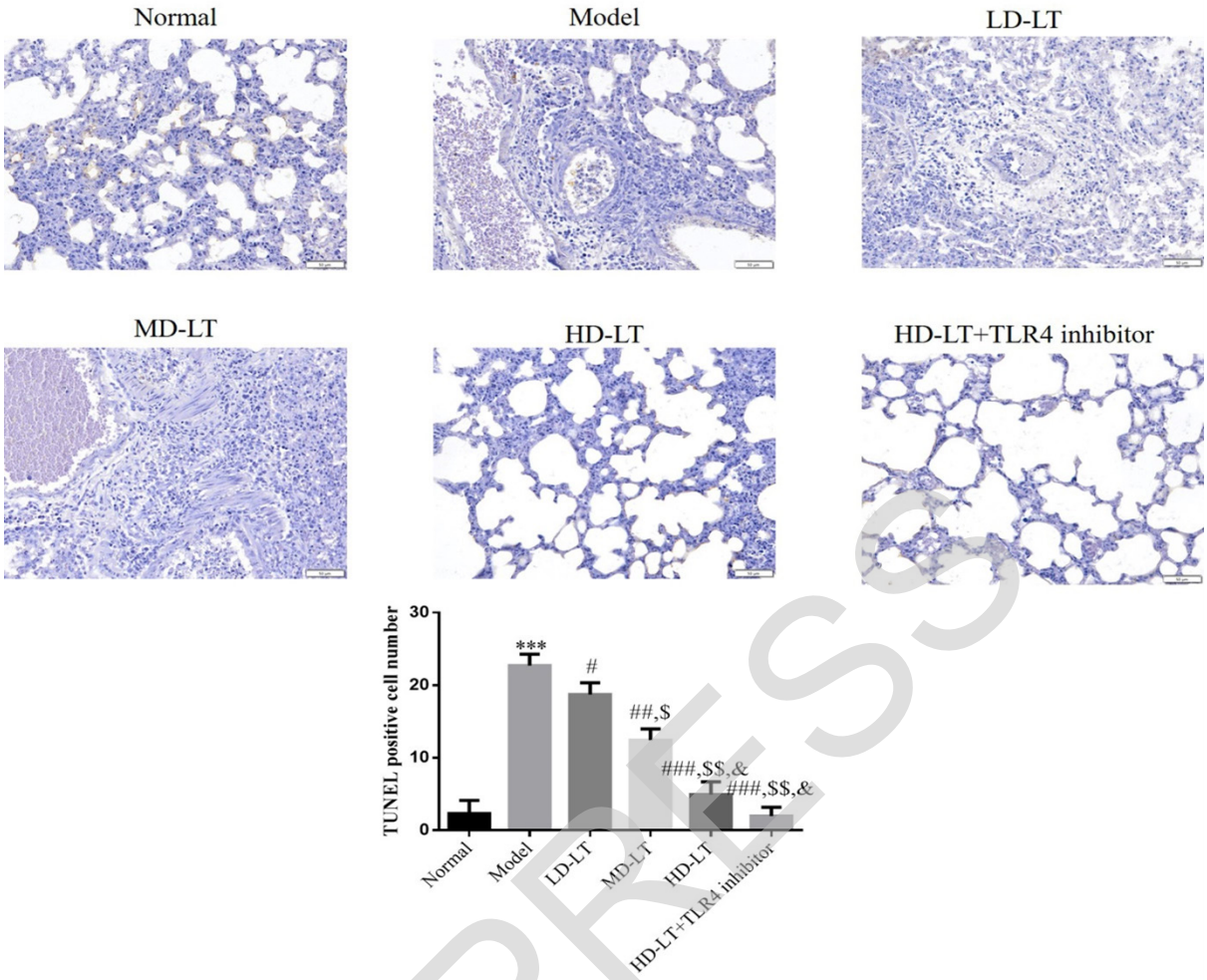


Figure 3: Apoptosis cell number in lung tissues by TUNEL assay (200×).

***: $p<0.001$, compared with Normal group; #: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$, compared with Model group; $: $p<0.05$, $$: $p<0.01$, compared with LD-LT group; &: $p<0.05$, compared with MD-LT group.

The effect of LT on the levels of SDO, MDA, and NO in serum and lung tissue of young rats

Compared with the Normal group, the MDA and NO content in serum and lung tissue of the Model group significantly increased ($p<0.001$, Figures 4 and 5), while SOD activity significantly decreased ($p<0.001$, Figures 4 and 5); Compared with the Model group, the LD-LT, MD-LT, HD-LT, and HD-LT+TLR4 inhibitor groups showed a significant decrease in serum and lung tissue MDA and NO content, while SOD activity significantly increased ($p<0.05$, Figures 4 and 5); There was a significant dose-response relationship ($p<0.05$, Figures 4 and 5) between the LT intervention groups in terms of MDA and NO content and SOD activity; There was no significant difference between the HD-LT group and the HD-LT+TLR4 inhibitor group.

Detection of ROS levels in lung tissue of young mice by flow cytometry

Compared with the Normal group, the ROS level in the lung tissue of the Model group significantly increased ($p<0.001$, Figure 6). This result indicates that after OVA intervention, the oxidative stress response of young mice intensifies. Compared with the Model group, the LD-LT, MD-LT, HD-LT, and HD-LT+TLR4 inhibitor groups all significantly reduced ROS levels in the lung tissue of young rats ($p<0.05$, Figure 6). In terms of ROS levels, LD-LT, MD-LT, and HD-LT groups showed a significant dose-response relationship ($p<0.05$, Figure 6); There was no significant difference between the HD-LT and HD-LT+TLR4 inhibitor groups.

Detected by RT-qPCR

The results of RT-qPCR revealed that, compared to the Normal group, the Model group exhibited a significant upregulation in mRNA levels of TLR4 and NF-κB(p65) ($p<0.001$, Figure 7). And compared to the Model group, the LD-LT, MD-LT, HD-LT, and HD-LT+TLR4 inhibitor groups showed a significant decrease in expression of TLR4 and NF-κB(p65) mRNAs ($p<0.05$, Figure 7). Moreover, a significant dose-response relationship was observed among the LD-LT, MD-LT, and HD-LT groups ($p<0.05$, Figure

IN PRESS

7), but no significant difference between the HD-LT group and the HD-LT+TLR4 inhibitor group.

Effect of LT on the Expression of TLR4 and p-NF-κB(p65) Proteins in Lung Tissues as Detected by IHC

The results of the IHC demonstrated that, compared to the Normal group, the Model group exhibited a significant increase in protein levels of TLR4 and p-NF-κB(p65) (p<0.001, Figures 8 and 9). And compared to the Model group, the LD-LT, MD-LT, HD-LT, and HD-LT+TLR4 inhibitor groups showed a significant decrease in the expression levels of TLR4 and p-NF-κB(p65) proteins (p<0.05, Figures 8 and 9). Similarly, a significant dose-response relationship was observed among the LD-LT, MD-LT, and HD-LT groups (p<0.05, Figures 8 and 9), but no significant difference between the HD-LT group and the HD-LT+TLR4 inhibitor group.

Effect of LT on TLR4, NF-κB(p65), and p-NF-κB(p65) protein levels in Pulmonary Tissues as Detected by WB

The results of the WB revealed that, compared to the Normal group, the Model group exhibited a significant increase in the levels of TLR4 protein and the p-NF-κB(p65)/NF-κB(p65) ratio (p<0.001, Figure 10). Compared to the Model group, the LD-LT, MD-LT, HD-LT, and HD-LT+TLR4 inhibitor groups showed a significant decrease in the levels of TLR4 protein and the p-NF-κB(p65)/NF-κB(p65) ratio (p<0.05, Figure 10). Again, significant dose-response relationship was observed among the LD-LT, MD-LT, and HD-LT groups (p<0.05, Figure 10), but there was no significant difference between the HD-LT group and the HD-LT+TLR4 inhibitor group.

DISCUSSION

Asthma affects 18% of global population, according to the recently revised Global Asthma Assessment Report, approximately 334 million people worldwide suffer from asthma, leading to 346,000

IN PRESS

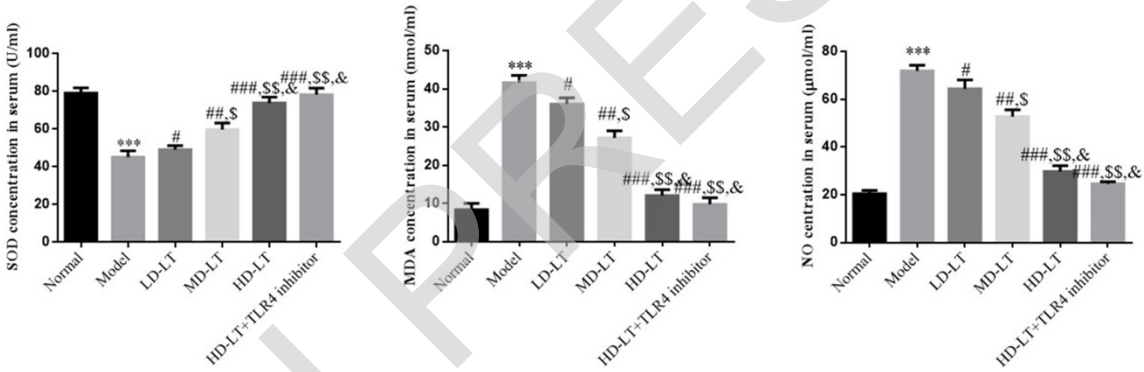


Figure 4: SOD, MDA and NO levels in serum.

***: p<0.001, compared with Normal group; #: p<0.05, ##: p<0.01, ###: p<0.001, compared with Model group; $: p<0.05, $$: p<0.01, compared with LD-LT group; &: p<0.05, compared with MD-LT group.

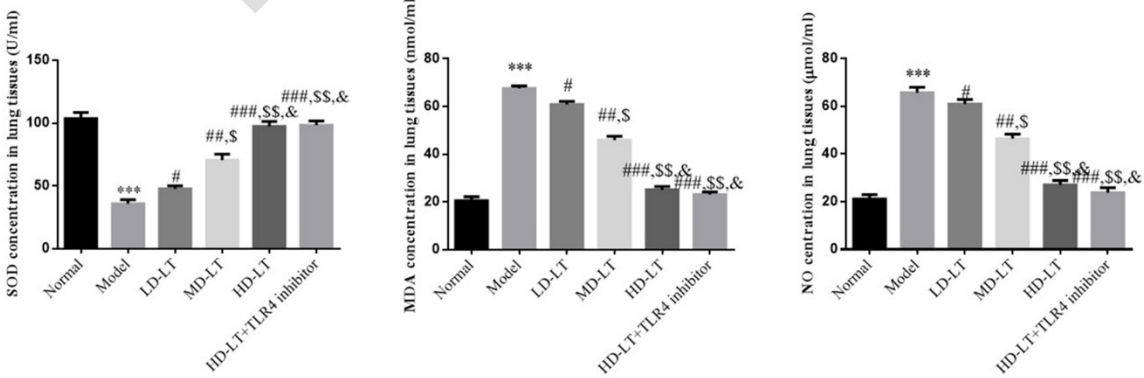


Figure 5: SOD, MDA and NO levels in lung tissues.

***: p<0.001, compared with Normal group; #: p<0.05, ##: p<0.01, ###: p<0.001, compared with Model group; $: p<0.05, $$: p<0.01, compared with LD-LT group; &: p<0.05, compared with MD-LT group.

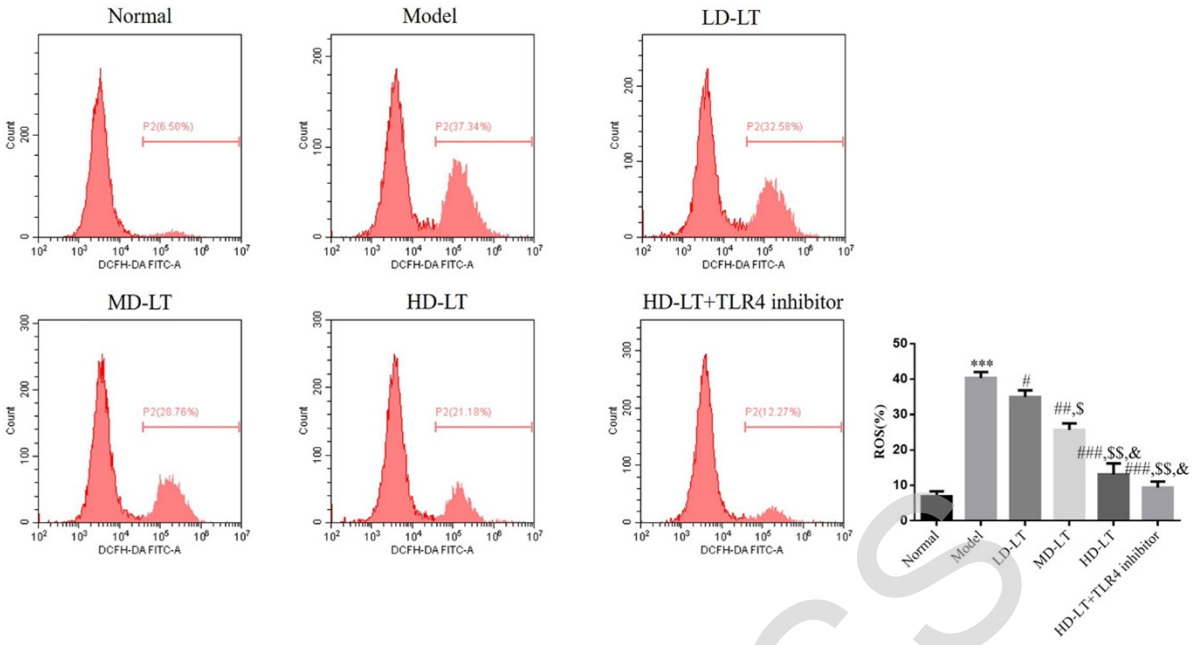


Figure 6: Detection of ROS levels in lung tissue by flow cytometry.

***: $p<0.001$, compared with Normal group; #: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$, compared with Model group; $: $p<0.05$, $$: $p<0.01$, compared with LD-LT group; &: $p<0.05$, compared with MD-LT group.

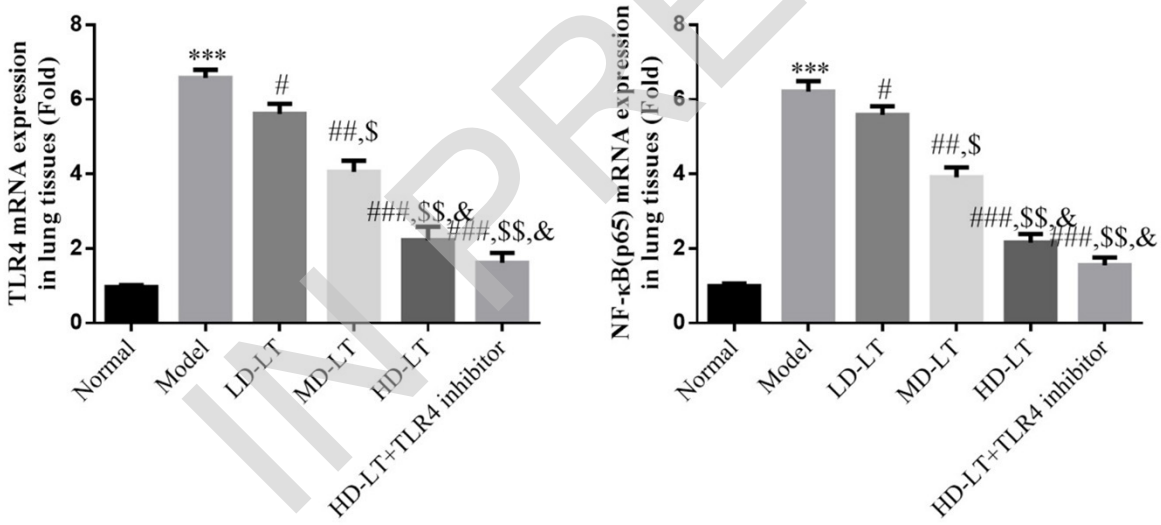


Figure 7: Relative mRNA expression in lung tissues.

***: $p<0.001$, compared with Normal group; #: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$, compared with Model group; $: $p<0.05$, $$: $p<0.01$, compared with LD-LT group; &: $p<0.05$, compared with MD-LT group.

deaths every year.[12] It is a common allergic disease characterized by airway inflammation. Despite effective relief of asthma symptoms with corticosteroids, most patients still experience recurrences, and the long-term efficacy of medications is often unsatisfactory. Safe and effective drugs to treat asthma are urgently needed.[13] This study utilized an OVA-induced asthma rats' model to investigate the effects of LT on TLR4/NF-κB(p65) and oxidative stress, aiming to provide a theoretical basis for the clinical application of LT in the treatment of asthma.

Significant infiltration of eosinophils is commonly observed in the airways of asthma patients. Studies have found that activated eosinophils release a large amount of cationic proteins, causing respiratory inflammation, lung damage, cell apoptosis, and a decline in lung function.[14] Additionally, eosinophils release peroxidases, causing oxidative damage to alveolar cells.[15] Ovalbumin (OVA) can induce airway inflammation, excessive proliferation of goblet cells, increased infiltration of inflammatory cells in the submucosa, particularly eosinophils. Therefore, it is often used to construct animal models of asthma.[12] In this study, an OVA-induced rats' model of asthma was employed. The rat exhibited symptoms of asthma, including accelerated breathing, sneezing, and cyanosis of the nose and

INPRESS

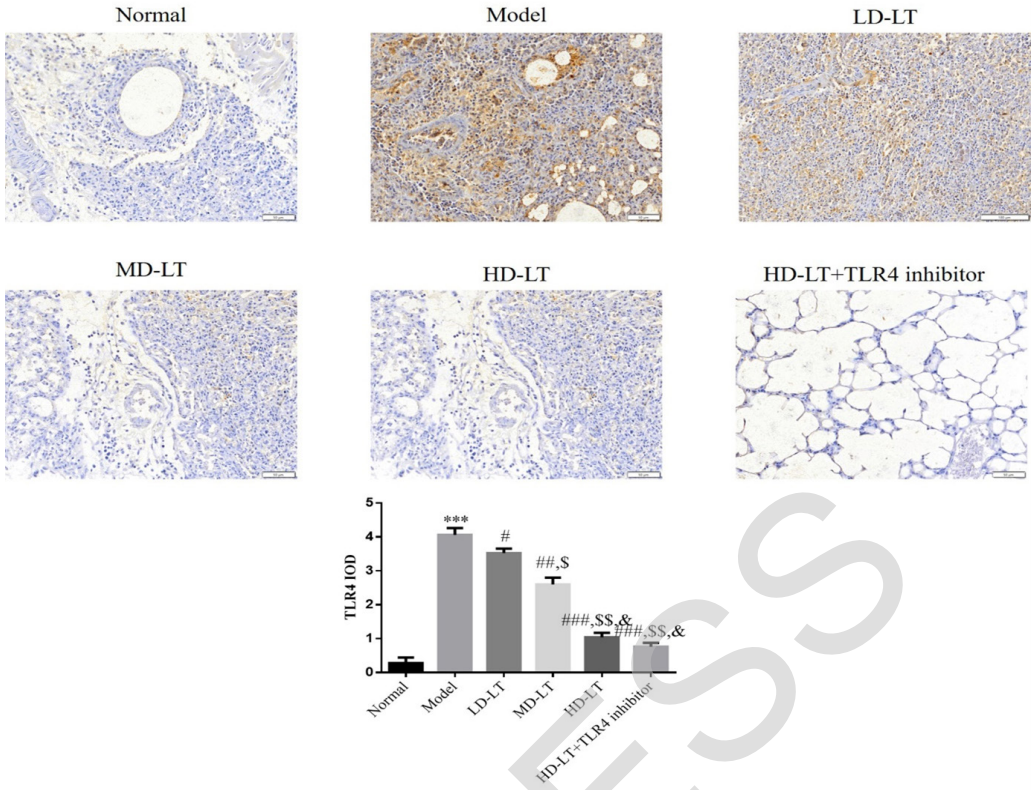


Figure 8: TLR4 protein expression by IHC assay in lung tissues (200×).

***: $p<0.001$, compared with Normal group; #: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$, compared with Model group; $: $p<0.05$, $$: $p<0.01$, compared with LD-LT group; &: $p<0.05$, compared with MD-LT group.

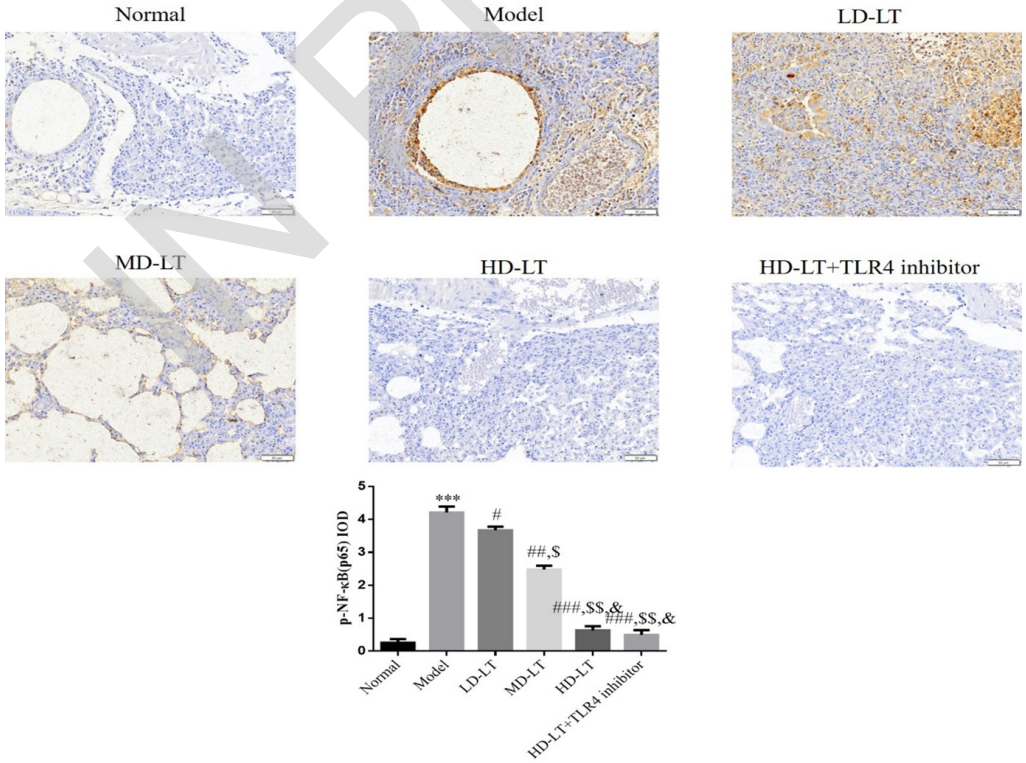


Figure 9: p-NF-κB(p65) protein expression by IHC assay in lung tissues (200×).

***: $p<0.001$, compared with Normal group; #: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$, compared with Model group; $: $p<0.05$, $$: $p<0.01$, compared with LD-LT group; &: $p<0.05$, compared with MD-LT group.

IN PRESS

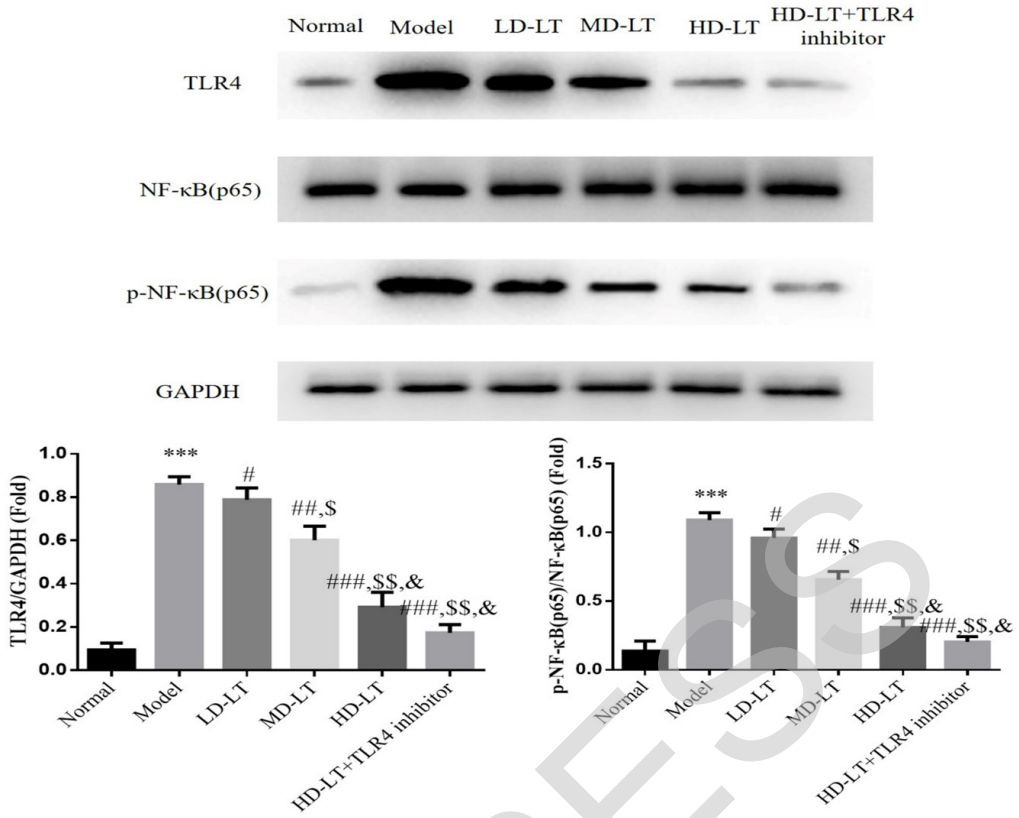


Figure 10: Relative proteins levels in lung tissues by WB assay.

***: $p<0.001$, compared with Normal group; #: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$, compared with Model group; $: $p<0.05$, $$: $p<0.01$, compared with LD-LT group; &: $p<0.05$, compared with MD-LT group.

mouth. Histological staining of lung tissues with Hematoxylin and Eosin (HE) revealed structural disruptions, irregular cell arrangement, bronchial epithelial cell shedding, edema, and significant infiltration of inflammatory cells in the model group, which indicate successful construction of the asthma rat's model. Following LT treatment, asthma symptoms in the rat alleviated, and the number of eosinophils in the Bronchoalveolar Lavage Fluid (BALF) decreased. This suggests that LT effectively alleviated asthma symptoms and reduced the infiltration of inflammatory cells, with most significant improvement observed in the high-dose group.

Epithelial cells in the airways can resist invasion by microbes and allergens, reducing entry of harmful substances into the lungs. Exposure to allergens increases the production of harmful substances such as Reactive Oxygen Species (ROS), leading to an imbalance between oxidative stress and antioxidant reactions. This imbalance triggers oxidative stress reactions, resulting in airway remodeling, increased smooth muscle thickness, and cellular damage in lung tissue.[16] Additionally, stimulation by allergens activates airway epithelial cells, leading to the release of cytokines and chemokines. This activation induces immune cells to enter the lungs, releasing more inflammatory factors and exacerbating lung inflammation and damage. Moreover, inflammatory epithelial cells induce the upregulation of the expression of reductive coenzyme II and nitric oxide synthase oxidase, promoting the production of ROS and NO, which further activate the TLR4/NF-κB(p65) pathway and further enhance the expression of TNF-α, IL-1β, and IL-6, aggravating lung damage.[17] Research has found that after OVA intervention, oxidative stress in the lungs is characterized by a decrease in Superoxide Dismutase (SOD) activity and glutathione content, along with a significant increase in Malondialdehyde (MDA) content (all $p<0.05$). Meanwhile, activated p-NF-κB translocates from the cytoplasm to the cell nucleus, leading to inflammatory reactions.[18] Consistent with these findings, our study suggests that inhibiting oxidative stress and inflammatory responses may play a crucial role in asthma treatment. In this study, after intervention with LT, SOD activity increased, and MDA and NO levels significantly decreased (all $p<0.05$). The levels of ROS, TLR4, and p-NF-κB proteins in lung tissue markedly reduced (all $p<0.05$), indicating that LT can protect lung tissue by inhibiting oxidative stress and inflammatory signaling pathways. When simultaneously administering a high dose of LT and TAK-242 (a TLR4 inhibitor), the effect was like the HD-LT group. Therefore, we infer that LT improves asthma-induced lung damage through the TLR4/NF-κB(p65) signaling pathway.

IN PRESS

CONCLUSION

In summary, LT can effectively alleviate asthma symptoms, reduce apoptosis damage in bronchial epithelial cells in lung tissue, mitigate inflammation cell infiltration, and lower ROS levels. Its mechanism of action may be associated with the inhibition of oxidative stress reactions and the TLR4/NF-κB(p65) signaling pathway.

ACKNOWLEDGEMENT

Not applicable

ABBREVIATIONS

BAL: Bronchoalveolar Lavage; **BALF:** Bronchoalveolar Lavage Fluid; **BCA:** Bicinchoninic Acid; **BSA:** Bovine Serum Albumin; **cDNA:** Complementary Deoxyribonucleic Acid; **COPD:** Chronic Obstructive Pulmonary Disease; **DAB:** 2,2'-Diaminobenzidine; **DMEM:** Dulbecco's Modified Eagle Medium; **EP tubes:** Eppendorf tubes; **FBS:** Fetal Bovine Serum; **GAPDH:** Glyceraldehyde 3-phosphate Dehydrogenase; **HE:** Hematoxylin and Eosin; **HD-LT:** High-Dose Luteolin Group; **HD-LT+TLR4 inhibitor group:** High-Dose Luteolin combined with TLR4 inhibitor group; **HRP:** Horseradish Peroxidase; **IHC:** Immunohistochemistry; **IL-6:** Interleukin-6; **IL-1β:** Interleukin-1 beta; **IOD:** Integrated Optical Density; **IFN-γ:** Interferon-gamma; **IgE:** Immunoglobulin E; **LD-LT:** Low-Dose Luteolin Group; **LPS:** Lipopolysaccharide; **LSD test:** Least Significant Difference test; **LT:** Luteolin; **MDA:** Malondialdehyde; **MD-LT:** Medium-Dose Luteolin Group; **mRNA:** Messenger Ribonucleic Acid; **NF-κB:** Nuclear Factor kappa-light-chain-enhancer of activated B cells; **NO:** Nitric Oxide; **OVA:** Ovalbumin; **p-NF-κB(p65):** Phosphorylated NF-κB (p65 subunit); **PAGE gel:** Polyacrylamide Gel Electrophoresis gel; **PBS:** Phosphate-Buffered Saline; **PCR:** Polymerase Chain Reaction; **PDE:** Phosphodiesterase; **PVDF membrane:** Polyvinylidene Fluoride membrane; **RIPA lysis buffer:** Radioimmunoprecipitation Assay lysis buffer; **RNA:** Ribonucleic Acid; **ROS:** Reactive Oxygen Species; **RT-qPCR:** Real-time Quantitative Polymerase Chain Reaction; **SD rat:** Sprague Dawley rat; **SD:** Standard Deviation; **SOD:** Superoxide Dismutase; **SPSS:** Statistical Package for the Social Sciences; **TLR4:** Toll-like Receptor 4; **TNF-α:** Tumor Necrosis Factor-alpha; **TUNEL:** Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; **WB:** Western Blotting.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

All procedures were conducted in accordance with the Guidelines for the Use and Care of Laboratory Animals (NOM-062-ZOO-1999, Ministry of Agriculture and Livestock, SAGARPA, Mexico), and the protocol was approved by the Institutional Committee for the Care and Use of Laboratory Animals. This study was approved by Ethics Committee of Huai'an Maternal and Children's Health Care Hospital.

PATIENT CONSENT FOR PUBLICATION

Not applicable.

SUMMARY

Using Ovalbumin (OVA) induced young rats to make asthma model, and dividing the young rats into Normal, Model, LD-LT, MD-LT, HD-LT and HD-LT+TLR4 inhibitor groups. Except for the normal control group, all other groups used OVA to construct asthma models, LD-LT, MD-LT and HD-LT groups were treated with 25 mg/kg, 50 mg/kg and 100 mg/kg LT, HD-LT+TLR 4 inhibitor group were treated with 100 mg/kg LT and TAK-242, which were TLR 4 inhibitor based on OVA induced asthma young rat model. Compared with Model group, with LT treatment, total cell number, Eosinophis significantly reduced and Neutrophis and Macrophage significantly increased in BALF (p<0.05); MDA and NO concentration were down-regulation (p<0.05), apoptosis cell number significantly depressed (p<0.05); SOD concentration significantly increased (p<0.01) and ROS concentration significantly down-regulated (p<0.05) with TLR4/NF-κB(p65) gene and protein expression significantly suppressed. HD-LT's effects were similar as HD-LT+TLR 4 inhibitor group. LT improved Ovalbumin-Induced Asthma young rat via regulation TLR4/NF-κB(p65) pathway *in vivo*.

REFERENCES

1. Pawankar R. Allergic diseases and asthma: a global public health concern and a call to action. World Allergy Organ J. 2014;7:12. DOI: 10.1186/1939-4551-7-12
2. Thornton DJ, Rousseau K, McGuckin MA. Structure and function of the polymeric mucins in airways mucus. Annu Rev Physiol. 2008;70:459-86; DOI: 10.1146/annurev.physiol.70.113006.100702
3. Coutts J, Fullarton J, Morris C, *et al.* Association between respiratory syncytial virus hospitalization in infancy and childhood asthma. Pediatr Pulmonol. 2020;55:1104-10; DOI: 10.1002/ppul.24676
4. Lin CC, Wang YY, Chen SM, *et al.* Shegan-Mahuang Decoction ameliorates asthmatic airway hyperresponsiveness by downregulating Th2/Th17 cells but upregulating CD4+FoxP3+ Tregs. J Ethnopharmacol. 2020;253:112656; DOI: 10.1016/j.jep.2020.112656
5. Helal MG, Megahed NA, Abd Elhameed AG. Saxagliptin mitigates airway inflammation in a mouse model of acute asthma via modulation of NF-kB and TLR4. Life Sci. 2019. 239:117017; DOI: 10.1016/j.lfs.2019.117017

IN PRESS

6. Mishra V, Banga J, Silveyra P. Oxidative stress and cellular pathways of asthma and inflammation: Therapeutic strategies and pharmacological targets. Pharmacol Ther. 2018, 181:169-182; DOI: 10.1016/j.pharmthera.2017.08.011
7. Adejayan AA, Ozolua RI, Uwaya DO, *et al.* Evaluation of the anti-asthmatic and antitussive potentials of methanol leaf extract of *Napoleona vogelii* in rodents. Biomed Pharmacother. 2019, 109:120-126; DOI: 10.1016/j.biopha.2018.10.058
8. Seelinger G, Merfort I, Wölfle U, *et al.* Anti-carcinogenic effects of the flavonoid luteolin. Molecules. 2008, 13:2628-2651; DOI: 10.3390/molecules13102628
9. Hamid I, Janbaz KH, Iqbal R, Akhtar MF, *et al.* Therapeutic potential of *Ceratonia siliqua* extract for the management of asthma and hypertension. Cell Mol Biol (Noisy-le-grand). 2022,67:6-15; DOI: 10.14715/cmb/2021.67.5.2
10. Huang Y, Zhang X. Luteolin alleviates polycystic ovary syndrome in rats by resolving insulin resistance and oxidative stress. Am J Physiol Endocrinol Metab. 2021,320:E1085-E1092; DOI: 10.1152/ajpendo.00034.2021
11. eng Y, Ju Y, Wu Q, *et al.* TAK-242, a toll-like receptor 4 antagonist, against brain injury by alleviates autophagy and inflammation in rats. Open Life Sci. 2023, 18:20220662; DOI: 10.1515/biol-2022-0662
12. Ingawale DK, Mandlik SK, Patel SS. Combination of Sarsasapogenin and Fluticasone attenuates ovalbumin-induced airway inflammation in a mouse asthma model. Immunopharmacol Immunotoxicol. 2020, 42:128-137; DOI: 10.1080/08923973.2020.1728541
13. Bourdin A, Adcock I, Berger P, *et al.* How can we minimise the use of regular oral corticosteroids in asthma? Eur Respir Rev. 2020, 29:190085; DOI: 10.1183/1600617.0085-2019
14. Liou CJ, Chen YL, Yu MC, *et al.* Sesamol Alleviates Airway Hyperresponsiveness and Oxidative Stress in Asthmatic Mice. Antioxidants (Basel). 2020, 9:295; DOI: 10.3390/antiox9040295
15. Yancey SW, Keene ON, Albers FC, *et al.* Biomarkers for severe eosinophilic asthma. J Allergy Clin Immunol. 2017, 140:1509-1518; DOI: 10.1016/j.jaci.2017.10.005
16. de Groot LES, van der Veen TA, Martinez FO, *et al.* Oxidative stress and macrophages: driving forces behind exacerbations of asthma and chronic obstructive pulmonary disease? Am J Physiol Lung Cell Mol Physiol. 2019, 316: L369-L384; DOI: 10.1152/ajplung.00456.2018
17. Roan F, Obata-Ninomiya K, Ziegler SF. Epithelial cell-derived cytokines: more than just signaling the alarm. J Clin Invest. 2019, 129:1441-1451; DOI: 10.1172/JCI124606
18. Cristofori F, Dargenio VN, Dargenio C, *et al.* Anti-Inflammatory and Immunomodulatory Effects of Probiotics in Gut Inflammation: A Door to the Body. Front Immunol. 2021, 12:578386; DOI: 10.3389/fimmu.2021.578386

Cite this article: Changqiang C, Li Z, Tailiang R, Xiaofei L. Effects of Luteolin (LT) on the TLR4/NF-κB Pathway and Oxidative Stress in Ovalbumin-Induced Asthma Young Rat Model. Indian J of Pharmaceutical Education and Research. 2027;61:10.5530/ijper.20272027.

IN PRESS