



Quercetin ameliorates lipopolysaccharide-caused inflammatory damage via down-regulation of miR-221 in WI-38 cells

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ABSTRACT

Background: Pneumonia is a common respiratory disease in pediatrics. Quercetin is a natural flavonoid widely distributed in many foods and drinks. Herein, we focused our investigation on the possible protective activity of quercetin in lipopolysaccharide (LPS)-caused inflammatory damage of WI-38 lung fibroblasts.

Methods: Viability and apoptosis of WI-38 were respectively tested using CCK-8 assay and Annexin V-FITC/PI staining. qRT-PCR was used to measure the expression levels of microRNA-221 (miR-221), IL-6 and TNF- α in WI-38. ELISA was conducted to determine the concentrations of IL-6 and TNF- α in culture supernatant of WI-38. miR-221 mimic was transfected to increase miR-221 expression. The protein levels of key molecules involving in cell apoptosis, inflammation, NF- κ B and JNK pathways were assessed using western blotting.

Results: LPS stimulation caused inflammatory damage of WI-38 lung fibroblasts via suppressing cell viability, inducing cell apoptosis and enhancing the production of inflammatory cytokines IL-6 and TNF- α . Quercetin treatment mitigated the LPS-caused inflammatory damage of WI-38 lung fibroblasts via enhancing cell viability, inhibiting cell apoptosis and reducing the production of inflammatory cytokines IL-6 and TNF- α . Moreover, quercetin ameliorated LPS-caused up-regulation of miR-221 in WI-38. The effects of quercetin on LPS-caused inflammatory damage of WI-38 were reversed by miR-221 overexpression. Furthermore, quercetin inactivated NF- κ B and JNK pathways in LPS-treated WI-38 via down-regulation of miR-221.

Conclusion: This research verified the protective effects of quercetin on lung fibroblasts inflammatory damage. We revealed that quercetin ameliorated LPS-caused inflammatory damage of WI-38 lung fibroblasts might be through down-regulation of miR-221 and inactivation of NF- κ B and JNK pathways.

1. Introduction

Respiratory diseases are common in pediatrics (Lichenstein et al., 2003). Pneumonia, a serious respiratory disease, is the main reason for the dying of infants and children all around the world (Walker et al., 2013). Its main clinical manifestations include fever, cough, dyspnea, somnolence, loss of appetite, and even breathing disorder and heart failure (Hunter et al., 2016; Lee et al., 2015). Bacteria, viruses, fungi and parasites are potential pathogens for pneumonia (Hooven and Polin, 2017). Antibiotics treatment is the most common therapeutic strategy for pneumonia (Shah et al., 2016). However, the use of antibiotics has considerable negative effects on patient's respiratory system and immune system (Russell et al., 2016). Due to inflammatory damage of lung fibroblasts has been demonstrated to be implicated in the occurrence and development of pneumonia (Amenomori et al., 2010), it is worthy believing that searching for other compounds that can alleviate

inflammatory damage of lung fibroblasts may provide more safe and effective medicines for pneumonia treatment.

Quercetin (CAS number: 117–39-5) is a natural flavonoid widely distributed in many dietary foods (Kitson and Kitson, 2000). During the last few years, pharmacological researches have proved that quercetin possesses a number of biological beneficial activities, including anti-cancer (Massi et al., 2017), anti-inflammation (Li et al., 2016c), immune-modulation (Kobori et al., 2016), neuro-protection (Costa et al., 2016). The application of quercetin in the treatment of diabetes (Eid and Haddad, 2017), obesity (Seo et al., 2015) and cardiovascular diseases (Gormaz et al., 2015) have also been proposed. Taslidere et al. reported that quercetin could exert positive effects on the treatment of pulmonary diseases characterized by edema, inflammation and fibrosis (Taslidere et al., 2014). Takashima et al. and Huang et al. indicated that quercetin could alleviate lipopolysaccharide (LPS)-caused pulmonary damage in mice and rats (Huang et al., 2015; Takashima et al., 2014).

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However, the specific effect of quercetin on inflammatory damage of lung fibroblasts in pneumonia still remains unclear and waits studying.

As a group of regulatory RNAs in cells, microRNAs (miRNAs) have been demonstrated to participate in the inflammatory regulation of multiple cells, including inflammatory damage of lung fibroblasts in pneumonia (McMillan et al., 2013; Sonkoly et al., 2008). miRNA-221 (miR-221) is a well-known pro-inflammatory RNA in cells, that can promote inflammatory cytokines expression by activating the inflammation-related signaling pathways, such as Nuclear factor-kappa B (NF- κ B) and c-Jun N-terminal kinase (JNK) pathways (Qian et al., 2017; Zhao et al., 2016).

In the current research, human embryonic lung fibroblasts WI-38 were treated by LPS to simulate inflammatory damage of lung fibroblasts in pneumonia. Then, we focused our investigation on the possible protective activity of quercetin on LPS-caused inflammatory damage of WI-38 lung fibroblasts, testing the roles of miR-221, NF- κ B pathway and JNK pathway. The findings of our research will have great implication for understanding the anti-inflammatory roles of quercetin in lung fibroblasts damage in pneumonia.

2. Materials and methods

2.1. Cell culture and treatment

Human embryonic lung fibroblasts WI-38 were obtained from Cell Bank of Chinese Academy of Science (Shanghai, China) and cultured in Minimum Essential Medium (MEM, Gibco, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% (v/v) penicillin-streptomycin (Sigma-Aldrich, St Louis, MO, USA) in 75 cm² flask (Corning Incorporation, New York, NY, USA). Flask was placed in a humidity incubator (Sanyo, Jencons, United Kingdom) at 37 °C with 5% CO₂.

LPS was purchased from Sigma-Aldrich (catalog number: L2630) and dissolved into ultrapure water to a storage concentration of 5 mg/ml. WI-38 cells were treated by 2.5–10 μ g/ml LPS for 6 h in this research.

Quercetin was also purchased from Sigma-Aldrich (catalog number: Q4951) and dissolved into dimethyl sulfoxide (DMSO) to a storage concentration of 100 mM. WI-38 cells were pre-incubated by 10–50 μ M quercetin for 24 h before LPS treatment. The chemical skeleton structure of quercetin was displayed in Fig. 1.

2.2. miRNA transfection

miR-221 mimic and its negative control (NC mimic) were designed and synthesized by GenePharma Corporation (Shanghai, China). The sequences of miR-221 mimic were: 5'-ACCUGGCAUACAAUGUAGA UUU-3' (sense) and 5'-AUCUACAUUGU AUGCCAGGUUU-3' (anti-sense). miR-221 mimic or NC mimic was transfected into WI-38 cells with the help of lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). Transfection efficiency was verified using quantitative reverse transcription PCR (qRT-PCR).

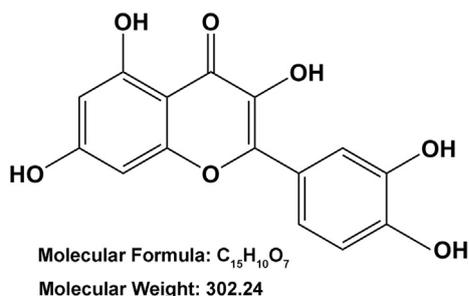


Fig. 1. The chemical skeleton structure of quercetin.

2.3. qRT-PCR

Total RNAs in WI-38 cells were isolated using TRIzol™ Plus RNA Purification kit (Invitrogen). *mirVana*™ qRT-PCR miRNA Detection kit (Invitrogen) was conducted to measure the expression level of miR-221 and the expression level of U6 acted as internal control. SuperScript™ IV One-Step RT-PCR system (Invitrogen) was conducted to measure the expression levels of interleukin 6 (IL-6) and tumor necrosis factor α (TNF- α). The expression level of β -actin acted as internal control. The primer sequences for miR-221 were: 5'-TGCGGAGCTACATTGTCTGC TGG-3' (Forward) and 5'-CCAGTGCAGGGTCCGAGGT-3' (Reverse). The primer sequences for U6 were: 5'-GCTTCGGCAGCACATATACTAA AAT-3' (Forward) and 5'-CGCTTCACG AATTTGCGTGTGCAT-3' (Reverse). The primer sequences for IL-6 were: 5'-AC AGCCACTCACC TCTTCAG-3' (Forward) and 5'-CCATCTTTTTCAGCCATCT TT-3' (Reverse). The primer sequences for TNF- α were: 5'-CCCAGGTGACA AGCCTGTAG-3' (Forward) and 5'-GATGGCAGAGAGAGAGGGTT GAC-3' (Reverse). The primer sequences for β -actin were: 5'-GCACC ACA CTCTACAATG-3' (Forward) and 5'-TGCTTGCTGA TCCACAT CTG-3' (Reverse). Data were quantified using 2^{- $\Delta\Delta$ Ct} method (Ish-Shalom and Lichter, 2010).

2.4. Cell counting kit-8 (CCK-8) assay

Viability of WI-38 cells was detected with the help of CCK-8 assay. Briefly, transfected or non-transfected WI-38 cells were seeded into 96-well plate (Corning Incorporation) with 5 \times 10³ cells per well and treated by LPS and/or quercetin. Then, 10 μ l CCK-8 solution was added into the culture medium of each well and the plate was placed at 37 °C in incubator for 1 h. Subsequently, the absorbance of each well at 450 nm was measured by Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). Cell viability (%) was calculated by mean absorbance of treatment (transfection) group/mean absorbance of control group \times 100%.

2.5. Cell apoptosis assay

Apoptosis of WI-38 cells was assessed with the help of Annexin V-FITC/PI apoptosis detection kit (Yeasen Biotechnology, Shanghai, China). Briefly, transfected or non-transfected WI-38 cells were seeded into 6-well plate (Corning Incorporation) with 1 \times 10⁵ cells per well and treated by LPS and/or quercetin. Then, cells in each group were collected and disposed following steps: washed with phosphate buffer saline (PBS) for three times, stained using 5 μ l Annexin V-FITC solution and 10 μ l PI solution for 20 min at room temperature in the dark, washed with PBS for three times and subjected to flow cytometry analysis (Beckman Coulter, Fullerton, CA, USA). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

2.6. Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to test the concentrations of IL-6 and TNF- α in culture supernatant of WI-38 cells. Briefly, transfected or non-transfected WI-38 cells were seeded into 6-well plate with 1 \times 10⁵ cells per well and treated by LPS and/or quercetin. Then, culture supernatant of each group was collected. The concentrations of IL-6 and TNF- α in culture supernatant were tested using Human IL-6 ELISA kit and Human TNF- α ELISA kit (Beyotime Biotechnology, Shanghai, China), respectively.

2.7. Western blotting

After different treatment and/or transfection, total proteins in WI-38 cells were extracted using RIPA Lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) and quantified with the help of BCA Protein assay (Thermo Fisher Scientific). Western blotting was

performed as previously described (Zhang et al., 2018). The following antibodies were used: Pro-caspase 3 (ab32150), Cleaved-caspase 3 (ab2302), Bcl-2 (ab32124), Bax (ab53154), IL-6 (ab6672), TNF- α (ab220210), I κ B α (ab7217), phosphorylated-I κ B α (ab133462), p65 (ab16502), phosphorylated-p65 (ab86299), JNK (ab179461), phosphorylated-JNK (ab124956), β -actin (ab8226), Goat Anti-Rabbit IgG H & L (ab205718) and Goat Anti-Mouse IgG H&L (ab205719, Abcam Biotechnology, Cambridge, MA, USA). The signals of proteins were captured using Bio-Rad ChemiDoc™ XRS system (Bio-Rad Laboratories, Hercules, CA, USA). The intensities of bands were analyzed by using Image Lab™ software (Bio-Rad Laboratories).

2.8. Statistical analysis

All experiments were repeated three times in triplicate. Results of multiple experiments were expressed as mean \pm standard deviation (SD). Graphpad 6.0 software was used for statistical analysis. *P*-values were calculated using one-way analysis of variance (ANOVA). *P* < .05 was considered to be significant difference.

3. Results

3.1. LPS caused inflammatory damage of WI-38 lung fibroblasts

Firstly, the viability, apoptosis and inflammatory cytokines expression of WI-38 lung fibroblasts after LPS treatment were detected. Results in Fig. 2A showed that 2.5–10 μ g/ml LPS treatment significantly suppressed the viability of WI-38 in a concentration-dependent manner (*P* < .05, *P* < .01 or *P* < .001). 5 μ g/ml LPS stimulation reduced the viability of WI-38 to 50.4 \pm 2.63%, which was chosen for further experiments. Fig. 2B displayed that 5 μ g/ml LPS treatment obviously promoted WI-38 apoptosis (*P* < .01). The expression levels of pro-apoptotic proteins, Cleaved-caspase 3 and Bax, were both increased in WI-38 after 5 μ g/ml LPS treatment (Fig. 2C). The expression level of anti-apoptotic protein Bcl-2 was decreased in WI-38 after 5 μ g/ml LPS

treatment. Moreover, Fig. 2D and E presented that 5 μ g/ml LPS stimulation enhanced the mRNA and protein expression levels of IL-6 and TNF- α in WI-38 (*P* < .01 or *P* < .001 in mRNA level). Fig. 2F illustrated that the concentrations of IL-6 and TNF- α in culture supernatant of WI-38 were also increased after 5 μ g/ml LPS treatment (*P* < .01 or *P* < .001). These above results suggested that LPS could cause inflammatory damage of WI-38 lung fibroblasts via inhibiting cell viability, promoting cell apoptosis and enhancing production of inflammatory cytokines.

3.2. Quercetin mitigated LPS-caused inflammatory damage of WI-38 lung fibroblasts

Next, the possible protective effects of quercetin on LPS-caused inflammatory damage of WI-38 lung fibroblasts were assessed. Fig. 3A displayed that 10–50 μ M quercetin incubation had no significant effects on WI-38 viability. The results in Fig. 3B presented that 30–50 μ M quercetin pre-incubation remarkably mitigated the LPS-caused WI-38 viability inhibition (*P* < .05 or *P* < .01). 40 μ M quercetin pre-incubation was selected for further experiments. Fig. 3C showed that 40 μ M quercetin pre-incubation notably attenuated the LPS-caused apoptosis of WI-38 (*P* < .05). Compared to LPS group, the protein expression levels of Cleaved-caspase 3 and Bax in WI-38 were both decreased, as well as the protein expression level of Bcl-2 was increased in LPS + quercetin group (Fig. 3D). In addition, the LPS-caused enhancement of mRNA and protein expression levels of IL-6 and TNF- α in WI-38 were both alleviated by 40 μ M quercetin pre-incubation (Fig. 3E and F, *P* < .05 or *P* < .01 in mRNA level). Fig. 3G illustrated that compared to LPS group, the concentrations of IL-6 and TNF- α in culture supernatant of WI-38 were both reduced in LPS + quercetin group (*P* < .05 or *P* < .01). These above findings indicated that quercetin could mitigate LPS-caused inflammatory damage of WI-38 lung fibroblasts via enhancing cell viability, inhibiting cell apoptosis and reducing production of inflammatory cytokines.

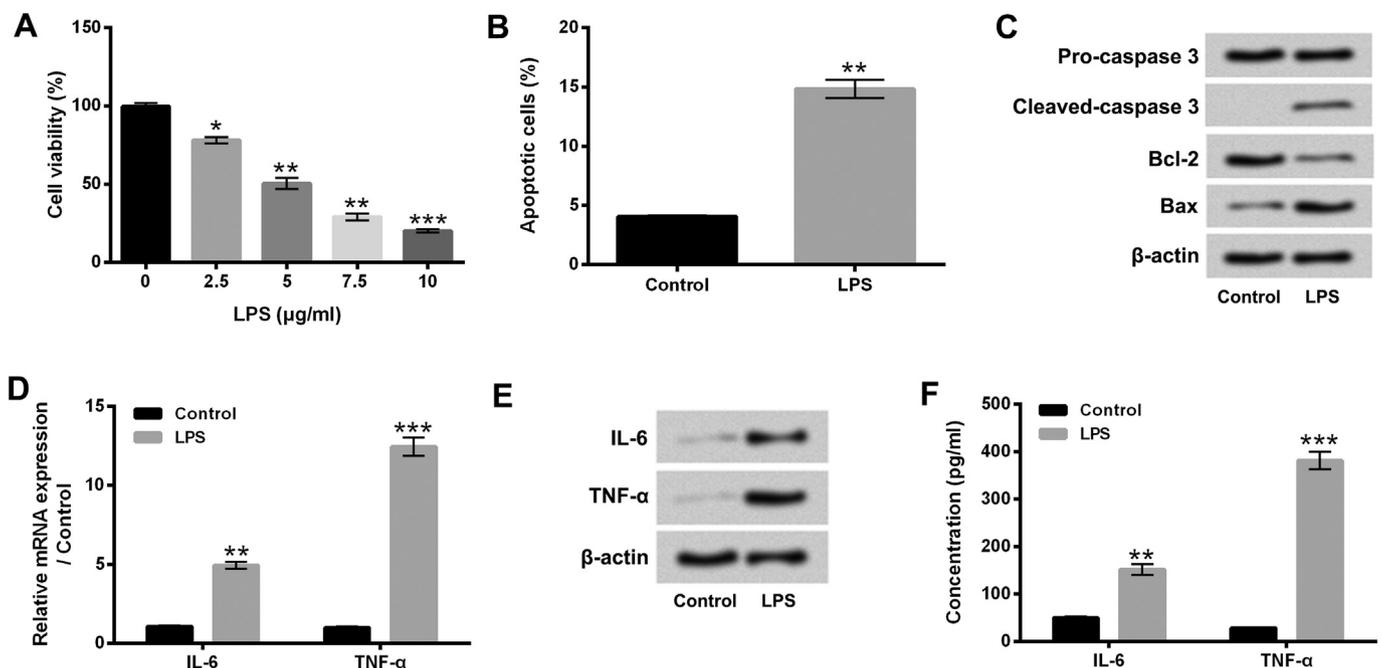


Fig. 2. LPS caused inflammatory damage of WI-38 lung fibroblasts. After 2.5–10 μ g/ml LPS treatment for 6 h, (A) CCK-8 assay was conducted to detect the viability of WI-38 cells. After 5 μ g/ml LPS treatment, (B) Annexin V-FITC/PI apoptosis detection kit was used for assessing the apoptosis of WI-38 cells; (C) the protein expression levels of Pro-caspase 3, Cleaved-caspase 3, Bcl-2 and Bax in WI-38 cells were measured with the help of western blotting; (D and E) qRT-PCR and western blotting were performed to measure the mRNA and protein expression levels of IL-6 and TNF- α in WI-38 cells; (F) the concentrations of IL-6 and TNF- α in culture supernatant of WI-38 cells were determined with the help of ELISA. LPS: Lipopolysaccharide; IL-6: Interleukin 6; TNF- α : Tumor necrosis factor α . *N* = 3. **P* < .05; ***P* < .01; ****P* < .001.

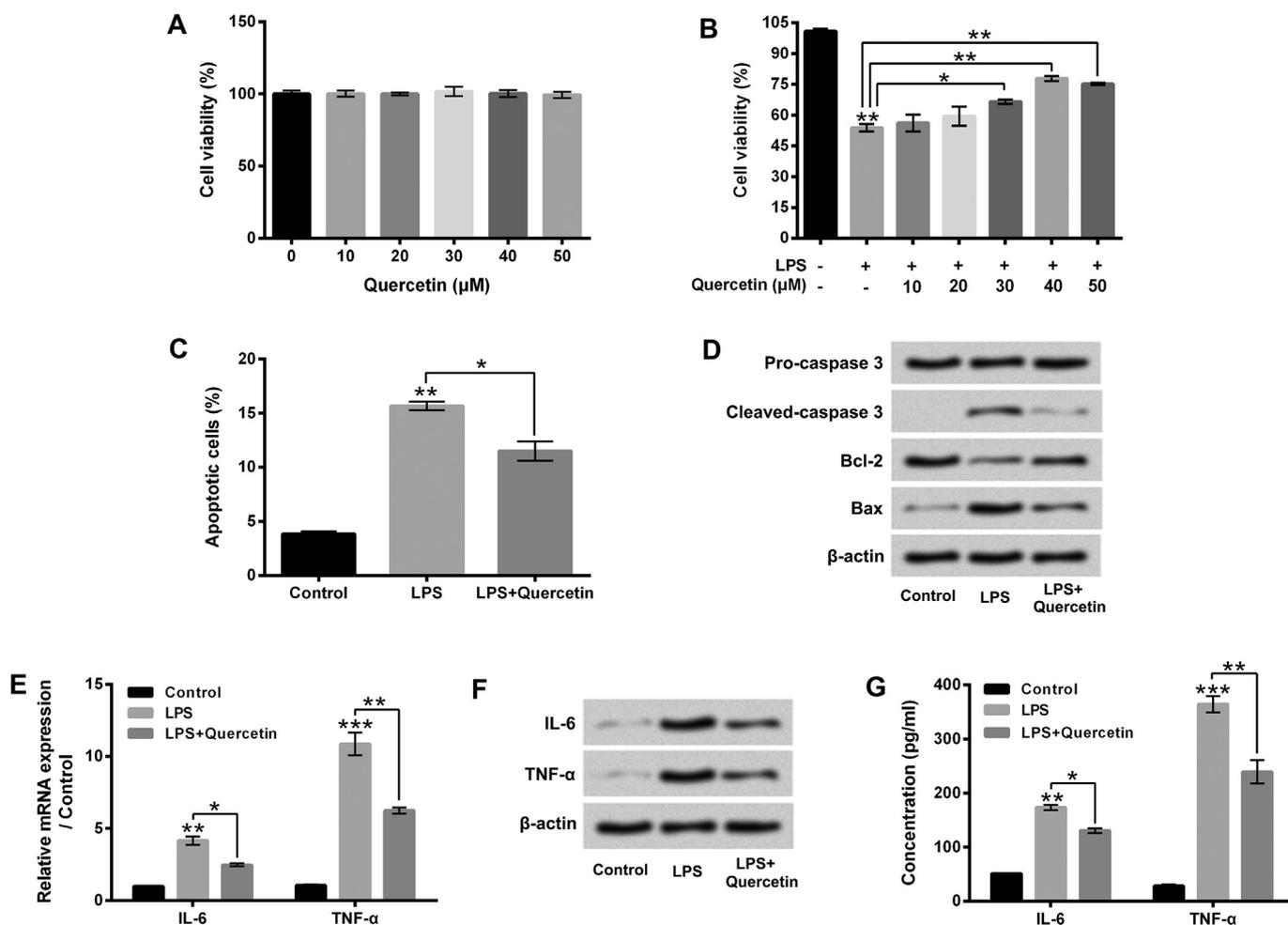


Fig. 3. Quercetin mitigated LPS-caused inflammatory damage of WI-38 lung fibroblasts. (A) After 10–50 μM quercetin treatment for 24 h, CCK-8 assay was conducted to detect the viability of WI-38 cells. (B) After 5 μg/ml LPS and/or 10–50 μM quercetin treatment, CCK-8 assay was conducted to detect the viability of WI-38 cells. After 5 μg/ml LPS and/or 40 μM quercetin treatment, (C) Annexin V-FITC/PI apoptosis detection kit was used for assessing the apoptosis of WI-38 cells; (D) the protein expression levels of Pro-caspase 3, Cleaved-caspase 3, Bcl-2 and Bax in WI-38 cells were measured with the help of western blotting; (E and F) qRT-PCR and western blotting were performed to measure the mRNA and protein expression levels of IL-6 and TNF-α in WI-38 cells; (G) the concentrations of IL-6 and TNF-α in culture supernatant of WI-38 cells were determined with the help of ELISA. LPS: Lipopolysaccharide; IL-6: Interleukin 6; TNF-α: Tumor necrosis factor α. N = 3. * $P < .05$; ** $P < .01$; *** $P < .001$.

3.3. Quercetin alleviated LPS-caused up-regulation of miR-221 expression in WI-38 cells

Then, we measured the expression level of miR-221 in WI-38 lung fibroblasts after LPS and/or quercetin treatment. As shown in Figs. 4, 5

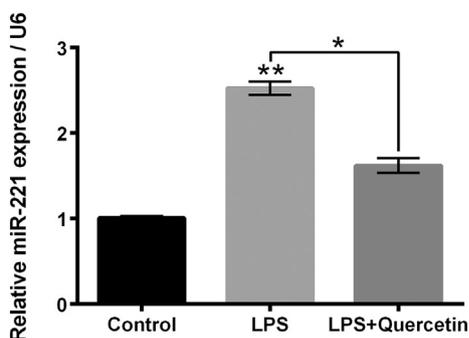


Fig. 4. Quercetin alleviated LPS-caused up-regulation of miR-221 expression in WI-38 cells. After 5 μg/ml LPS and/or 40 μM quercetin treatment, qRT-PCR was used for measuring the expression level of miR-221 in WI-38 cells. LPS: Lipopolysaccharide; miR-221: MicroRNA-221. N = 3. * $P < .05$; ** $P < .01$.

μg/ml LPS stimulation significantly up-regulated the expression level of miR-221 in WI-38 ($P < .01$), while 40 μM quercetin pre-incubation notably alleviated the LPS-caused up-regulation of miR-221 in WI-38 ($P < .05$). This finding implied that miR-221 might be involved in the protective effects of quercetin on LPS-caused inflammatory damage of WI-38 lung fibroblasts.

3.4. miR-221 was involved in the effects of quercetin on LPS-caused inflammatory damage of WI-38 lung fibroblasts

miR-221 mimic was transfected into WI-38 lung fibroblasts to enhance the expression level of miR-221 (Fig. 5A, $P < .01$). After that, the viability, apoptosis and inflammatory cytokines expression of WI-38 treated by LPS and/or quercetin were determined. Fig. 5B and C showed that miR-221 mimic transfection obviously reversed the protective effects of quercetin on LPS-caused WI-38 viability inhibition and cell apoptosis ($P < .05$). Compared to LPS + quercetin + NC mimic group, the protein expression levels of Cleaved-caspase 3 and Bax in WI-38 were increased, as well as the protein expression level of Bcl-2 in WI-38 was decreased in LPS + quercetin + miR-221 mimic group (Fig. 5D). Furthermore, the mRNA and protein expression levels of IL-6 and TNF-α in WI-38 were both increased in LPS + quercetin + miR-221 mimic

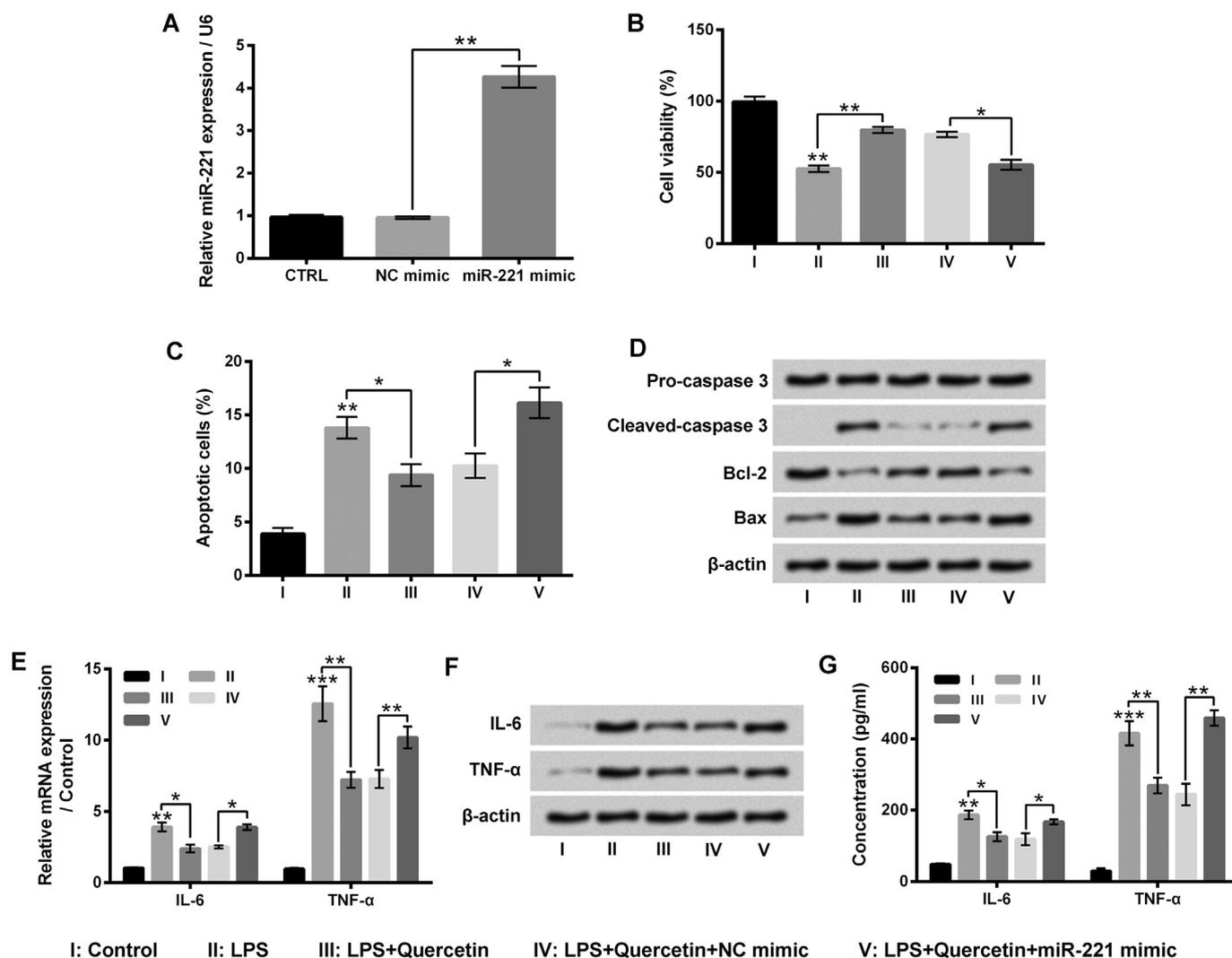


Fig. 5. miR-221 was involved in the effects of quercetin on LPS-caused inflammatory damage of WI-38 lung fibroblasts. (A) After NC mimic or miR-221 mimic transfection, the expression level of miR-221 in WI-38 cells was measured with the help of qRT-PCR. After 5 μ g/ml LPS and/or 40 μ M quercetin treatment or miR-221 mimic transfection, (B) CCK-8 assay was conducted to detect the viability of WI-38 cells; (C) Annexin V-FITC/PI apoptosis detection kit was used for assessing the apoptosis of WI-38 cells; (D) the protein expression levels of Pro-caspase 3, Cleaved-caspase 3, Bcl-2 and Bax in WI-38 cells were measured with the help of western blotting; (E and F) qRT-PCR and western blotting were performed to measure the mRNA and protein expression levels of IL-6 and TNF- α in WI-38 cells; (G) the concentrations of IL-6 and TNF- α in culture supernatant of WI-38 cells were determined with the help of ELISA. LPS: Lipopolysaccharide; miR-221: MicroRNA-221; IL-6: Interleukin 6; TNF- α : Tumor necrosis factor α . N = 3. * P < .05; ** P < .01; *** P < .001.

group, relative to LPS + quercetin + NC mimic group (Fig. 5E and F, P < .05 or P < .01 in mRNA level). Fig. 5G presented that the concentrations of IL-6 and TNF- α in culture supernatant of WI-38 were also enhanced in LPS + quercetin + miR-221 mimic group, relative to LPS + quercetin + NC mimic group (P < .05 or P < .01). Taken together, these above findings indicated that quercetin mitigated LPS-caused inflammatory damage of WI-38 lung fibroblasts might be through down-regulation of miR-221.

3.5. Quercetin inactivated NF- κ B and JNK pathways in LPS-treated WI-38 cells via down-regulation of miR-221

NF- κ B and JNK pathways are two important signaling pathways in cells that participate in the regulation of inflammatory response (Li et al., 2016b; Tak and Firestein, 2001). Finally, the effects of LPS, quercetin and miR-221 on NF- κ B and JNK pathways in WI-38 lung fibroblasts were evaluated. Fig. 6A and B presented that LPS stimulation remarkably activated NF- κ B and JNK pathways in WI-38 lung fibroblasts by up-regulating the expression levels of phosphorylated-I κ B α , phosphorylated-p65 and phosphorylated-JNK (P < .01). Quercetin

pre-incubation notably mitigated the LPS-caused activation of NF- κ B and JNK pathways in WI-38 lung fibroblasts by down-regulating the expression levels of rates of phosphorylated-I κ B α , phosphorylated-p65 and phosphorylated-JNK (P < .05). Moreover, miR-221 mimic transfection obviously alleviated the effects of quercetin on LPS-caused activation of NF- κ B and JNK pathways in WI-38 lung fibroblasts (P < .05). These findings suggested that quercetin could inactivate NF- κ B and JNK pathways in LPS-treated WI-38 cells via down-regulation of miR-221.

4. Discussion

Pneumonia is a common respiratory disease in pediatrics with high fatality rate (O'Brien et al., 2009; Walker et al., 2013). Searching for safe and effective medicines for pneumonia treatment has become one of the most important research subjects for biological and medical researchers worldwide (Rello and Perez, 2016). Herein, LPS was used to damage human embryonic WI-38 lung fibroblasts to simulate lung fibroblasts inflammatory damage in pneumonia and we revealed that quercetin could mitigate the LPS-caused inflammatory damage of WI-

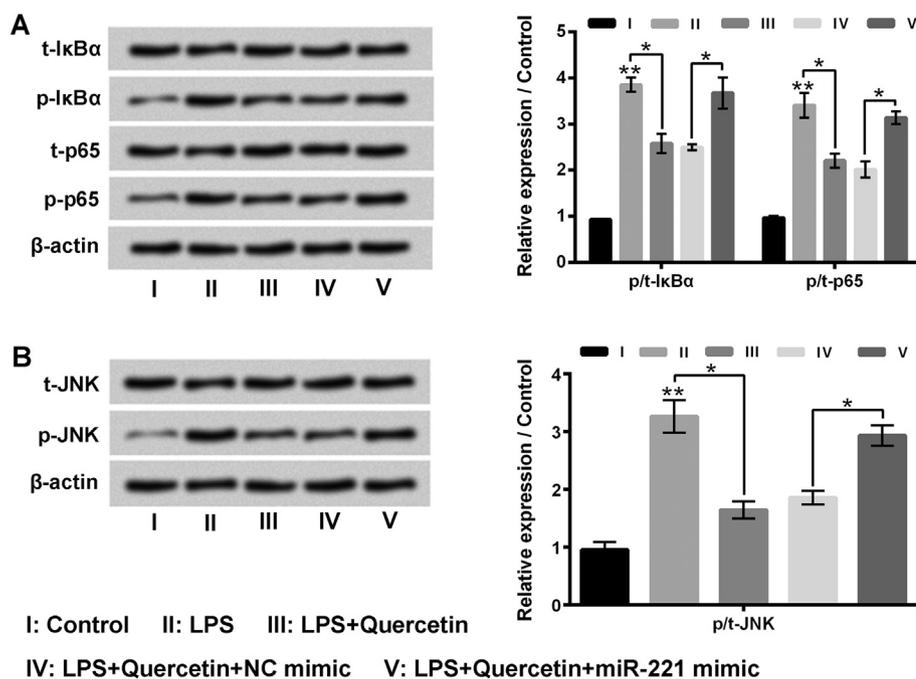


Fig. 6. Quercetin inactivated NF- κ B and JNK pathways in LPS-treated WI-38 cells via down-regulation of miR-221. (A and B) After 5 μ g/ml LPS and/or 40 μ M quercetin treatment or miR-221 mimic transfection, western blotting was used for measuring the protein expression levels of t-I κ B α , p-I κ B α , t-p65, p-p65, t-JNK and p-JNK in WI-38 cells. LPS: Lipopolysaccharide; miR-221: MicroRNA-221; NF- κ B: Nuclear factor-kappa B; JNK: c-Jun N-terminal kinase; I κ B α : NF- κ B inhibitor alpha. N = 3. * P < .05; ** P < .01.

38 lung fibroblasts. More importantly, we found that miR-221 played critical roles in the protective effects of quercetin on LPS-caused inflammatory damage of WI-38 lung fibroblasts. Besides, quercetin could inactivate NF- κ B and JNK pathways in LPS-treated WI-38 lung fibroblasts via down-regulation of miR-221.

Previous study reported that LPS-caused inflammatory damage of lung fibroblasts could be used as a cell-model of pneumonia (Liu et al., 2018). As a common stimulator of cell inflammatory response, LPS can suppress lung fibroblasts proliferation and induced inflammatory cytokines production (Zhang et al., 2011). In this research, we found that LPS stimulation significantly suppressed WI-38 cell viability, induced cell apoptosis and promoted the production of inflammatory cytokines IL-6 and TNF- α , which suggested that the inflammatory damage model of lung fibroblasts caused by LPS stimulation was established successfully and could be used to test the possible protective effects of quercetin.

A number of plant-derived medicines, such as artemisinin (Tu, 2016), berberine (Kumar et al., 2015) and vincristine (Sugalski et al., 2016), have made their own contribution on the treatment of many human diseases with high safety and efficient. Quercetin is one of the most important dietary flavonoids in many foods and drinks (Kitson and Kitson, 2000). It may be a mediator of the biological beneficial actions attributed to healthy diets (Gormaz et al., 2015). Previous studies proved that quercetin could exert protective activity on pulmonary diseases characterized by inflammation and attenuate LPS-caused pulmonary inflammatory damage in mice and rats (Huang et al., 2015; Takashima et al., 2014; Taslidere et al., 2014). In consistent with the previous studies, we discovered that quercetin treatment notably mitigated the LPS-caused inflammatory damage of WI-38 lung fibroblasts via enhancing cell viability, reducing cell apoptosis and inhibiting production of inflammatory cytokines IL-6 and TNF- α , which indicated that quercetin also could exert protective activity on inflammatory damage of lung fibroblasts. We proposed that quercetin might be as a novel therapeutic medicine for pneumonia.

miRNAs are small and non-coding RNA molecules that have been linked to many human diseases, including pneumonia (Abd-El-Fattah et al., 2013; Sonkoly et al., 2008). Increasing numbers of experimental and clinical investigations pointed out that miRNAs could serve as molecular targets for the diagnosis and treatment of multiple human diseases (Barwari et al., 2016; Shin and Chu, 2014). One of the most

important findings in this research was that miR-221 participated in the protective effects of quercetin on LPS-caused inflammatory damage of WI-38 lung fibroblasts. miR-221 has been demonstrated to play pro-inflammatory roles in a series of human diseases by promoting production of inflammatory cytokines (Chen et al., 2015; Qian et al., 2017). In the current study, we found that LPS stimulation obviously up-regulated the expression level of miR-221 in WI-38 lung fibroblasts, while quercetin treatment notably alleviated the LPS-caused up-regulation of miR-221 in WI-38 lung fibroblasts. More importantly, we discovered that the protective effects of quercetin on LPS-caused inflammatory damage of WI-38 lung fibroblasts were remarkably reversed by overexpression of miR-221, which suggested that miR-221 participated in the protective effects of quercetin on inflammatory damage of lung fibroblasts caused by LPS. Previous studies reported that quercetin could regulated the expression of multiple miRNAs, including miR-21, miR-146, miR-200b, miR-27a, miR-125b, miR let-7c, miR-181c, miR-155, miR-122, miR-217, miR-145, miR-16, miR-503, miR-546, miR-1283, miR-3717 and miR-6767 in different cells (Boesch-Saadatmandi et al., 2011; Chuammitri et al., 2017; Joven et al., 2012; Li et al., 2014; Nwaeburu et al., 2017; Nwaeburu et al., 2016; Park et al., 2019; Sonoki et al., 2015; Tao et al., 2015; Wein et al., 2015; Zhang et al., 2016a; Zhang et al., 2015; Zhou et al., 2015). Among them, miR-21, miR-200b, miR-125b, miR-181c, miR-122 and miR-145 have been found to exert anti-inflammatory roles (Diao et al., 2017; Ge et al., 2016; Li et al., 2016a; Matsui et al., 2018; Noh et al., 2017; Yuan et al., 2017), while miR-146, miR-27a and miR-155 have been found to exert pro-inflammatory roles in cells (Ammari et al., 2018; Wang et al., 2017; Wang et al., 2014). We propose that quercetin perhaps participate in the regulation of multiple miRNAs in WI-38 lung fibroblasts, which may form a very complex network. Further experiments are still needed to explore whether other miRNAs are also involved in the effects of quercetin on LPS-caused inflammatory damage of WI-38 lung fibroblasts.

NF- κ B and JNK pathways have been found to play critical regulatory roles in cell inflammatory response (Li et al., 2016b; Tak and Firestein, 2001). Moreover, the activation of NF- κ B and JNK pathways has been demonstrated to contribute to pro-inflammatory activity of miR-221 in cells (Qian et al., 2017; Zhao et al., 2016). Zhang et al. reported that quercetin could ameliorate LPS-caused inflammatory damage of human peripheral blood mononuclear cells by inhibition of NF- κ B pathway

(Zhang et al., 2016b). Park et al. indicated that quercetin-3-O- β -D-glucuronide, a glycoside derivative of quercetin, could inhibit LPS-caused activation of JNK pathway in macrophage RAW264.7 (Park et al., 2016). So, in the present research, we also investigated the effects of LPS, quercetin and miR-221 on NF- κ B and JNK pathways in WI-38 lung fibroblasts. We found that LPS stimulation dramatically activated NF- κ B and JNK pathways in WI-38 lung fibroblasts, while quercetin treatment mitigated the LPS-caused activation of NF- κ B and JNK pathways in WI-38 lung fibroblasts. Besides, overexpression of miR-221 alleviated the effects of quercetin on LPS-caused activation of NF- κ B and JNK pathways in WI-38 lung fibroblasts. These findings implied that quercetin exerted protective effects on LPS-caused inflammatory damage of WI-38 lung fibroblasts might be via down-regulating miR-221 and then inactivating NF- κ B and JNK pathways.

Previous study reported that after absorption, quercetin becomes metabolized in various organs including the small intestine, colon, liver and kidney (Li et al., 2016c). Metabolites formed in the small intestine and liver by biotransformation enzymes include the methylated, sulfo-substituted and glucuronidated forms (Day et al., 2000). A study regarding the tissues distribution in rats and pigs has shown that the highest accumulation of quercetin and its metabolites are found in (rat) lung and (pig) liver and kidney (de Boer et al., 2005). Continuous intake of diet containing quercetin accumulated in blood and significantly increased quercetin concentration in plasma, which was notably correlated to its dietary content (Koli et al., 2010). Isorhamnetin and glucoside acid-sulfated derivatives of quercetin account for 91.5% of its metabolites (Morand et al., 1998). These above findings suggested that quercetin could be metabolized in many positions of body after absorption. Further experiments are still needed to explore the effects of metabolized quercetin on LPS-caused inflammatory damage of WI-38 lung fibroblasts in the future, which will be helpful for its potential application as a pharmacological drug.

5. Conclusions

In conclusion, this research verified the protective effects of quercetin on inflammatory damage of lung fibroblasts. Quercetin ameliorated LPS-caused inflammatory damage of WI-38 lung fibroblasts might be through down-regulation of miR-221 and inactivation of NF- κ B and JNK pathways. The findings of our research offer experimental evidences for understanding the anti-inflammatory roles of quercetin in lung fibroblasts damage in pneumonia. Quercetin may be as a novel therapeutic medicine for pneumonia, in spite of further safety evaluation, metabolism analysis and clinical experiments are still needed.

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Conflict of interest

The authors declare no conflict of interest.

References

Abd-El-Fattah, A.A., et al., 2013. Differential microRNAs expression in serum of patients with lung cancer, pulmonary tuberculosis, and pneumonia. *Cell Biochem. Biophys.* 67, 875–884.

Amenomori, M., et al., 2010. HSP47 in lung fibroblasts is a predictor of survival in fibrotic nonspecific interstitial pneumonia. *Respir. Med.* 104, 895–901.

Ammari, M., et al., 2018. Delivery of miR-146a to Ly6C(high) monocytes inhibits pathogenic bone Erosion in inflammatory arthritis. *Theranostics.* 8, 5972–5985.

Barwari, T., et al., 2016. MicroRNAs in cardiovascular disease. *J. Am. Coll. Cardiol.* 68, 2577–2584.

Boesch-Saadatmandi, C., et al., 2011. Effect of quercetin and its metabolites isorhamnetin and quercetin-3-glucuronide on inflammatory gene expression: role of miR-155. *J. Nutr. Biochem.* 22, 293–299.

Chen, C.F., et al., 2015. MicroRNA-221 regulates endothelial nitric oxide production and inflammatory response by targeting adiponectin receptor 1. *Gene.* 565, 246–251.

Chuammitri, P., et al., 2017. The effects of quercetin on microRNA and inflammatory gene expression in lipopolysaccharide-stimulated bovine neutrophils. *Vet. World.* 10, 403–410.

Costa, L.G., et al., 2016. Mechanisms of Neuroprotection by Quercetin: counteracting oxidative stress and more. *Oxidative Med. Cell. Longev.* 2016 2986796.

Day, A.J., et al., 2000. Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radic. Biol. Med.* 29, 1234–1243.

de Boer, V.C., et al., 2005. Tissue distribution of quercetin in rats and pigs. *J. Nutr.* 135, 1718–1725.

Diao, W., et al., 2017. MicroRNA-125b-5p modulates the inflammatory state of macrophages via targeting B7-H4. *Biochem. Biophys. Res. Commun.* 491, 912–918.

Eid, H.M., Haddad, P.S., 2017. The Antidiabetic potential of Quercetin: underlying mechanisms. *Curr. Med. Chem.* 24, 355–364.

Ge, X., et al., 2016. miR-21-5p alleviates leakage of injured brain microvascular endothelial barrier in vitro through suppressing inflammation and apoptosis. *Brain Res.* 1650, 31–40.

Gormaz, J.G., et al., 2015. Cardiovascular disease: a target for the pharmacological effects of Quercetin. *Curr. Top. Med. Chem.* 15, 1735–1742.

Hooven, T.A., Polin, R.A., 2017. Pneumonia. *Semin. Fetal Neonatal Med.* 22, 206–213.

Huang, R., et al., 2015. Quercetin protects against lipopolysaccharide-induced acute lung injury in rats through suppression of inflammation and oxidative stress. *Arch. Med. Sci.* 11, 427–432.

Hunter, M., et al., 2016. Clinical manifestations of organizing pneumonia. *Medicina (B Aires).* 76, 338–342.

Ish-Shalom, S., Lichter, A., 2010. Analysis of fungal gene expression by real time quantitative PCR. *Methods Mol. Biol.* 638, 103–114.

Joven, J., et al., 2012. Plant-derived polyphenols regulate expression of miRNA paralogs miR-103/107 and miR-122 and prevent diet-induced fatty liver disease in hyperlipidemic mice. *Biochim. Biophys. Acta* 1820, 894–899.

Kitson, T.M., Kitson, K.E., 2000. The effect of quercetin, a widely distributed flavonoid in food and drink, on cytosolic aldehyde dehydrogenase: a comparison with the effect of diethylstilboestrol. *Biochim. Biophys. Acta* 1481, 247–254.

Kobori, M., et al., 2016. Quercetin suppresses immune cell accumulation and improves mitochondrial gene expression in adipose tissue of diet-induced obese mice. *Mol. Nutr. Food Res.* 60, 300–312.

Koli, R., et al., 2010. Bioavailability of various polyphenols from a diet containing moderate amounts of berries. *J. Agric. Food Chem.* 58, 3927–3932.

Kumar, A., et al., 2015. Current knowledge and pharmacological profile of berberine: an update. *Eur. J. Pharmacol.* 761, 288–297.

Lee, J.K., et al., 2015. Clinical manifestations of pneumonia according to the causative organism in patients in the intensive care unit. *Korean J. Intern. Med.* 30, 829–836.

Li, W., et al., 2014. Combination of quercetin and hyperoside has anticancer effects on renal cancer cells through inhibition of oncogenic microRNA-27a. *Oncol. Rep.* 31, 117–124.

Li, X., et al., 2016a. Exosome derived from human umbilical cord Mesenchymal stem cell mediates MiR-181c attenuating burn-induced excessive inflammation. *EBioMedicine.* 8, 72–82.

Li, X.M., et al., 2016b. Study on JNK/AP-1 signaling pathway of airway mucus hypersecretion of severe pneumonia under RSV infection. *Eur. Rev. Med. Pharmacol. Sci.* 20, 853–857.

Li, Y., et al., 2016c. Quercetin, inflammation and immunity. *Nutrients.* 8, 167.

Lichenstein, R., et al., 2003. Pediatric pneumonia. *Emerg. Med. Clin. North Am.* 21, 437–451.

Liu, M., et al., 2018. Long noncoding RNA HAGLROS regulates cell apoptosis and autophagy in lipopolysaccharides-induced WI-38 cells via modulating miR-100/NF- κ B axis. *Biochem. Biophys. Res. Commun.* 500, 589–596.

Massi, A., et al., 2017. Research Progress in the modification of Quercetin leading to anticancer agents. *Molecules.* 22.

Matsui, S., et al., 2018. MiR-200b attenuates IL-6 production through IKK β and ZEB1 in human gingival fibroblasts. *Inflamm. Res.* 67, 965–973.

McMillan, D.H., et al., 2013. Attenuation of inflammatory mediator production by the NF- κ B member RelB is mediated by microRNA-146a in lung fibroblasts. *Am. J. Phys. Lung Cell. Mol. Phys.* 304, L774–L781.

Morand, C., et al., 1998. Plasma metabolites of quercetin and their antioxidant properties. *Am. J. Phys.* 275, R212–R219.

Noh, K., et al., 2017. miR-122-SOCS1-JAK2 axis regulates allergic inflammation and allergic inflammation-promoted cellular interactions. *Oncotarget.* 8, 63155–63176.

Nwaeburu, C.C., et al., 2016. Up-regulation of microRNA let-7c by quercetin inhibits pancreatic cancer progression by activation of Numb. *Oncotarget.* 7, 58367–58380.

Nwaeburu, C.C., et al., 2017. Quercetin-induced miR-200b-3p regulates the mode of self-renewing divisions in pancreatic cancer. *Mol. Cancer* 16, 23.

O'Brien, K.L., et al., 2009. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet.* 374, 893–902.

Park, J.Y., et al., 2016. Quercetin-3-O-beta-D-Glucuronide suppresses lipopolysaccharide-induced JNK and ERK phosphorylation in LPS-challenged RAW264.7 cells. *Biomol. Ther. (Seoul).* 24, 610–615.

Park, S., et al., 2019. Quercetin inhibits proliferation of endometriosis regulating cyclin D1 and its target microRNAs in vitro and in vivo. *J. Nutr. Biochem.* 63, 87–100.

Qian, L.B., et al., 2017. Exacerbation of diabetic cardiac hypertrophy in OVE26 mice by

- angiotensin II is associated with JNK/c-Jun/miR-221-mediated autophagy inhibition. *Oncotarget*. 8, 106661–106671.
- Rello, J., Perez, A., 2016. Precision medicine for the treatment of severe pneumonia in intensive care. *Expert Rev. Respir. Med.* 10, 297–316.
- Russell, C.J., et al., 2016. The use of inhaled antibiotic therapy in the treatment of ventilator-associated pneumonia and tracheobronchitis: a systematic review. *BMC Pulm Med.* 16, 40.
- Seo, M.J., et al., 2015. The inhibitory effects of quercetin on obesity and obesity-induced inflammation by regulation of MAPK signaling. *J. Nutr. Biochem.* 26, 1308–1316.
- Shah, S.S., et al., 2016. Intravenous versus Oral antibiotics for Postdischarge treatment of complicated pneumonia. *Pediatrics*. 138.
- Shin, V.Y., Chu, K.M., 2014. MiRNA as potential biomarkers and therapeutic targets for gastric cancer. *World J. Gastroenterol.* 20, 10432–10439.
- Sonkoly, E., et al., 2008. MicroRNAs and immunity: novel players in the regulation of normal immune function and inflammation. *Semin. Cancer Biol.* 18, 131–140.
- Sonoki, H., et al., 2015. Quercetin decreases Claudin-2 expression mediated by up-regulation of microRNA miR-16 in lung adenocarcinoma A549 cells. *Nutrients*. 7, 4578–4592.
- Sugalski, J., et al., 2016. NCCN's commitment to medication safety: the vincristine initiative. *J. Natl. Compr. Cancer Netw.* 14, 959–960.
- Tak, P.P., Firestein, G.S., 2001. NF-kappaB: a key role in inflammatory diseases. *J. Clin. Invest.* 107, 7–11.
- Takashima, K., et al., 2014. Protective effects of intratracheally administered quercetin on lipopolysaccharide-induced acute lung injury. *Respir. Res.* 15, 150.
- Tao, S.F., et al., 2015. Quercetin inhibits proliferation and invasion acts by up-regulating miR-146a in human breast cancer cells. *Mol. Cell. Biochem.* 402, 93–100.
- Taslidere, E., et al., 2014. Protective effects of melatonin and quercetin on experimental lung injury induced by carbon tetrachloride in rats. *Exp. Lung Res.* 40, 59–65.
- Tu, Y., 2016. Artemisinin-a gift from traditional Chinese medicine to the world (Nobel lecture). *Angew. Chem. Int. Ed. Eng.* 55, 10210–10226.
- Walker, C.L.F., et al., 2013. Global burden of childhood pneumonia and diarrhoea. *Lancet*. 381, 1405–1416.
- Wang, Z., et al., 2014. miR-27a is up regulated and promotes inflammatory response in sepsis. *Cell. Immunol.* 290, 190–195.
- Wang, C., et al., 2017. Macrophage-derived mir-155-containing Exosomes suppress fibroblast proliferation and promote fibroblast inflammation during cardiac injury. *Mol. Ther.* 25, 192–204.
- Wein, S.A., et al., 2015. Quercetin induces hepatic gamma-glutamyl hydrolase expression in rats by suppressing hepatic microRNA rno-miR-125b-3p. *J. Nutr. Biochem.* 26, 1660–1663.
- Yuan, M., et al., 2017. MiR-145-5p regulates hypoxia-induced inflammatory response and apoptosis in cardiomyocytes by targeting CD40. *Mol. Cell. Biochem.* 431, 123–131.
- Zhang, J., et al., 2011. Inhibited proliferation of human lung fibroblasts by LPS is through IL-6 and IL-8 release. *Cytokine*. 54, 289–295.
- Zhang, X., et al., 2015. Quercetin enhances Cisplatin sensitivity of human osteosarcoma cells by modulating microRNA-217-KRAS Axis. *Mol. Cell* 38, 638–642.
- Zhang, L., et al., 2016a. Hyperoside ameliorates glomerulosclerosis in diabetic nephropathy by downregulating miR-21. *Can. J. Physiol. Pharmacol.* 94, 1249–1256.
- Zhang, M., et al., 2016b. Quercetin ameliorates LPS-induced inflammation in human peripheral blood mononuclear cells by inhibition of the TLR2-NF-kappaB pathway. *Genet. Mol. Res.* 15.
- Zhang, W., et al., 2018. Propofol inhibits proliferation, migration and invasion of gastric cancer cells by up-regulating microRNA-195. *Int. J. Biol. Macromol.* 120, 975–984.
- Zhao, D., et al., 2016. MiR-221 activates the NF-kappaB pathway by targeting A20. *Biochem. Biophys. Res. Commun.* 472, 11–18.
- Zhou, J., et al., 2015. Quercetin induces the apoptosis of human ovarian carcinoma cells by upregulating the expression of microRNA-145. *Mol. Med. Rep.* 12, 3127–3131.