

Fisetin administration improves LPS-induced acute otitis media in mouse *in vivo*

PENG LI¹, DAN CHEN² and YANG HUANG³

¹Department of Otorhinolaryngology, The Affiliated Hospital of Sun Yat-sen University; ²Department of Otolaryngology, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong 510630; ³Department of Otolaryngology, The First People's Hospital of Yunnan Province, Xishan, Kunming 650032, P.R. China

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Abstract. Acute otitis media is one of the most common infectious diseases worldwide in spite of the widespread vaccination. The present study was conducted to explore the effects of fisetin on mouse acute otitis media models. The animal models were established by lipopolysaccharide (LPS) injection into the middle ear of mice via the tympanic membrane. Fisetin was administered to mice for ten days through intragastric administration immediate after LPS application. Hematoxylin and eosin (H&E) staining was performed and the pro-inflammatory cytokines, including interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), IL-6 and VEGF, were measured through enzyme-linked immunosorbent assay (ELISA) method and RT-qPCR analysis. Toll-like receptor 4 (TLR4)/nuclear factor- κ B (NF- κ B) signaling pathway was detected by immunoblotting assays. Reactive oxygen species (ROS) generated levels were determined through assessment of anti-oxidants, and TXNIP/MAPKs signaling pathways were explored to reveal the possible molecular mechanism for acute otitis media progression and the function of fisetin. Fisetin reduced mucosal thickness caused by LPS. In fisetin-treated animals, pro-inflammatory cytokine release was downregulated accompanied with TLR4/NF- κ B inactivation. ROS production was significantly decreased in comparison to the LPS-treated group. The TXNIP/MAPKs signaling pathway was inactivated for fisetin treatment in LPS-induced mice with acute otitis media. The above results indicated that fisetin improved acute otitis media through inflammation and ROS suppression via inactivating TLR4/NF- κ B and TXNIP/MAPKs signaling pathways.

Introduction

Acute otitis media is reported as one of the most common diseases due to viral, or fungal pathogens and bacterial infection (1,2). The progression, pathophysiology as well as pathogenesis of acute otitis media are influenced by various factors, such as pathogenicity of the pathogens, reactive oxygen species (ROS) generation and inflammatory cytokines secretion (3,4). Presently, ~10-20% children experience recurrence and persistence of otitis media with long-term loss of hearing (5). Finding effective therapy is urgent for clinical treatment.

Studies before have indicated that a large number of cytokines or chemokines participate in acute otitis media pathogenesis (6). Lipopolysaccharides (LPS) are from gram-negative bacteria, which has been suggested to induce pro-inflammatory cytokine release, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and IL-6 (7). It has been reported that TNF- α can regulate the activity and expression of Toll-like receptors (TLRs), which is also important for TLR down-stream signaling pathway, including the nuclear factor- κ B (NF- κ B) (8). Recently, studies have suggested an essential function of TLR4 signaling pathway through MyD88-dependent pathway during the middle ear inflammatory responding to bacteria infection (9). Further, they are also crucial for the recovery process from otitis media (10).

Thioredoxin-interacting protein (TXNIP) is evidenced as a binding partner to the Nod-like receptor protein 3 (NLRP3) inflammasome, which is involved in activation of ROS-related NLRP3 inflammasome (11). NLRP3 inflammasome is known as a protein complex, consisting of an NLRP3 inflammasome sensor, the adaptor protein ASC and caspase-1 (12). TXNIP/NLRP3 signaling pathway is well reported to regulate inflammatory response through regulating NF- κ B transcription to induce IL-1 β pro-inflammatory cytokines release (13). ERK1/2, and p38 MAPK are well introduced for their role in ROS generation (14). Previously, suppression of MAPKs was reported to improve otitis media, indicating its role in otitis media progression.

Fisetin, a natural polyphenol found in various plants, including vegetables and fruits, has been shown to be able to defend both inflammation response and oxidative stress in

Correspondence to: Professor Yang Huang, Department of Otolaryngology, The First People's Hospital of Yunnan Province, 157 JinBi Road, Xishan, Kunming 650032 P.R. China
E-mail: huangyangkmerke@foxmail.com

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cells, exhibiting potent anti-inflammatory and anti-oxidative properties in various conditions (15,16). Analyses of the related signaling pathways modulated by the polyphenol have indicated that fisetin has the ability to suppress the activation of NF- κ B, ERK1/2, p38 MAPK, and the secretion of pro-inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α in a variety of different cells (17). Thus, we supposed that fisetin may be of potential value in treating LPS-induced acute otitis media by inflammation and ROS inhibition. The present study evaluated the role of fisetin in acute otitis media induced by LPS. We also investigated the signaling pathways underlying the possible inflammatory responses and ROS in the disease in mice, as well as the function of fisetin during the acute otitis media formation.

Materials and methods

The establishment of animal models. Sixty male, 8-week-old, C57BL/6 mice weighing 18–22 g were purchased from Shanghai Laboratory Animal Research Center (Shanghai, China). The mice were housed in a constant temperature of 22 \pm 2°C and relative humidity of 60 \pm 10% environment under 12 h light/dark cycles at the Third Affiliated Hospital of Sun Yat-sen University. The animals were permitted free access to water and chow, and were housed and fed in the specific pathogen-free facility. The mouse experiments were conducted minimizing animal suffering following the Guide for the Care and Use of Laboratory Animals, issued by the National Institutes of Health in 1996. All animal procedures were done following the guidelines for Care and Use of Laboratory Animals approved by Sun Yat-sen University. The acute otitis media mouse model was established through LPS injection (1.0 mg/ml; Sigma, St. Louis, CA, USA) into the middle ear of the mice via tympanic membrane in the right ear only for 24 h. The mice (n=15) without LPS treatment served as the control (Con). Forty-three mice were induced with acute otitis media successfully. After LPS exposure for 24 h, 36 mice with acute otitis media were randomly divided into two groups. Eighteen mice after LPS treatment were given low dose of 10 mg/kg of fisetin (FisL) dissolved in 5% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS) by intragastric administration every day for 10 days, and the other 18 mice after LPS treatment were given high dose of 20 mg/kg of fisetin (FisH) dissolved in 5% DMSO in PBS through intragastric administration every day for 10 days. Finally, the mice were sacrificed and the eyeball blood was collected and centrifuged at 15,000 \times g for 20 min prepared for following research. Middle ear lavage fluids (MELF) were collected, and then centrifuged at 500 \times g for 15 min, and the single-use aliquots of MELF were stored at -80°C. The cell pellets were then washed twice for further detections. Middle ear effusions from mice were harvested with 10 μ l saline (\times 3) first, and then the middle ears were washed with 200 μ l physiologic saline.

Enzyme-linked immunosorbent assay (ELISA). Concentrations of cytokines of IL-1 β , TNF- α , IL-6 and VEGF in the MEE and serum of mice were determined by the ELISA with the mouse enzyme immunoassay sets (R&D Systems, Inc., Minneapolis,

MN, USA) following the manufacturer's instructions. The samples were performed in duplicate.

Assessment of bioindicators. All the serum stored at -80°C were removed and maintained in glacial table. SOD activity and MDA levels in serum and MEE were tested using biochemical kit, Superoxide Dismutase assay kit and Maleic Dialdehyde assay kit, respectively (Jiancheng Biotech Co., Ltd., Nanjing, China) following the manufacturer's instruction.

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA from the middle ear tissue samples was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The cDNA was synthesized using SuperScript II reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative PCR was performed with SYBR-Green Real-Time PCR Master mix (Thermo Fisher Scientific). Finally, the quantitative expression data were collected and analyzed by a 7900 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primers were designed to determine endogenous genes and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. The crossing point of target genes with GAPDH was calculated using the formula $2^{-(\text{target gene} - \text{GAPDH})}$ and the relative amounts were quantified. Forward TNF- α , (5'-3') CTT CGT ACT ATC ATG TAG GTC GAG and reverse primers, (5'-3') ATG CTC GGC ATG TTT GAA CTA GTC; forward IL-1 β , (5'-3') ATG CGG TAG CAA GTT ACG AC and reverse primers, (5'-3') GAG GTG GCT GTT GGT ATT GAT; forward IL-6, (5'-3') CTA AGG ACG GAG AGT CAG T and reverse primers, (5'-3') CTG TCG TCG ATG AGA TTA TG; forward VEGF, (5'-3') CCT CCT GGC GCT CTG ATA TGT and reverse, (5'-3') GTG AGT GTG TAG GTG TGC GC; forward TXNIP, (5'-3') CTT GCC GTC ATC TTC CTA CA and reverse primers, (5'-3') GCC AAG GTA AAG GAG GCA CT; forward NLRP3, (5'-3') GCT CAC TCT TAT CTA TCC CGA and reverse primers, (5'-3') ACA AGG CGT AGC AGA GAG CT; forward GAPDH, (5'-3') AGT CTA CGT GCA ATC AAC AGA ATG and reverse primers, (5'-3') CGC CCT AAC AAC ACG ACA TCC AT.

Western blot assays. The middle ear tissues were harvested and frozen in liquid nitrogen immediately, and then were stored in -80°C until homogenization. Proteins were extracted from the middle ear tissue samples using T-PER tissue protein extraction reagent kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Protein concentrations were determined by BCA protein assay kit (Thermo Fisher Scientific) following the manufacturer's instruction, and equal amounts (40 μ g) of protein were loaded per well on a 10% sodium dodecyl sulphatepolyacrylamide gel. Subsequently, proteins were transferred onto polyvinylidene difluoride membrane. The resulting membrane was blocked with Tris-buffered saline containing 0.05% Tween-20 (TBS-T), supplemented with 5% skim milk (Sigma) at room temperature for 2 h on a rotary shaker, and followed by TBS-T washing. The specific primary antibody, diluted in TBST, was incubated with the membrane at 4°C overnight. Subsequently, the membrane was washed with TBS-T followed by incubation with the horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. The

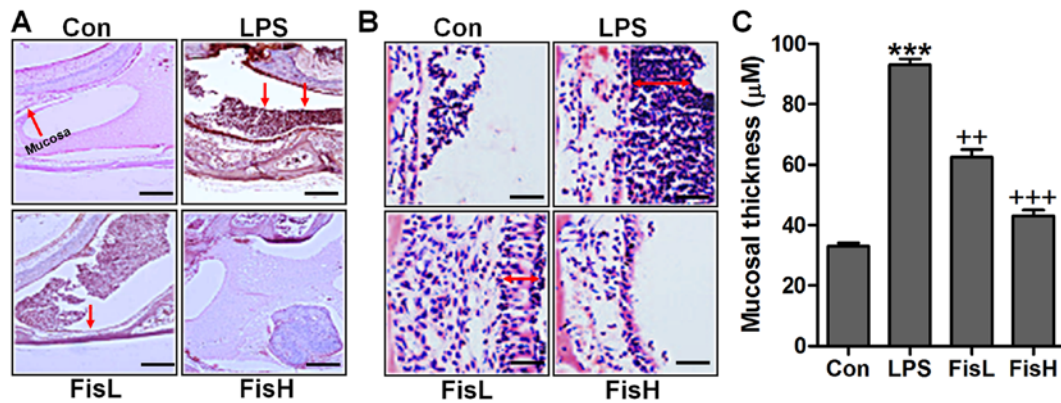


Figure 1. Fisetin ameliorates the middle ear injury of mice after lipopolysaccharide (LPS) exposure. (A) H&E staining analysis of the middle ear sections obtained from mice under different conditions (scale bar, 500 μ m). (B) The representative images of ME histopathology in LPS-treated mice with or without fisetin administration exhibited by H&E staining (scale bar, 100 μ m) (A). (C) The quantification of mucosa thickness is shown. Data are expressed as the mean \pm SEM (n=10). * p <0.05, ** p <0.01 and *** p <0.001 vs. the control (Con) group; + p <0.05, ++ p <0.01 and +++ p <0.001 vs. the LPS group.

immunoactive proteins were detected by using an enhanced chemiluminescence western blot detection kit. Western blot bands were observed using GE Healthcare ECL western blotting analysis system (GE Healthcare, Logan, UT, USA) and exposed to Kodak X-ray film. The primary antibodies used in our study are as follows: rabbit anti-GAPDH (1:1,000; Cell Signaling Technology, Beverly, MA, USA), anti-TLR-4 (1:1,000), mouse rabbit anti-MyD88 (1:1,000), rabbit anti-p-IKK α (1:1,000) (all from Abcam, Cambridge, MA, USA), rabbit anti-p-IkB α (1:1,000), rabbit anti-p-NF- κ B (1:1,000), rabbit anti-NF- κ B (1:1,000), rabbit anti-caspase-3 (1:1,000) (all from Cell Signaling Technology), rabbit anti-PARP (1:1,000; Abcam), rabbit anti-Bax (1:1,000; Cell Signaling Technology), rabbit anti-Bad (1:1,000), mouse anti-Bcl-2 (1:1,000), rabbit anti-Bcl-xL (1:1,000) (all from Abcam), rabbit anti-SOD1 (1:1,000), rabbit anti-SOD2 (1:1,000) (both from Cell Signaling Technology), rabbit anti-HO-1 (1:1,000), rabbit anti-Nrf2 (1:1,000), rabbit anti-TXNIP (1:1,000), rabbit anti-NLRP3 (1:1,000), rabbit anti-ASC (1:1,000) (all from Abcam), rabbit anti-caspase-1 (1:1,000; Cell Signaling Technology), rabbit anti-p-ERK1/2 (1:1,000), rabbit anti-ERK1/2 (1:1,000) (both from Abcam), rabbit anti-p38 (1:1,000), and rabbit anti-p-p38 (1:1,000) (both from Cell Signaling Technology).

Flow cytometry analysis. The cells in MELF were centrifuged at 500 \times g for 10 min, the cell pellets were washed twice with cold PBS, and next the Fc receptors were fine blocked using the Mouse BD Fc Block purchased from BD Biosciences (Franklin Lakes, NJ, USA). Then, the cells were stained using specific immune (CD11b; BD Biosciences) markers on cell surface, followed by the staining parameters: cells experiencing early apoptosis marked as Annexin V-positive and propidium iodide-negative, and the cells during late apoptosis described as Annexin V-positive and propidium iodide-positive.

Immunohistochemical assays. Histopathologic evaluation was performed on mice. Mouse middle ear tissue samples were fixed with 10% buffered formalin, imbedded in paraffin, and sliced into 5 μ m sections. After hematoxylin and eosin (H&E) staining, pathological changes of the tissues were observed under a light microscope. For fluorescent analysis, the mouse

middle ear tissue samples were carefully isolated and fixed in 4% paraformaldehyde for 16 h after cold 4% paraformaldehyde perfusion. Then, optimum cutting temperature (OCT) package tissues were sliced as 20-30 μ m sections. The tissues were incubated with primary antibodies (TXNIP, NLRP3, p-p38 and p-ERK1/2) at 4°C overnight after deparaffinized and rehydrated. Fluorophore-conjugated secondary antibodies were treated 1 h at 25°C thermostat. The Alexa Fluor 488 labeled anti-rabbit or anti-mouse secondary antibodies (Invitrogen) were used. Sections were subjected to immunofluorescence staining via epifluorescence microscopy (Sunny Co., Shanghai, China). Leica TCS SP5 confocal microscope (Leica, Richmond Hill, ON, Canada) was used to obtain images and carried out in a blinded manner with respect to treatment groups.

Statistical analysis. Data are expressed as means \pm SEM. Treated tissues and the corresponding controls were compared using GraphPad Prism (version 6.0; GraphPad Software, La Jolla, CA, USA) by one-way ANOVA with Dunn's least significant difference tests. Differences between groups were considered significant at p <0.05.

Results

Fisetin ameliorates the middle ear injury of mice after LPS exposure. In order to prove the successful establishment of acute otitis media in the mouse model, the histologic sections and the mucosa thickness in the mouse middle ear were observed through H&E staining. As shown in Fig. 1A, the normal control groups showed no signs of inflammatory response, while in the LPS-treated mice, the cells exhibited brown color suggesting significant inflammation, which was comparable to the control group. Of note, fisetin administration apparently reduced the number of inflammatory cells, mainly including monocytes, macrophages, leukocyte, neutrophil and eosinophils (18,19). In addition, the photomicrographs indicated the mucosa in middle ear of mice with LPS induction. By assessment, the mucosa in the roof of LPS-treated mice with acute otitis media was much thicker compared to the control ones, which was reduced for fisetin treatment at different concentrations (Fig. 2B and C).

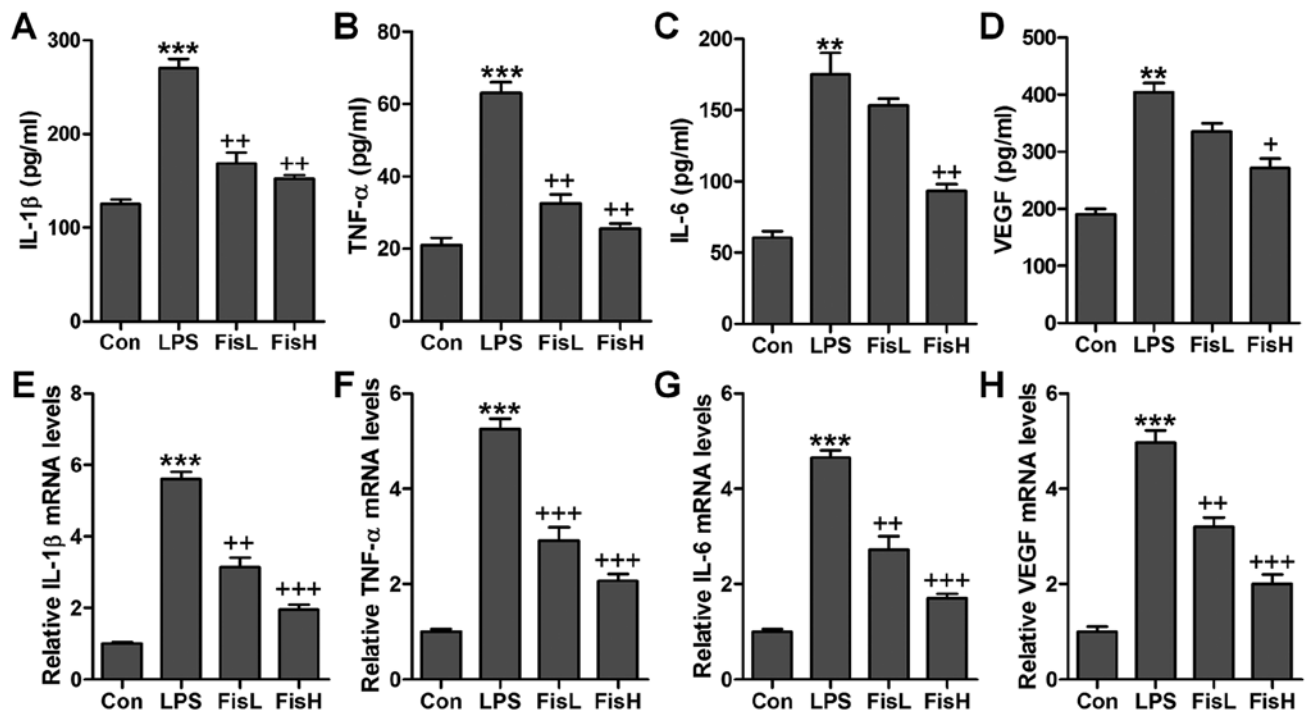


Figure 2. Fisetin reduced pro-inflammatory cytokine release in lipopolysaccharide (LPS)-induced mice with acute otitis media. The pro-inflammatory cytokines in serum of mice were calculated through enzyme-linked immunosorbent assay (ELISA) methods, including (A) interleukin-1 β (IL-1 β), (B) tumor necrosis factor- α (TNF- α), (C) IL-6 and (D) VEGF. The pro-inflammatory cytokines in the middle ear effusions of mice were assessed via ELISA kits, including (E) IL-1 β , (F) TNF- α , (G) IL-6 and (H) VEGF. The quantification is displayed. Data are expressed as the mean \pm SEM (n=10). *p<0.05, **p<0.01 and ***p<0.001 vs. the control (Con) group; +p<0.05, ++p<0.01 and +++p<0.001 vs. the LPS group.

The findings above indicated that LPS indeed induced acute otitis media in mice, and interestingly, fisetin showed ameliorated effects on LPS-triggered acute otitis media in the middle ear of mice.

Fisetin reduces pro-inflammatory cytokine release in LPS-induced mice with acute otitis media. Inflammation response has been suggested to be of great importance to cause injury in the middle ear under different conditions, such as bacteria exposure (20). According to the observations above, the ear injury was induced in mice after LPS exposure, which is well known to induce inflammation response in various diseases (21). Thus, here we attempted to investigate whether fisetin-improved acute otitis media was associated with inflammation suppression, pro-inflammatory cytokines were measured in the serum and middle ear tissue samples obtained from mice after LPS treatment. The pro-inflammatory cytokines, IL-1 β , TNF- α , IL-6 and VEGF, were highly accumulated in LPS-treated mice, which were reduced for fisetin administration with significant difference in comparison to the LPS group (Fig. 2A-D). Further, these factors in the dissected tissue samples of middle ear were calculated through RT-qPCR assays. The results illustrated that IL-1 β , TNF- α , IL-6 and VEGF gene abundance occurred in LPS-treated group in the absence of fisetin. However, fisetin administration dramatically downregulated IL-1 β , TNF- α , IL-6 and VEGF mRNA levels in a dose-dependent manner (Fig. 2E-H). The data above demonstrated that inflammation response was induced in the middle ear of mice with LPS treatment, and fisetin exhibited effective role in reversing pro-inflammatory cytokine secretion.

Fisetin ameliorates LPS-induced inflammation in the middle ear of mice through TLR4/NF- κ B signaling pathway. As our above data indicated, inflammation response was indeed induced in LPS-treated mice with acute otitis media in the middle ear. Fisetin was evidenced to be repressive for pro-inflammatory cytokine release. TLR4/NF- κ B signaling pathway is well reported to be essential for inflammation response through transcription factor activity regulation, contributing to pro-inflammatory cytokine secretion (22). Hence, in this regard, we attempted to explore if the classic TLR4/NF- κ B signaling pathway participated in fisetin-improved acute otitis media in LPS-induced mice. As shown in Fig. 3A and B, TLR4 protein levels in the middle ear tissue samples were found to be highly expressed due to LPS treatment by the use of western blot analysis, leading to the downstream signal MyD88 upregulation. Notably, fisetin reduced TLR4 and MyD88 protein expression levels, which was comparable to the LPS group. Also, RT-qPCR assays showed similar results that fisetin had a suppressive role in TLR4 and MyD88 activity induced by LPS (Fig. 3C and D). NF- κ B phosphorylation is crucial for inflammation response regulated by TLR4/MyD88 signaling pathway. LPS treatment considerably increased IKK α and I κ B α phosphorylation, which improved NF- κ B phosphorylation (Fig. 3E-G). In contrast, fisetin administration apparently restrained IKK α , I κ B α and NF- κ B activity, in line with the results of pro-inflammatory cytokine alteration mentioned above. The results suggested that fisetin-improved acute otitis media caused by LPS was dependent on TLR4/NF- κ B signaling pathway suppression.

Fisetin inhibits apoptosis in the middle ear of mice treated with LPS. Previous studies indicated that apoptosis was

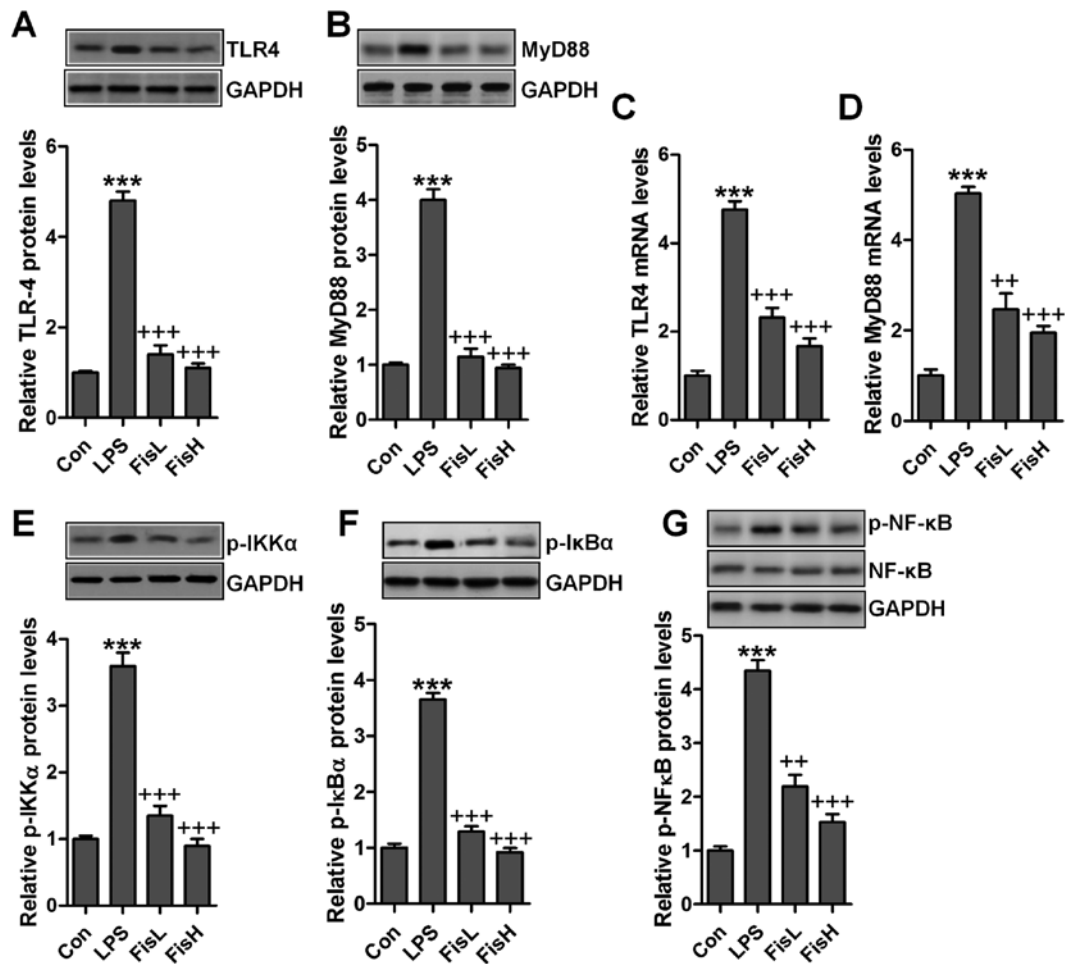


Figure 3. Fisetin ameliorates lipopolysaccharide (LPS)-induced inflammation in the middle ear of mice through Toll-like receptor 4 (TLR4)/nuclear factor-κB (NF-κB) signaling pathway. Western blot analysis was conducted to explore (A) TLR4 and (B) MyD88 protein expression levels. (C) TLR4 and (D) MyD88 mRNA levels were measured via RT-qPCR assays, and the relative fold quantification is exhibited. The immunoblotting analysis was performed to determine (E) p-IKKα, (F) p-IκBα and (G) p-NF-κB protein levels in the middle ear of mice. Data are expressed as the mean ± SEM (n=10). *p<0.05, **p<0.01 and ***p<0.001 vs. the control (Con) group; +p<0.05, ++p<0.01 and +++p<0.001 vs. the LPS group.

closely related to acute otitis media induced by bacterial (23). Thus, the neutrophil apoptosis may be involved in fisetin against LPS challenging in acute otitis media. Here, flow cytometry analysis showed that cells in the MELF obtained from LPS-treated mice underwent significant apoptosis, while fisetin-treated groups at different concentrations displayed markedly downregulated rate (Fig. 4A and B). Caspase-3 enhances apoptotic response via PARP activation (24). Pro-apoptotic members and anti-apoptotic molecules are in homeostasis under normal condition, which will be disrupted for different stress exposure, including LPS. In this study, we found that caspase-3 and PARP cleavage was highly upregulated for LPS induction, contributing to cell death in the middle ear of mice (Fig. 4C-E). Additionally, pro-apoptotic signals, Bax and Bad were also expressed in abundance from the protein levels, which was in agreement with cleaved caspase-3 and PARP (Fig. 4F and G). On the contrary, anti-apoptotic members, Bcl-2 and Bcl-xL protein levels were downregulated due to LPS (Fig. 4H and I). Fisetin treatment decreased caspase-3 and PARP activation, accompanied with reduced Bax and Bad, whereas Bcl-2 and Bcl-xL were obviously increased. Moreover, RT-qPCR assays showed that Bax and Bad mRNA levels were augmented in LPS group, which

was comparable with the control ones, while being impeded after fisetin treatment (Fig. 4J and K). Consistent with preprotein alterations, Bcl-2 and Bcl-xL gene levels were reduced for LPS, and fisetin reversed Bcl-2 and Bcl-xL expression in a dose-dependent manner (Fig. 4L and M). Taken together, the data illustrated that fisetin could improve acute otitis media through apoptosis suppression via inactivating caspase-3 signaling pathway.

Fisetin upregulates anti-oxidant levels in LPS-exposed mice with acute otitis media. ROS generation with decreased anti-oxidants is considered to induce tissue injury or organ dysfunction for various reasons, including LPS induction (25). In this regard, ROS-related signals were calculated to reveal that if fisetin-improved acute otitis media was related to ROS suppression. As shown in Fig. 5A, SOD activity in serum was found to be decreased for LPS treatment, which recovered highly for fisetin administration. In contrast, LPS-triggered higher MDA levels were significantly reduced for fisetin treatment (Fig. 5B). Consistently, in MEE, SOD activity and MDA levels for LPS challenge were similar to that in serum, and fisetin reversed expression of the two indicators to near normal levels (Fig. 5C and D). For further confirmation,

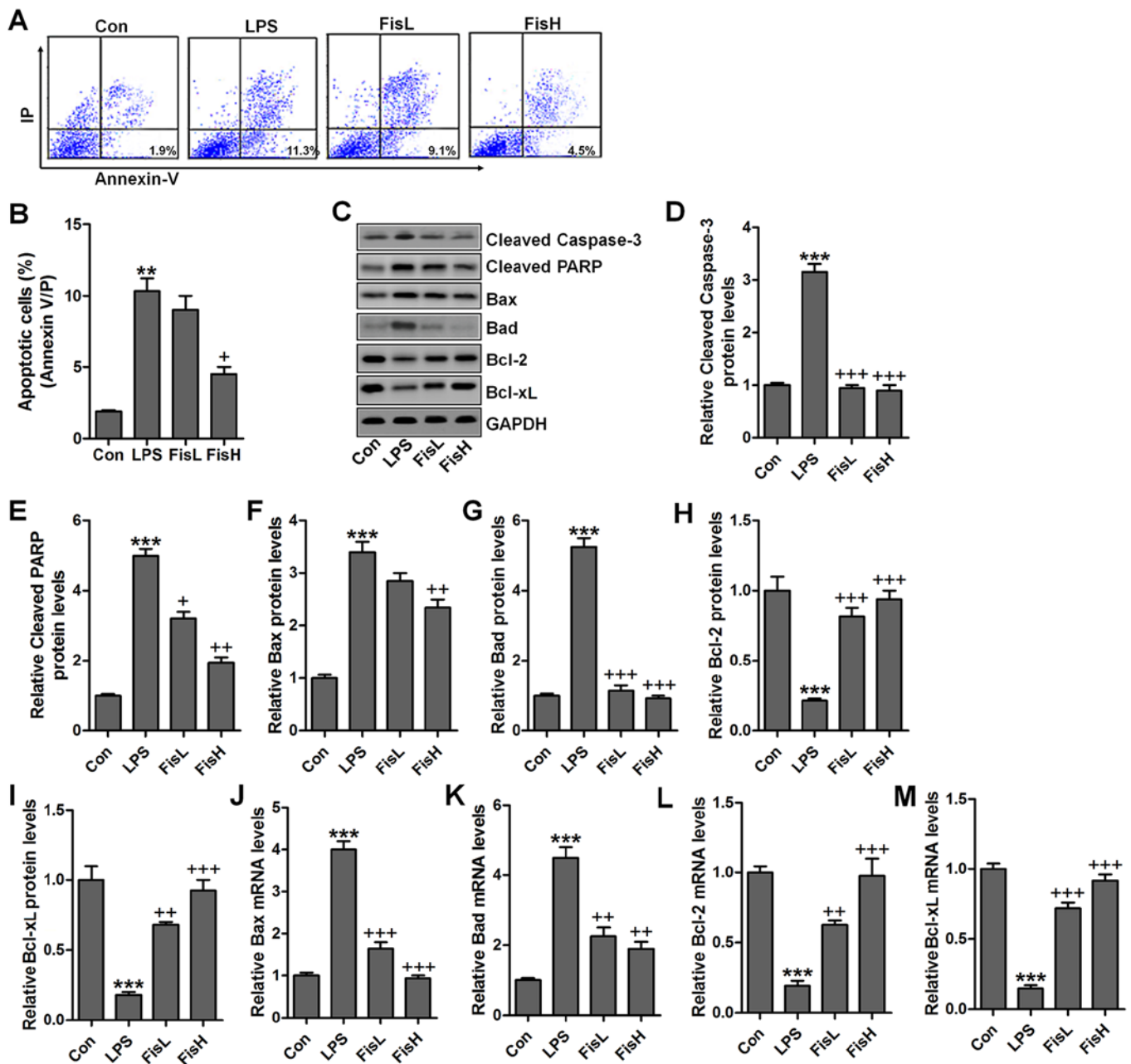


Figure 4. Fisetin inhibits apoptosis in the middle ear of mice treated with lipopolysaccharides (LPS). (A) Flow cytometry was used to examine apoptosis in lipopolysaccharide (LPS)-treated mice with acute otitis media after fisetin administration at different concentrations. (B) The percentage of apoptotic cells following flow cytometry analysis was shown. (C) The representative images of cleaved caspase-3, cleaved PARP, Bax, Bad, Bcl-2, and Bcl-xL were shown through western blot analysis. The quantification of (D) cleaved caspase-3, (E) cleaved PARP, (F) Bax, (G) Bad, (H) Bcl-2, and (I) Bcl-xL is exhibited. RT-qPCR assays were used to determine (J) Bax, (K) Bad, (L) Bcl-2 and (M) Bcl-xL gene levels. Data are expressed as the mean \pm SEM (n=10). * p <0.05, ** p <0.01 and *** p <0.001 vs. the control (Con) group; * p <0.05, ** p <0.01 and *** p <0.001 vs. the LPS group.

western blot analysis was performed to determine SOD1, SOD2, HO-1 and Nrf2 protein levels in the middle ear of mice under different conditions. As shown in Fig. 5E and F, SOD1, SOD2, HO-1 and Nrf2 protein levels were apparently reduced in LPS administration, which were highly improved by fisetin treatment, in a dose-dependent manner. In conclusion, the data here indicated that ROS production induced by LPS could be decreased in fisetin treatment to improve acute otitis media in mice.

Fisetin-improved acute otitis media triggered by LPS is associated with TXNIP and MAPKs signaling pathways. According

to previous studies, TXNIP and MAPKs are involved in inflammation and ROS production through NLRP3 and p38/ERK1/2 activation (26,27). Hence, western blot analysis was used to calculate TXNIP/NLRP3 and MAPKs signaling pathways. As shown in Fig. 6A and B, TXNIP and NLRP3 protein expression levels were highly upregulated in LPS exposure, leading to ROS generation largely. After fisetin administration, TXNIP and NLRP3 were downregulated. TXNIP/NLRP3 signaling pathway activation is a key to induce ASC expression, causing caspase-1 cleavage and enhancing NF- κ B transcription to induce pro-inflammatory cytokines secretion (28). Similarly, in this study, we found that ASC was increased following

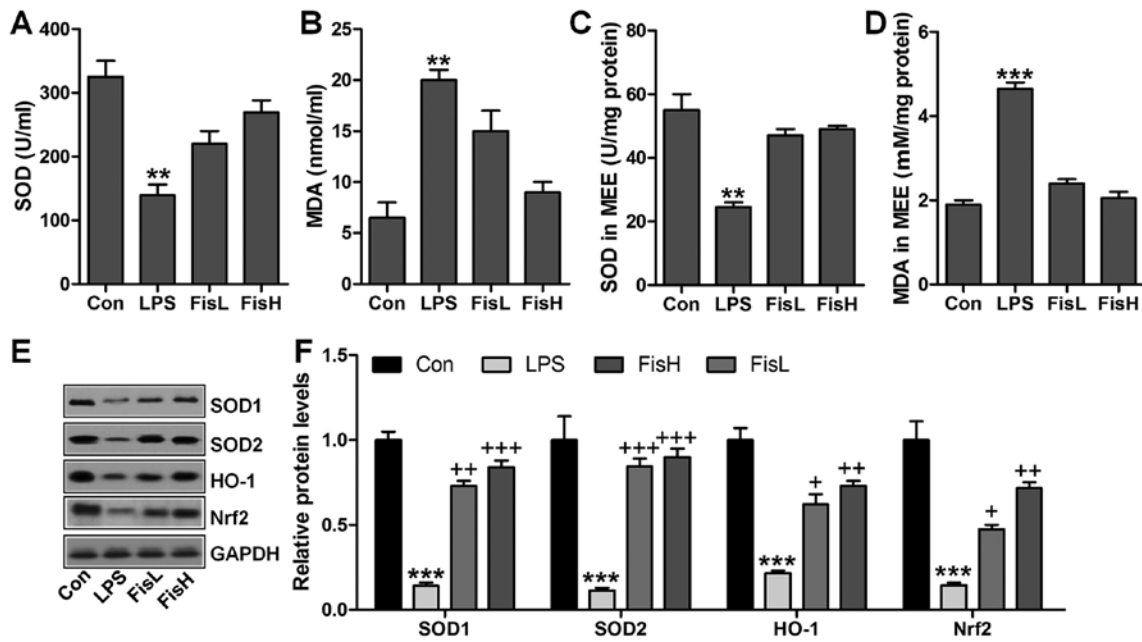


Figure 5. Fisetin upregulates anti-oxidants levels in lipopolysaccharide (LPS)-exposed mice with acute otitis media. (A) SOD activity, and (B) MDA levels in serum were measured. (C) SOD activity and (D) MDA levels in the middle ear tissues were analyzed. Western blot analysis was included to detect SOD1, SOD2, HO-1 and Nrf2 protein expression levels in the middle ear tissue of mice with LPS induction in the presence or absence of fisetin at different doses. (E) Representative images of western blot analysis are displayed. (F) SOD1, SOD2, HO-1 and Nrf2 protein levels were quantified following western blot results. Data are expressed as the mean \pm SEM (n=10). *p<0.05, **p<0.01 and ***p<0.001 vs. the control (Con) group; +p<0.05, ++p<0.01 and +++p<0.001 vs. the LPS group.

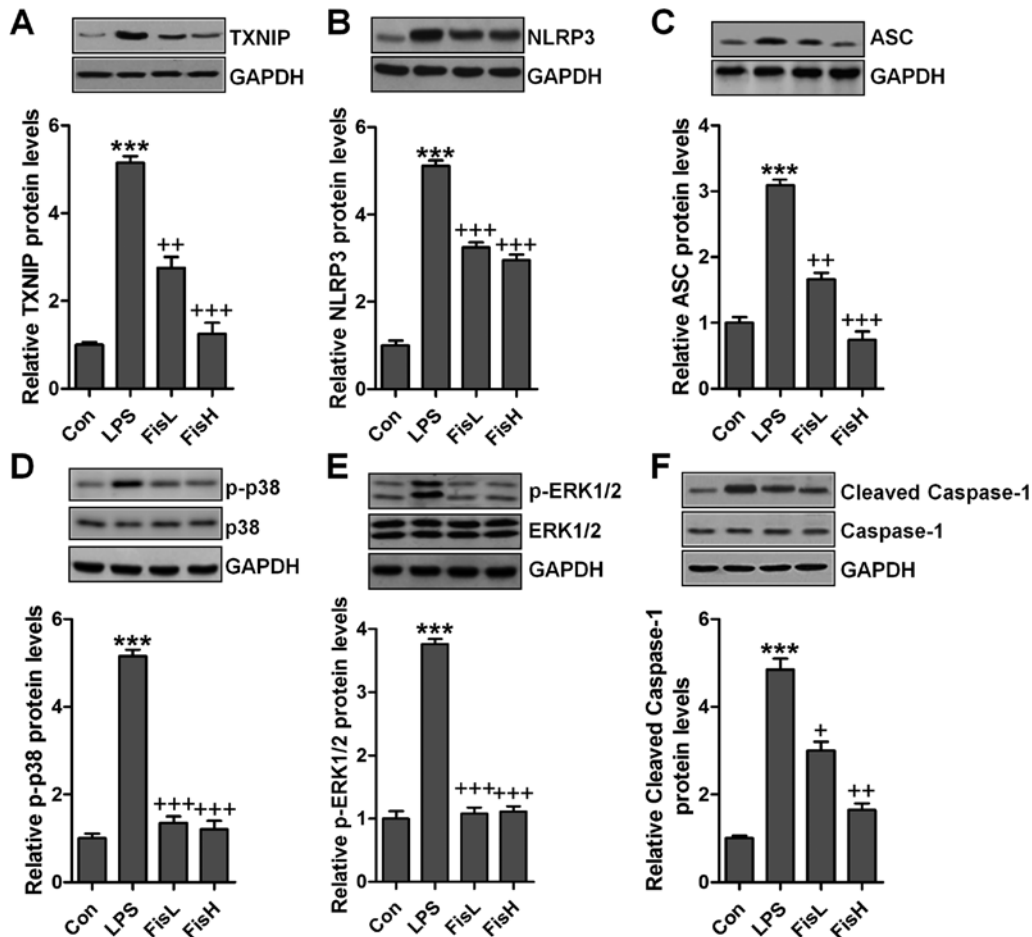


Figure 6. Fisetin improves acute otitis media triggered by lipopolysaccharides (LPS) is associated with TXNIP and MAPKs signaling pathways. Western blot assays were conducted to assess (A) TXNIP, (B) NLRP3, (C) ASC, (D) cleaved caspase-1, (E) phosphorylated ERK1/2 and (F) phosphorylated p38 levels in the middle ear of mice after LPS treatment. Data are expressed as the mean \pm SEM (n=10). *p<0.05, **p<0.01 and ***p<0.001 vs. the control (Con) group; +p<0.05, ++p<0.01 and +++p<0.001 vs. the LPS group.

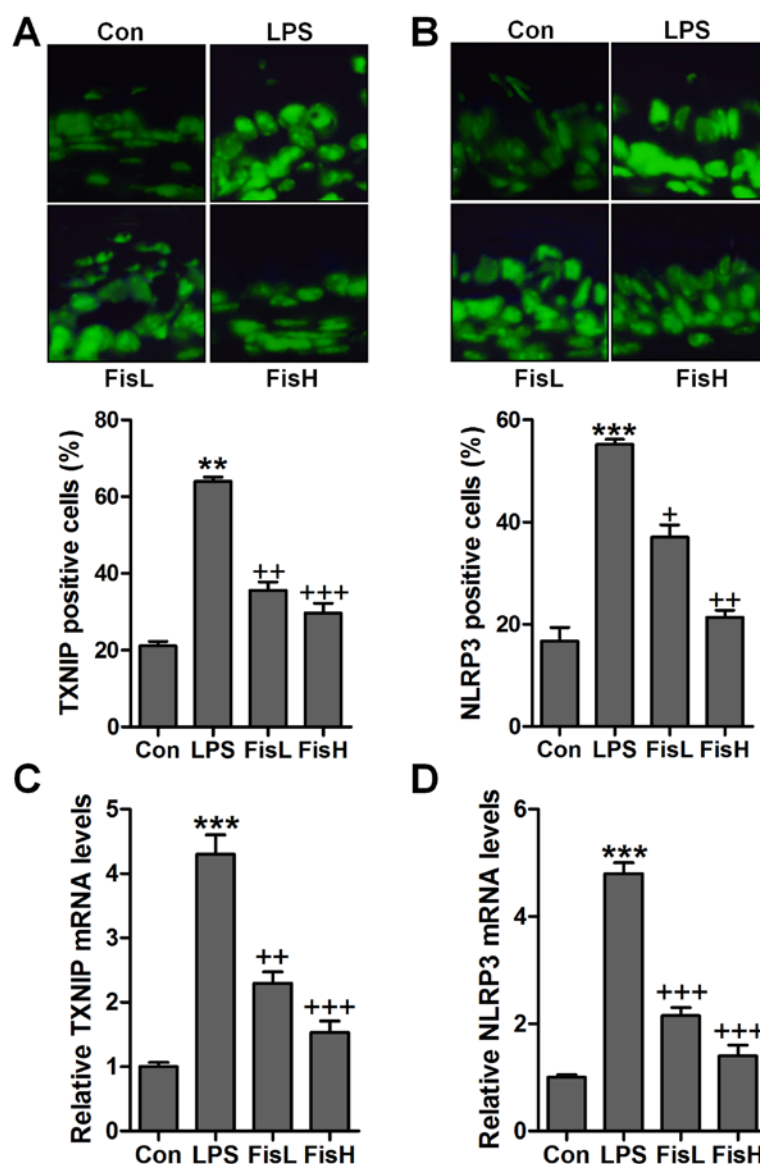


Figure 7. Fisetin ameliorates the acute otitis media in the middle ear of mice through TXNIP/NLRP3 suppression. The immunofluorescent analysis was used to analyze (A) TXNIP and (B) NLRP3 levels in the middle ear sections with the specific antibodies. The quantification of fluorescent intensity is displayed. (C) TXNIP and (D) NLRP3 mRNA levels were calculated through RT-qPCR assays. Data are expressed as the mean \pm SEM (n=10). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. the control (Con) group; + $p < 0.05$, ++ $p < 0.01$ and +++ $p < 0.001$ vs. the lipopolysaccharides (LPS) group.

TXNIP and NLRP3 upregulation in LPS-treated group, which was reduced by fisetin addition (Fig. 6C). Subsequently, cleaved caspase-1 was also stimulated for LPS, reduced by fisetin administration (Fig. 6D). Finally, MAPKs, ERK1/2 and p38, phosphorylated levels were measured through western blot analysis. As shown in Fig. 6E and F, we found that ERK1/2 and p38 phosphorylated levels were highly stimulated in LPS-treated group, while in fisetin-treated groups, both ERK1/2 and p38 phosphorylation were restrained.

To further prove TXNIP and NLRP3 in acute otitis media formation, immunofluorescent analysis was used to calculate TXNIP and NLRP3 alterations in fisetin-treated mice induced by LPS. Apparently high immunofluorescent intensity was observed in LPS-treated group, which was weakened for fisetin treatment (Fig. 7A and B). Additionally, RT-qPCR assays further indicated that TXNIP and NLRP3 mRNA levels were reduced by LPS, reversed by fisetin and shown in a dose-dependent manner (Fig. 7C and D). Moreover, p-p38 and

p-ERK1/2 were expressed with extremely high fluorescent intensity in the middle ear tissue of mice after LPS exposure, which was blocked by fisetin administration (Fig. 8). The data indicated that fisetin-improved acute otitis media induced by LPS was highly related to TXNIP/NLRP3 and MAPKs signaling pathway repression.

Discussion

Acute otitis media is reported as one of the most common infectious diseases for children. Acute otitis media could develop into otitis media chronically, leading to hearing loss (1,29). Severe complications include language disability and intellectual impairment (30). However, until now the specific molecular mechanism of acute otitis media is poorly known, and finding effective therapeutic strategy is urgently required. Fisetin has been reported to be effective in anti-inflammation and anti-oxidation through various signaling

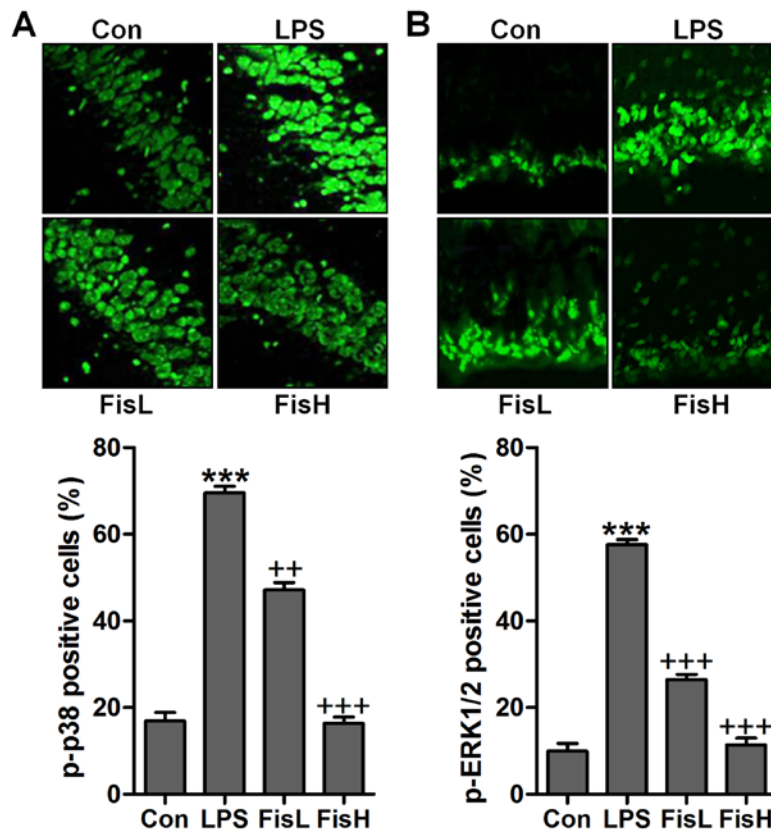


Figure 8. Fisetin ameliorates the acute otitis media in the middle ear of lipopolysaccharides (LPS)-treated mice by inhibiting MAPK signaling pathway. (A) p-p38 and (B) p-ERK1/2 levels were determined via the immunofluorescent analysis. The quantification of phosphorylated p38 and ERK1/2 is shown. Data are expressed as the mean \pm SEM (n=10). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs. the control (Con) group; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs. the LPS group.

pathways (31). A previous study found that acute otitis media development has a close relationship with inflammatory response and oxidative stress (32). Moreover, apoptosis is a cellular process regulating different cell types, leading to cell death eventually (33). Apoptosis-induced cell dysfunction has been reported in a variety of diseases, including acute lung injury, acute renal injury and even the acute otitis media (34). LPS, as inflammatory response inducer, is considered to be feasible for acute otitis media establishment in mice (35). In this study, LPS was used to induce acute otitis media in C57BL/6 mice by inflammation induction, apoptosis accumulation and ROS generation through TLR4/NF- κ B, caspase-3/PARP and TXNIP/NLRP3 signaling pathways. Of note, it was the first time that fisetin was applied to ameliorate acute otitis media induced by LPS injection into the middle ear of mice. The thickened mucosa confirmed the successful establishment of acute otitis media in mice, indicating the inflammatory severity. Importantly, fisetin showed activity to reduce the thickened mucosa induced by LPS, which demonstrated that fisetin could improve LPS-triggered acute otitis media in the middle ear of mice.

Earlier studies have reported that fisetin suppresses pro-inflammatory cytokine release is protective against acute organ or tissue injuries (36,37). The suppressive role of fisetin in acute otitis media could inhibit LPS-induced injury in mice. Blocking pro-inflammatory cytokines secretion could be an effective target for otitis media treatment. In order to investigate the restraining of pro-inflammatory cytokines in fisetin-treated groups, TNF- α , IL-1 β , IL-6 and VEGF molecules were

assessed through ELISA method and RT-qPCR analysis. We found that pro-inflammatory cytokines, TNF- α , IL-1 β , IL-6 and VEGF were highly expressed in LPS group, which was in line with the histological study. Fisetin impeded secretion of these factors in a dose-dependent manner, illustrating that fisetin could be a potential natural compound for acute otitis media treatment through inhibiting pro-inflammatory cytokine release.

TLR4/NF- κ B has been well investigated in activating inflammatory response (38). TLR4 once activated by LPS could stimulate its down-streaming signal MyD88, leading to IKK α phosphorylation (39). Subsequently, I κ B α and NF- κ B complex is disassociated through phosphorylation, contributing to NF- κ B translocation into the nucleus and leading to pro-inflammatory cytokine release (40). Similarly, in this study, we found that TLR4/NF- κ B signaling pathway was highly activated by LPS induction, which was suppressed due to fisetin administration. In addition, TXNIP/NLRP3 is an important regulator for inflammation response (41). TXNIP could bind to NLRP3 inflammasome (42). Once TXNIP/NLRP3 is activated under different conditions, NLRP3 is ligated with ASC, which in turn combines to pro-caspase-1, leading to its transformation to the cleaved caspase-1. Cleaved caspase-1 modulates the maturation of pro-inflammatory cytokines, including IL-1 β and IL-18 (43). Previously, NLRP3 caused renal injury has been reported (44). We found that TXNIP and NLRP3 signaling pathway was upregulated for LPS treatment, followed by ASC and caspase-1 cleavage increase, which was in line with the inflammation response.

Apoptotic response is important in regulating cellular process through inducing cell death (45). Caspase-3 activation induced PARP cleavage, contributing to apoptosis and causing cell injury (46). Bcl-2, belonging to anti-apoptotic family, is known to suppress apoptosis. In contrast, pro-apoptotic members, Bad and Bax, which also include in Bcl-2 family, are apoptosis inducers (47). Here, we found that apoptosis was stimulated for LPS, resulting in cell death and acute otitis media in the middle ear of mice eventually, which was conformed by caspase-3 and PARP cleavage, as well as increased Bax and Bad. In contrast, decreased Bcl-2 and Bcl-xL was observed. Fisetin suppressed apoptosis through caspase-3 and PARP inactivation. Furthermore, ROS generation is another cellular stress, leading to cell injury and contributing to inflammation response (48). ROS production is highly related to anti-oxidants and oxidant accumulation. Previous studies have suggested that ROS over-production is a key point, causing injuries under various situations, such as liver, heart and lung injury (49). Further, acute otitis media is reported to be associated with ROS levels. MAPKs, p38 and ERK1/2, are reported to be of great importance in promoting ROS generation (50). It has been reported that MAPKs activate the pathological process of various diseases (51). In this study, MAPK signaling pathway was activated, accompanied with high expression of phosphorylated p38 and ERK1/2 after LPS stimulation, which was reduced by fisetin administration, indicating that fisetin-improved acute otitis media is related to MAPK suppression.

Our results indicated that pro-inflammatory cytokine levels, apoptosis and ROS generation were significantly upregulated in acute otitis media mouse model induced by LPS. Fisetin significantly reduced the levels of pro-inflammatory cytokines, apoptosis and ROS. Therefore, fisetin is a promising therapeutic strategy for the treatment of acute otitis media.

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Availability of data and material

Data can be available on the request.

Authors' contributions

PL, DC and YH designed and performed the experiments. PL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This work was approved by the Department of Otorhinolaryngology, The First Affiliated Hospital of Jinan University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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