Anti-inflammatory activity of resveratrol prevents inflammation by inhibiting NF-κB in animal models of acute pharyngitis

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Abstract. Recent studies have demonstrated that resveratrol can reduce blood sugar, improve insulin resistance, regulate abnormalities in lipid metabolism, and lower the secretion and expression of inflammatory factors. The present study investigated the anti-inflammatory effects of resveratrol in animal models of acute pharyngitis, and its possible mechanisms. Commercial ELISA kits were used to measure tumor necrosis factor-α, interleukin (IL)-6, macrophage inflammatory protein-2, cyclooxygenase-2 levels and caspase-3/9 activity. Toll-like receptor (TLR)-4, myeloid differentiation primary response protein MyD88, phosphorylated (p)-nuclear factor (NF)-κB and p-IκB were analyzed using western blotting. In a rabbit model of acute pharyngitis, it was demonstrated that resveratrol inhibited tumor necrosis factor-α and interleukin-6 serum levels, macrophage inflammatory protein-2 and cyclooxygenase-2 activity levels, reactive oxygen species production and caspase-3/9 activity. Resveratrol suppressed NACHT, LRR and PYD domains-containing protein 3 and caspase-1 protein expression, and reduced IL-1β and IL-18 protein expression in animal models of acute pharyngitis. Additionally, resveratrol suppressed TLR4 and myeloid differentiation primary response protein 88 protein expression, and reduced p-NF-κB and increased p-IκB protein expression in animal models of acute pharyngitis. In conclusion, these findings indicated that the anti-inflammatory activity of resveratrol prevents acute pharyngitis-induced inflammation by inhibiting NF-κB in animal models. Therefore, these data suggested an important clinical application of resveratrol in preventing acute pharyngitis.

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Introduction

Acute pharyngitis is a type of upper respiratory tract infection, with the most common symptom of an acute sore throat (1). Acute pharyngitis refers to acute inflammation occurring in nasopharyngeal mucosa and submucosal tissues, and usually involves pharyngeal lymphoid tissues (2). It may occur alone or secondary to acute rhinitis and acute tonsillitis (2). Acute pharyngitis is a common and frequently-occurring disease, characterized by rapid incidence and development (3). The main causes of acute pharyngitis include bacterial and virus infection, as well as non-infectious factors (such as breathing through the mouth, allergic reaction, gastro-esophageal reflux, smoking, alcohol intake, high temperature, dust, smoke and pungent gas) (4).

The activation and transcriptional regulation of the nuclear factor (NF)-κB signaling pathway has been a primarily research focus (5). In general, NF-κB binds to inhibitor (I) κB in the cytoplasm under a resting state to form a resting complex, which blocks the DNA binding sites of the Rel dimer. Under certain stimulation, IκB will be deactivated through phosphorylation by IκB kinase (6). NF-κB is released into the nucleus to bind to specific sites in DNA, serving a transcription factor role. Notably, the base sequences of κB sites are not exactly the same; additionally, the Rel dimer cannot fully activate target genes alone (7). NF-κB not only serves an important role in the regulation of inflammatory response, but also participates in many cellular activities, including proliferation and apoptosis of cells. (8).

Resveratrol, a natural polyphenol compound (Fig. 1), exists in grapes, peanuts, berries and other plants. Previous studies have demonstrated that resveratrol regulates the activity of histone deacetylase, which determines lifespan (9). As an activator of deacetylase, resveratrol has been confirmed to prolong the lifespan of yeast, nematode and mouse (9). Available studies have confirmed that resveratrol has anti-inflammatory, anti-oxidant and anti-neoplastic effects as well as protecting the heart (9). Due to their extensive effects, rich and renewable sources and high compatibility with the environment, botanical bactericide has become a hotspot in the field of the research of pesticides (10). Many plants with antibacterial and bactericidal activity have been detected, and many active materials such as phenols, terpenoids and flavonoids have been isolated (10). Stilbenes, one kind of active components derived from many plants, has

certain antibacterial activity. Studies have demonstrated that resveratrol, a typical component of stilbenes, has extensively biological activities and pharmacological properties (10). The present study observed that the anti-inflammatory activity of resveratrol prevents inflammation in animal models of acute pharyngitis.

Materials and methods

Animals and acute pharyngitis treatments. Male adult New Zealand white rabbits (n=18; age, 4-6 months; weight, 2.2-2.5 kg, 6 rabbits/group) were provided by the Experimental Animal Center of Shandong University and all animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Liaocheng People's Hospital (Shandong, China). All rabbits were randomly assigned to three groups: Control, acute pharyngitis model and resveratrol treatment groups.

In the acute pharyngitis model and resveratrol treatment groups, rabbits were anesthetized with 30 mg/kg of pentobarbital sodium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), then fixed at operating table at 37˚C. The neck was cut, the weasand was separated and endotracheal intubation was executed at 3 μm from the cricoid cartilage. The weasand was cut and exposed, and croton oil (0.1 ml; 2%) was used to wipe the weasand and plated for 30 min. The wound was sterilized with iodophor and rabbit placed back in the cage. In the resveratrol treatment group, rabbits were treated with 4 mg/kg every two days (intraperitoneally) resveratrol for 2 weeks.

Commercial ELISA kits. After treatment with resveratrol, peripheral blood was acquired from the eye socket and was centrifuged at 2,000 x g for 10 min at 4˚C. ELISA kits were used to determine levels of tumor necrosis factor (TNF)-α (cat. no. H052), interleukin (IL)-6 (cat. no. H007), macrophage inflammatory protein-2 (MIP-2, cat. no. H112) and cyclooxygenase-2 (COX-2, cat. no. H200) activity, and ROS production (cat. no. E004) and caspase-3/9 activity (cat. no. G015/G018) in blood serum.

Western blot analysis. After treatment with resveratrol, rabbits were sacrificed and weasand tissue samples were washed with PBS and homogenized using ice-cold radioimmunoprecipitation assay buffer. Protein content was measured using a Bicinchoninic Acid protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). Total protein (50 μg) was separated by 6-10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (BD Biosciences, San Jose, CA, USA). The membranes were subsequently blocked with 5% nonfat dry milk for 1 h at room temperature and overnight at 4˚C with primary antibodies against NACHT, LRR and PYD domains-containing protein 3 (NLRP3; cat. no. ab214185; 1:1,000), caspase-1 (cat. no. ab108362; 1:1,000), IL-1β (cat. no. ab200478; 1:1,000), IL-18 (cat. no. ab71495; 1:1,000), toll-like receptor 4 (TLR4; cat. no. ab13556; 1:1,000), myeloid differentiation primary response protein 88 (MyD88; cat. no. ab2068; 1:1,000), phosphorylated (p)-NF-κB (cat. no. ab86299; 1:1,000), p-IκB (cat. no. ab75746; 1:1,000) and GAPDH (cat. no. ab8245; 1:2,000), all purchased from Abcam (Cambridge, MA, USA). The membranes were washed with TBST for 15 min and incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (cat. no. ab205718; 1:5,000, Abcam, Cambridge, MA, USA) for 1 h at room temperature, and the results were detected using enhanced chemiluminescence (Gibco; Thermo Fisher Scientific, Inc.). Band intensity was calculated using Image J software version 1.42q (National Institutes of Health, Bethesda, MA, USA).

Table I. Vocal cord and absorption indexes.

Group	Index of the vocal cords	Absorption index
Control	0	0
Model	28.91	36.77
Resveratrol	16.67	20.09

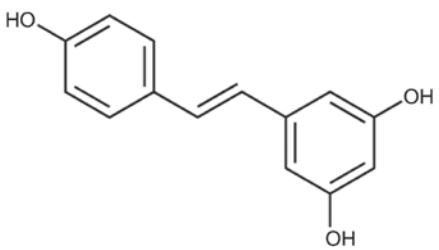

Figure 1. Chemical structure of resveratrol.

Statistical analysis. The results are expressed as the mean ± standard deviation using SPSS version 19.0 software (SPSS, Inc., Chicago, IL, USA). The data were analyzed using one-way analysis of variance followed by Duncan's multiple range test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Vocal cord and absorption indexes in animal models of acute pharyngitis. Firstly, the present study demonstrated that the index of the vocal cords and absorption index in acute pharyngitis model were higher compared with the control group (Table I). Resveratrol treatment significantly inhibited the index of the vocal cords and absorption index in acute pharyngitis rabbits, compared with the acute pharyngitis model group (Table I).

TNF-α and IL-6 serum levels in animal models of acute pharyngitis. There was a significant increase of TNF-α and IL-6 serum levels in the acute pharyngitis model, compared with control group (Fig. 2). Resveratrol treatment significantly reduced TNF-α and IL-6 serum levels in acute pharyngitis rabbits, compared with the acute pharyngitis model group (Fig. 2).

MIP-2 and COX-2 activity levels in animal models of acute pharyngitis. It was observed that MIP-2 and COX-2 activity levels of the acute pharyngitis model were significantly higher

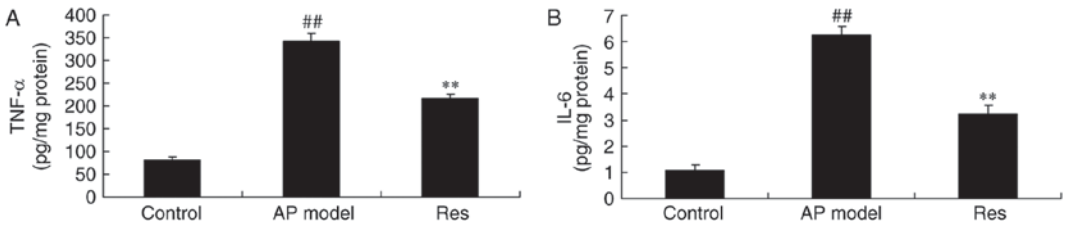

Figure 2. TNF-α and IL-6 serum levels in animal models of acute pharyngitis. (A) TNF-α and (B) IL-6 serum levels in animal models of acute pharyngitis. Data are expressed the mean ± standard deviation. ##P<0.01 vs. control group; **P<0.01 vs. AP model group. Control, control group; AP model, acute pharyngitis model group; Res, resveratrol treatment group; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

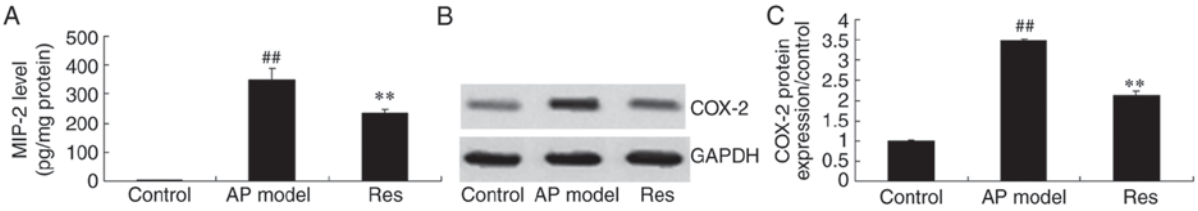

Figure 3. MIP-2 and COX-2 activity levels in animal models of acute pharyngitis. (A) MIP-2 levels. (B) Representative western blot images and (C) quantification of COX-2 activity levels in animal models of acute pharyngitis. Data are expressed the mean ± standard deviation. ##P<0.01 vs. control group; **P<0.01 vs. AP model group. Control, control group; AP model, acute pharyngitis model group; Res, resveratrol treatment group; MIP-2, macrophage inflammatory protein-2; COX-2, cyclooxygenase-2.

compared with the control group (Fig. 3). In acute pharyngitis rabbit model, treatment with resveratrol significantly inhibited MIP-2 and COX-2 activity levels in acute pharyngitis rabbits, compared with the acute pharyngitis model group (Fig. 3).

ROS production in animal models of acute pharyngitis. After treatment with resveratrol, the present study demonstrated that ROS production was significantly enhanced in the acute pharyngitis model group, compared with the control group (Fig. 4). Treatment with resveratrol significantly inhibited the induction of ROS production in acute pharyngitis rabbits, compared with the acute pharyngitis model group (Fig. 4).

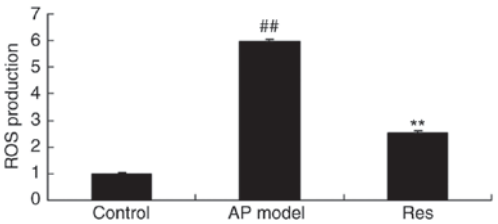

Figure 4. ROS production in animal models of acute pharyngitis. Data are expressed the mean ± standard deviation. ##P<0.01 vs. control group; **P<0.01 vs. AP model group. Control, control group; AP model, acute pharyngitis model group; Res, resveratrol treatment group; ROS, reactive oxygen species.

Caspase-3/9 activity in animal models of acute pharyngitis. To investigate the apoptotic status of acute pharyngitis by resveratrol, caspase-3/9 activity was measured using ELISA kits. As presented in Fig. 5, caspase-3/9 activity was significantly promoted in the acute pharyngitis model, compared with the control group. However, resveratrol significantly reduced the increase of caspase-3/9 activity in acute pharyngitis rabbits, compared with the acute pharyngitis model group (Fig. 5).

NLRP3 and caspase-1 protein expression in animal models of acute pharyngitis. The present study investigated the effects of resveratrol on the inflammasome in acute pharyngitis rabbits. NLRP3 and caspase-1 protein expression were measured using western blotting. The results demonstrated that NLRP3 and caspase-1 protein expression were significantly induced in the acute pharyngitis model, compared with the control group (Fig. 6). Resveratrol treatment significantly suppressed NLRP3 and caspase-1 protein expression in acute pharyngitis rabbits, compared with the acute pharyngitis model group (Fig. 6).

IL-1β and IL-18 protein expression in animal models of acute pharyngitis. The present study investigated the effects of resveratrol on IL-1β and IL-18 protein expression in animal models of acute pharyngitis using western blotting. The results demonstrated that the protein expression levels of IL-1β and IL-18 in the acute pharyngitis model group were significantly higher compared with the control group (Fig. 7). Treatment with resveratrol significantly suppressed the protein expression of IL-1β and IL-18 levels in acute pharyngitis rabbits, compared with the acute pharyngitis model group (Fig. 7).

TLR4 and MyD88 protein expression in animal models of acute pharyngitis. To further demonstrate the anti-inflammation effect of resveratrol on the TLR4/MyD88 signaling pathway, acute pharyngitis rabbits were treated with resveratrol. In the acute pharyngitis model group, there was a significantly increase of TLR4 and MyD88 protein expression levels, compared with the control group (Fig. 8). The induction of TLR4 and MyD88 protein expression was significantly suppressed by resveratrol in acute pharyngitis rabbits, compared with the acute pharyngitis model group (Fig. 8).

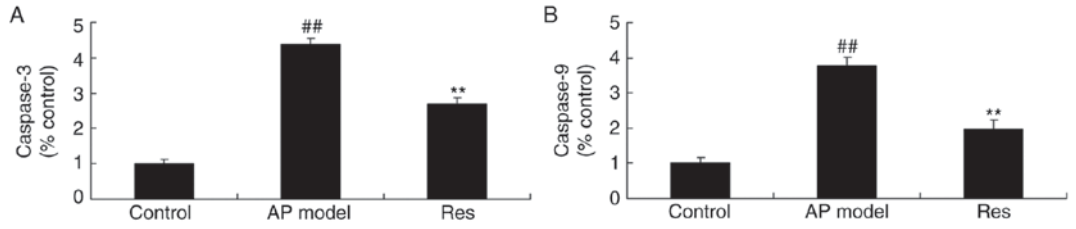

Figure 5. Caspase-3/9 activity in animal models of acute pharyngitis. (A) Caspase-3 and (B) -9 activity in animal models of acute pharyngitis. Data are expressed the mean ± standard deviation. ##P<0.01 vs. control group; **P<0.01 vs. AP model group. Control, control group; AP model, acute pharyngitis model group; Res, resveratrol treatment group.

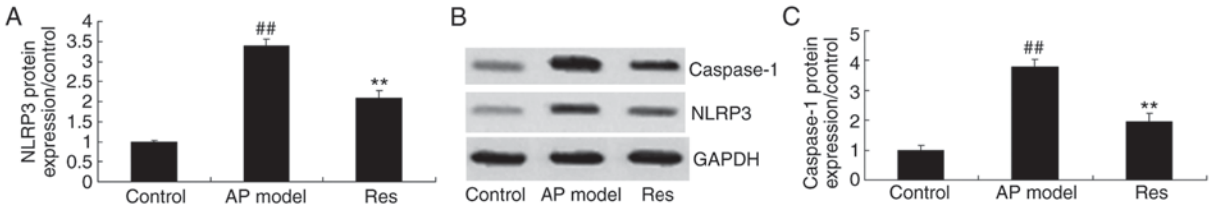

Figure 6. NLRP3 and caspase-1 protein expression in animal models of acute pharyngitis. (A) Quantification of NLRP3 protein expression levels, (B) representative western blot images of NLRP3 and caspase-1 protein expression levels, and (C) quantification of caspase-1 protein expression levels in animal models of acute pharyngitis. Data are expressed the mean ± standard deviation. ##P<0.01 vs. control group; **P<0.01 vs. AP model group. Control, control group; AP model, acute pharyngitis model group; Res, resveratrol treatment group; NLRP3, NACHT, LRR and PYD domains-containing protein 3.

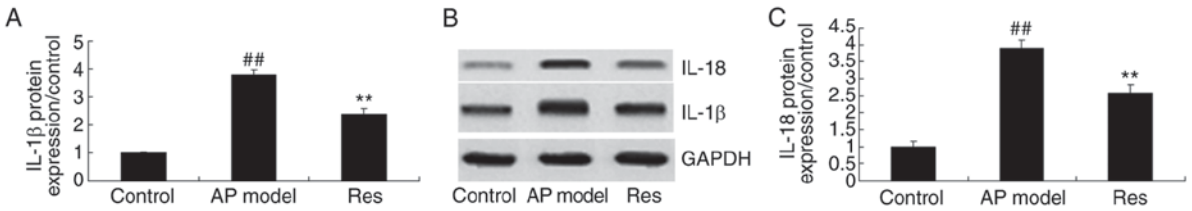

Figure 7. IL-1β and IL-18 protein expression in animal models of acute pharyngitis. (A) Quantification of IL-1β protein expression levels, (B) representative western blot images of IL-1β and IL-18 protein expression levels, and (C) quantification of IL-18 protein expression levels in animal models of acute pharyngitis. Data are expressed the mean ± standard deviation. ##P<0.01 vs. control group; **P<0.01 vs. AP model group. Control, control group; AP model, acute pharyngitis model group; Res, resveratrol treatment group; IL, interleukin.

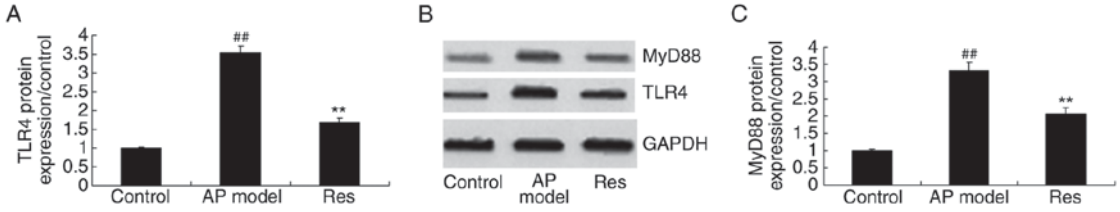

Figure 8. TLR4 and MyD88 protein expression in animal models of acute pharyngitis. (A) Quantification of TLR4 protein expression levels, (B) representative western blot images of TLR4 and MyD88 protein expression levels, and (C) quantification of MyD88 protein expression levels in animal models of acute pharyngitis. Data are expressed the mean ± standard deviation. ##P<0.01 vs. control group; **P<0.01 vs. AP model group. Control, control group; AP model, acute pharyngitis model group; Res, resveratrol treatment group; TLR4, toll-like receptor 4; MyD88, myeloid differentiation primary response protein 88.

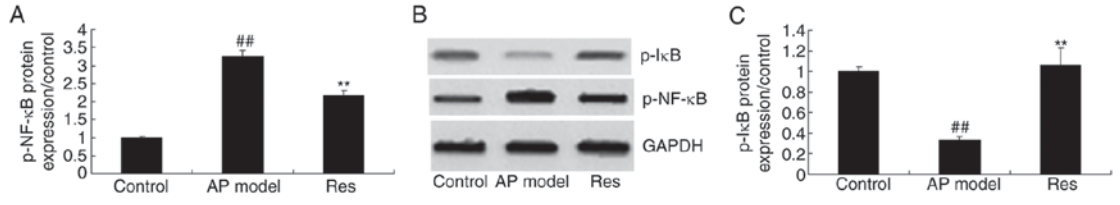

Figure 9. p-NF-κB and p-IκB protein expression in animal models of acute pharyngitis. (A) Quantification of p-NF-κB protein expression levels, (B) representative western blot images of p-NF-κB and p-IκB protein expression levels, and (C) quantification of p-IκB protein expression levels in animal models of acute pharyngitis. Data are expressed the mean ± standard deviation. ##P<0.01 vs. control group; **P<0.01 vs. AP model group. Control, control group; AP model, acute pharyngitis model group; Res, resveratrol treatment group; NF-κB, nuclear factor-κB; IκB, inhibitor of κB; p-, phosphorylated.

p-NF-κB and p-IκB protein expression in animal models of acute pharyngitis. To further investigate the anti-inflammation effects of resveratrol on the NF-κB signaling pathway, p-NF-κB and p-IκB protein expression were analyzed using western blotting. Compared with the control group, a significant increase of p-NF-κB protein expression and inhibition of p-IκB in the acute pharyngitis model group were observed (Fig. 9). Resveratrol significantly suppressed p-NF-κB and induced p-IκB protein expression in animal models of acute pharyngitis, compared with the acute pharyngitis model group (Fig. 9).

Discussion

Acute pharyngitis, a common exogenous disease, mainly occurs in the autumn and winter, as well as at the end of spring and the beginning of summer (4). It is generally caused by the decreased local or systemic immunity due to catching a cold, fatigue, or excessive alcohol intake and smoking, which create a favorable condition for pathogenic microorganisms (11). The preliminary symptoms mainly include throat itching, dry cough, a sore throat, hoarseness, fever, aversion to cold, malaise, joint pain, headache and loss of appetite (12). At present, patients with acute pharyngitis are mainly treated with symptomatic therapies such as western anti-inflammatory and antiviral medicines, as well as therapies of relieving cough and eliminating phlegm, with slow alleviation of symptoms (13). The results of the present study confirmed that resveratrol treatment significantly inhibited the reduced MIP-2 and COX-2 activity levels and ROS production, and suppressed the increase of caspase-3/9 activity in acute pharyngitis rabbits.

Macrophages and epithelium produce large amounts of cytokines and chemokines, as well as recruiting neutrophils, monocytes, macrophages and lymphocytes under mechanical stimulation, leading to lung inflammatory reaction, such as the activation of IL-1β, IL-18, IL-6, IL-2, MIP-2, TNF-a and NF-kB, which will be more severe if combined with inflammation (14,15). At the beginning of the activation of the NLRP3 inflammasome, the inflammasome complex is assembled, which requires the binding of protein domains apoptosis-associated speck-like protein containing a CARD (ASC) and NLRP3, as well as the structure domains ASC and pro-caspase-1. Subsequently, caspase-1 is activated, and IL-1β and IL-18 are released (16). The present study demonstrated that resveratrol significantly inhibited TNF-α and IL-6 serum levels and NLRP3/caspase-1/IL-1β/IL-18 protein expression in acute pharyngitis rabbits. Similarly, Sui *et al* (17) demonstrated that resveratrol protects against sepsis and inhibits NLRP3/IL-1β in microglia.

The TLR4/NF-κB signaling pathway also serves an critical role in the inflammatory response, especially the activation of macrophages in liver and adipose tissues (18). TLRs, a member of pattern recognition receptor family, serve a key role in the innate immune response (19). As the first TLR protein identified, TLR4 is associated with a variety of inflammatory reactions, and serves as the main receptor of the lipopolysaccharide response (19). TLR4 can activate multiple signaling pathways associated with inflammation, and induce the expression and secretion of various cytokines (20). Usually, TLR4 assists the binding of receptor cluster of differentiation 14 and lymphocyte antigen 96 to form a polymer, and then MyD88 will be recruited to the structure domain of toll/IL-1 receptor (TIR). The interaction between TLR4 and MyD88 in the structure domain of TIR triggers a cascade reaction of downstream signals, thus activating the NF-KB signaling pathway to promote cells to secrete a variety of inflammatory cytokines and chemokines (21). The present study demonstrated that resveratrol significantly suppressed TLR4 and MyD88 protein expression in acute pharyngitis rabbits. Zhang *et al* (22) indicated that resveratrol attenuates acute inflammatory injury through the TLR4 signaling pathway in experimental subarachnoid hemorrhage.

NF-κB participates in the regulation of many cellular functions, and serves an important role in the growth, proliferation and apoptosis of cells, closely associated with the innate and acquired immune response (23). NF-κB exists in the cytoplasm under resting state and binds to IκB, of the inhibitory protein family, to form a non-active complex (7). Nearly 20 years of studies have demonstrated that the NF-κB signaling pathway can be activated by various stimulating factors, which results in the aggregation of NF-κB in the nucleus; subsequently, NF-κB will be regulated by many signals in the nucleus, serving the role of transcription factors (7). The present study revealed that resveratrol significantly suppressed p-NF-κB and induced pn-IκB protein expression in animal models of acute pharyngitis. Tian *et al* (24) reported that resveratrol inhibited the NF-κB inflammation pathway in mice with fatty liver.

In conclusion, the findings of the present study support that the anti-inflammatory activity of resveratrol prevents acute pharyngitis and inflammation in a rabbit model of acute pharyngitis through suppression of NLRP3/caspase-1/IL-1β and IL-18, and of the TLR4/MyD88/NF-κB signaling pathway. These results implicate resveratrol as a novel drug for the treatment of acute pharyngitis.

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