Isosinensetin alleviates the injury of human bronchial epithelial cells induced by $PM_{2.5}$

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Abstract. Flavonoids which are extracted from citrus peel and pulp have been reported to have multiple beneficial effects on human health. Isosinensetin (ISO) is a type of flavonoid compound, which has several protective effects including anticancer, antioxidant, antiviral, anti-inflammatory and bacteriostatic. However, the molecular mechanism of its antioxidant and anti-inflammatory effects remain unclear. The present study aimed to investigate the intervention effect and possible mechanism of ISO on human bronchial epithelial cells injured by fine particular matter ≤2.5 μm in diameter ($PM_{2.5}$). In the present study, the cell viability was detected by Cell Counting Kit-8 method. The levels of pro-inflammatory cytokines were analyzed by ELISA. The level of reactive oxygen species (ROS) was detected by fluorescence probe. The expression levels of proliferating cell nuclear antigen (PCNA), nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor κB (NF-κB) proteins were detected by western blotting. The results revealed that ISO evidently increased the viability of 16-HBE cells and sharply decreased the levels of pro-inflammatory factors in cell culture supernatant. ISO significantly inhibited ROS release caused by $PM_{2.5}$. Moreover, the expression levels of PCNA, Nrf2 and NF-κB proteins were downregulated after ISO incubation. These results indicated that ISO alleviated 16-HBE-cell injury by $PM_{2.5}$ through the ROS-Nrf2/NF-κB signaling pathway.

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Introduction

In recent years, environmental pollution has become a major threat to human health. Increased attention has been paid to the effect of environmental factors on the occurrence and development of respiratory diseases. Fine particular matter ≤2.5 μm in diameter ($PM_{2.5}$) is the main pollutant in the atmosphere. It refers to fine particulate matter with an aerodynamic diameter under 2.5 μm. $PM_{2.5}$ inhalation can induce respiratory tract injury directly and aggravate a variety of diseases (1,2). A large number of epidemiological studies have revealed that $PM_{2.5}$ exposure is closely related to the morbidity and mortality of cardiovascular and respiratory systems as well as other diseases, such as arterial embolism, asthma, pneumonia, and lung cancer (3,4).

Flavonoids are natural compounds with a wide range of molecular diversity. Fruits, vegetables, herbs and other plant foods are the sources of flavonoids (5). Flavonoids have received increasing attention due to their anti-inflammatory, antimicrobial and anticancer activities (6). The important and beneficial effect of flavonoids is antioxidant activity for human body health, which is related with scavenging of reactive oxygen species (ROS) (7). Flavonoids can adjust the expression of inflammatory mediators including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and cyclooxygenase-2 (COX-2) through regulation of the Toll receptor (TLR)/NF-κB axis (8). The large number of natural compounds containing flavonoids used to treat, manage, and prevent human diseases have been widely studied. A typical example is usage of licorice derived from the dried roots and rhizomes of the Glycyrrhiza species. Licorice has been used to treat diabetes, tuberculosis, and other inflammatory disorders (9). In addition, some flavonoids (quercetin, apigenin and luteolin) have been revealed to reduce the expression and secretion of cytokines (10). However, the anti-inflammatory effect of isosinensetin (ISO) has not been reported. Therefore, in the present study, the intervention effect and possible mechanism of ISO on human bronchial epithelial cells injured by $PM_{2.5}$ were investigated.

Materials and methods

Materials. The human bronchial epithelial cell line, 16-HBE, was obtained from the American Type Culture Collection

(ATCC). DMEM medium was purchased from Hyclone; Cytiva. Epithelial cell medium (ECM; cat. no. 4101) containing fetal bovine serum (FBS; cat. no. 0010) and epithelial cell growth supplements (ECGs; cat. no. 4152) were purchased from ScienCell Research Laboratories, Inc. Anti-proliferating cell nuclear antigen (PCNA; product no. 2586) antibody was purchased from Cell Signaling Technology, Inc. Anti-nuclear factor erythroid 2-related factor 2 (Nrf2; cat. no. sc-365949), anti-p65 (cat. no. sc-8008), anti-IκB kinase (IKK; cat. no. sc52932) and anti-actin (cat. no. sc-81178) antibodies were purchased from Santa Cruz Biotechnology, Inc. 2',7'-Dicholorofluorescein-diacetate (DCFH-DA) probe (product no. D6883) was obtained from Sigma-Aldrich; Merck KGaA. Cell Counting Kit-8 (CCK-8; cat. no. AR1199) was purchased from Wuhan Boster Biological Technology, Ltd. IL-6, IL-1β and TNF-α ELISA kits (cat. nos. BMS213-2, BMS224-2 and BMS223-4, respectively) were purchased from Thermo Fisher Scientific, Inc. N-acetyl-L-cysteine (NAC; product no. S0077), trypsin (product no. C0202), PBS solution (product no. C0221A), and RIPA lysis solution (product no. P0013B) were obtained from Shanghai Beyotime Institute of Biotechnolgy.

Drug. Isosinensetin (99.5% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP; Beijing, China). ISO (2 mg) was firstly dissolved in 0.1 ml absolute ethyl alcohol. The concentrations used were 0, 20, 40, 80 or 160 μg/ml and the drug was stored at 4˚C (Fig. 1; structure of ISO). NAC (2 mg) was dissolved in 2 ml sterile deionized water and stored at 4˚C.

$PM_{2.5}$ sample. The collection and preparation of $PM_{2.5}$ sample were conducted according to our previously reported methods (11). Briefly, samples were collected on nitrocellulose filters using a high-volume sampler particle collector in the center of Shenyang city. $PM_{2.5}$ was extracted from the nitrocellulose filters by immersing in deionized water and then sonicating for 30 min. The $PM_{2.5}$ sample was stored at -80˚C.

Cell culture. 16-HBE cells were cultured in ECM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% penicillin/ECGs at 37˚C in an atmosphere of 5% CO_2 and 95% air. The cells were routinely sub-cultured every 2-3 days and it was confirmed that all of the cells were in the logarithmic phase before subsequent experimentation.

Cell viability assay. 16-HBE cells were seeded at a density of $1x10^4$ cells/ml in a 96-well plate. When confluence reached 70-80%, the cells were treated with serum-free medium for 12 h at 37˚C. Subsequently, the cells were incubated with $PM_{2.5}$ solution or $PM_{2.5}$ solution plus ISO for 6, 12, or 24 h at 37˚C. The cell viability was assessed by the CCK-8 assay. A total of 10 μl of CCK-8 reagent was added to each well, and incubated at 37˚C for 1 h. The absorbance was measured at 540 nm using a microplate spectrophotometer (Thermo MK3; Thermo Fisher Scientific, Inc.).

Contents of pro-inflammatory factors determination. The concentrations of IL-1β, IL-6 or TNF-α were detected by ELISA kits. 16-HBE cells were seeded at a density of

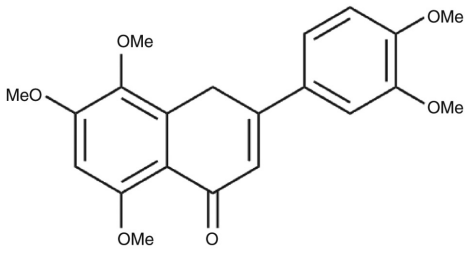

Figure 1. Structure of isosinensetin.

$2x10^5$ cells/ml in 6-well plates and cultured as aforementioned, followed by exposure to $PM_{2.5}$ solution or $PM_{2.5}$ solution plus ISO for 24 h. The supernatants were collected. Inflammatory factors were detected using IL-6, IL-1β and TNF-α ELISA kits (eBioscience; Thermo Fisher Scientific, Inc.) by measuring the absorbance at a 460-nm wavelength.

Assessment of ROS production. 16-HBE cells were cultured and treated as aforementioned. A DCFH-DA probe was used to determine the level of intracellular ROS production. A total of 10 μM DCFH-DA probe was added for 30 min at 37˚C in the dark. After incubation, the cells were washed three times with ice-cold PBS. The fluorescence was observed with an immunofluorescence microscope (excitation wavelength, 488 nm; emission wavelength, 525 nm) (Leica CTR 4000; Leica Microsystems GmbH).

Western blot analysis. The procedures and quantification methods were the same as those previously described (12). Briefly, the proteins in cell lysates were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidenefluoride (PVDF) membranes. The membranes were incubated with primary antibodies (anti-actin, 1:1,000 dilution; anti-PCNA, 1:1,000 dilution; anti-Nrf2, 1:800 dilution; anti-p65, 1:800 dilution; and anti-IKK, 1:800 dilution, respectively) for 12 h at 4˚C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution; cat. no. 62-6520; Pierce; Thermo Fisher Scientific, Inc.). The protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Amersham; Cytiva). The data were analyzed using the Quantity One software (v.4.6.7; Bio-Rad Laboratories, Inc.).

Statistical analysis. All data are presented as the mean ± standard deviation (SD) from three or more independent experiments. Differences between the groups were evaluated for significance by one-way ANOVA followed by Tukey's post hoc test for pairwise comparisons or by Dunnett's test for comparisons vs. a control, where appropriate. The statistical graphs were constructed using GraphPad Prism (version 5; GraphPad Software, Inc.) and photoshop (version 8.0; Adobe systems, Inc). $P<0.05$ was considered to indicate a statistically significant difference.

Results

$PM_{2.5}$ induces damage of human bronchial epithelial cells. A CCK-8 assay was used to assess the viability of 16-HBE cells

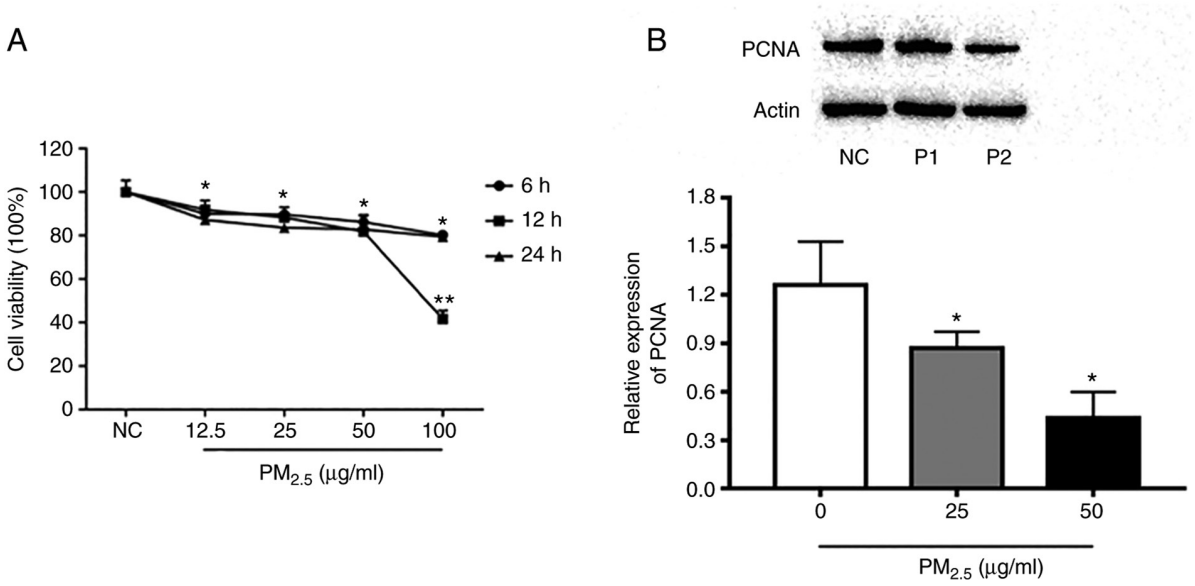

Figure 2. Exposure to $PM_{2.5}$ decreases cell viability in human bronchial epithelial cells. (A) 16-HBE cells were exposed to different doses of $PM_{2.5}$ for 6, 12 or 24 h respectively and the cell viability was detected by Cell Counting Kit-8 method. (B) 16-HBE cells were exposed to 25 or 50 μg/ml $PM_{2.5}$ for 24 h, and the intracellular proliferating cell nuclear antigen expression was detected by western blot assay. P1 indicates 25 μg/ml $PM_{2.5}$ solution treatment. P2 indicates 50 μg/ml $PM_{2.5}$ solution treatment. *P<0.05 and **P<0.01, compared to the NC group. $PM_{2.5}$, fine particular matter ≤2.5 μm in diameter; PCNA, proliferating cell nuclear antigen; NC, negative control.

in order to determine the effective dose of $PM_{2.5}$ on 16-HBE cells after $PM_{2.5}$ exposure for 6, 12 and 24 h, respectively. As revealed in Fig. 2, a dose greater than 25 μg/ml $PM_{2.5}$ posed a threat to cell viability of 16-HBE cells (P<0.05).

PCNA is a type of nucleoprotein, whose level can reflect the condition of cell growth and proliferation (13). Therefore, the expression of PCNA protein in 16-HBE cells was detected by western blotting. The results revealed that after exposure to 25 and 50 μg/ml $PM_{2.5}$, the expression of PCNA was significantly decreased compared with the control group of 16-HBE cells.

ISO increases cell viability of 16-HBE cells injured by $PM_{2.5}$. In order to investigate the role of ISO in alleviating $PM_{2.5}$-induced cell damage and select an optimal concentration to perform further experiments, the effects of various concentrations of ISO (0, 20, 40, 80 and 160 μg/ml) were investigated regarding the viability of 16-HBE cells exposed to $PM_{2.5}$ at 25 μg/ml which caused a 20-30%-decrease in cell viability (Fig. 3). The results indicated that the addition of ISO could attenuate the toxicity caused by $PM_{2.5}$ on 16-HBE cells. The optimum concentration observed in cells treated with ISO was 80 μg/ml. Compared with the cells exposed to $PM_{2.5}$ for 24h, ISO (80 μg/ml) significantly increased the expression of PCNA protein in 16-HBE cells.

ISO decreases the contents of pro-inflammatory factors. The concentrations of IL-1β, TNF-α and IL-6 were detected by ELISA kits. The concentrations of IL-1β, TNF-α and IL-6 in the $PM_{2.5}$ group were significantly higher compared with the negative control (NC) group (from 49.8±3.4, 79.8±3.9 and 62.5±3.3 to 25.6±1.8, 40.6±3.5 and 39.6±2.5, respectively). However, after treatment with 80 μg/ml ISO, the concentrations of IL-1β, TNF-α and IL-6 were decreased (36.3±2.1, 56.1±3.3 and 49.1±4.1, respectively) (Fig. 4) (P<0.05). These results indicated that ISO could alleviate the inflammatory effect induced by $PM_{2.5}$ in 16-HBE cells.

ISO inhibits the ROS generation induced by $PM_{2.5}$. ROS acts as a contributor to the toxicity of $PM_{2.5}$ and flavonoids are the antioxidants of nature (14). Fluorescence microscopy revealed that ISO significantly inhibited the ROS content caused by $PM_{2.5}$ (Fig. 5). The level of ROS was significantly decreased in the $PM_{2.5}$ + ISO co-incubation group in comparison with the $PM_{2.5}$ group. These data demonstrated that ISO played a restraining role against the toxic effect of $PM_{2.5}$ by reducing ROS.

ISO downregulates the expression of inflammatory-related proteins. The transcription factor NF-κB may be related with the anti-inflammatory properties of $PM_{2.5}$ exposure. The proteins p65 and IKK are the important subunits of the NF-κB family and their expression is correlated with NF-κB activity (15). Our results revealed that the expression levels of p65 and IKK in the $PM_{2.5}$ group were markedly higher than that in the NC group. However, the expression levels of p65 and IKK were significantly decreased after treatment with 80 μg/ml ISO.

Nrf2 protein is a critical signal molecule that regulates immunity and inflammation (16). The expression of Nrf2 is associated with inflammatory molecules. The results revealed that ISO downregulated the expression of Nrf2 protein. These data illustrated that ISO played a restraining role against the toxic effect of $PM_{2.5}$ through the ROS-Nrf2/NF-κB signaling pathway (Fig. 6).

Moreover, the results revealed after inhibiting ROS generation through ROS inhibitor NAC treatment, the expression levels of Nrf2, p65 and IKK proteins were

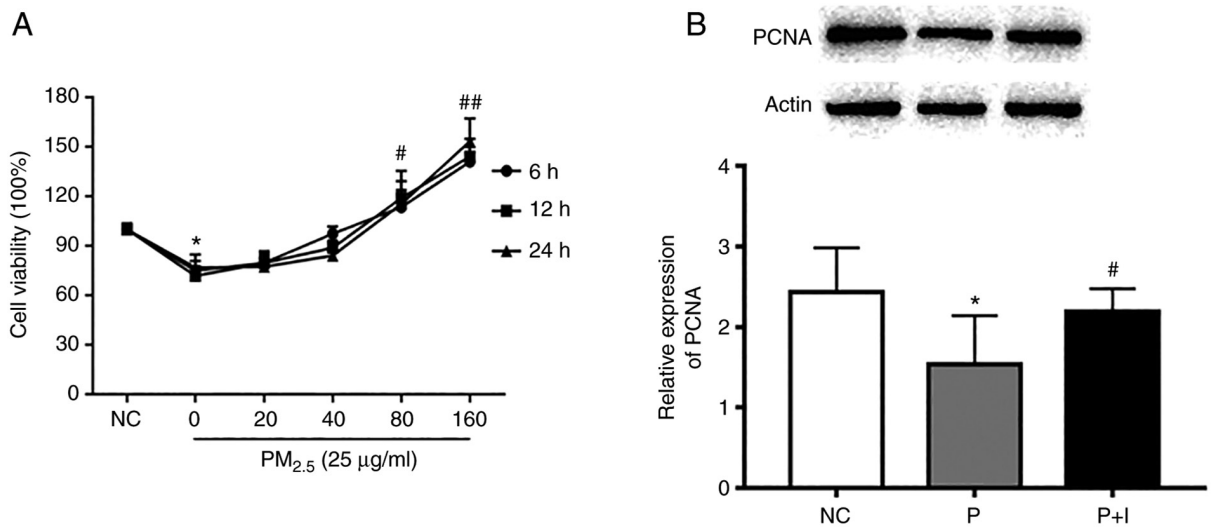

Figure 3. ISO treatment increases cell viability in human bronchial epithelial cells induced by $PM_{2.5}$ exposure. (A) 16-HBE cells were exposed to $PM_{2.5}$ (25 μg/ml, 24 h) or $PM_{2.5}$ (25 μg/ml) plus different doses of ISO (0, 20, 40, 80, or 160 μg/ml) for 6, 12 and 24 h and the cell viability was detected by Cell Counting Kit-8 method. (B) 16-HBE cells were exposed to $PM_{2.5}$ (25 μg/ml) solution or $PM_{2.5}$ (25 μg/ml) plus ISO (80 μg/ml) for 24 h. The intracellular proliferating cell nuclear antigen expression levels were detected by western blot analysis. P indicates 25 μg/ml $PM_{2.5}$ solution treatment. P + I indicates 25 μg/ml $PM_{2.5}$ solution + 80 μg/ml ISO treatment. *P<0.05 compared with NC group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 compared with the P group. ISO, isosinensetin; $PM_{2.5}$, fine particular matter ≤2.5 μm in diameter; PCNA, proliferating cell nuclear antigen; NC, negative control.

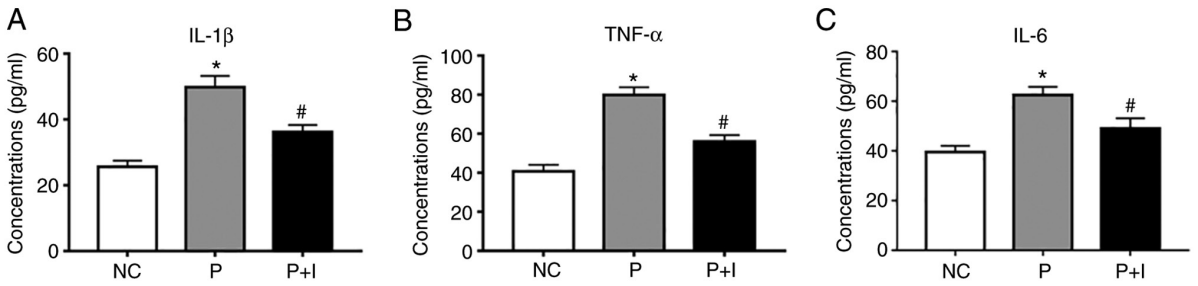

Figure 4. ISO treatment inhibits the increase in the production of IL-1β, TNF-α and IL-6 induced by $PM_{2.5}$ exposure. 16-HBE cells were exposed to $PM_{2.5}$ (25 μg/ml, 24 h) or $PM_{2.5}$ (25 μg/ml, 24 h) plus ISO (80 μg/ml) for 24 h. The (A) IL-1β, (B) TNF-α and (C) IL-6 concentrations were examined by ELISA kits. P indicates 25 μg/ml $PM_{2.5}$ solution treatment. P + I indicates 25 μg/ml $PM_{2.5}$ solution + 80 μg/ml ISO treatment. *P<0.05 compared with the NC group; $^{\#}$P<0.05 compared with the P group. ISO, isosinensetin; $PM_{2.5}$, fine particular matter ≤2.5 μm in diameter; IL, interleukin; TNF-α, tumor necrosis factor-α; NC, negative control.

downregulated compared with the $PM_{2.5}$ group. However, there was no statistical significant difference between the NAC + $PM_{2.5}$ group and NAC + $PM_{2.5}$ + ISO group (Fig. S1).

Discussion

In the present study, it was firstly indicated that ISO had protective effects on oxidative damage and decreased the expression levels of inflammatory factors induced by $PM_{2.5}$. ISO could alleviate the injury of human bronchial epithelial cells induced by $PM_{2.5}$.

$PM_{2.5}$, as one of the toxic and harmful components in the air, is correlated with various systemic diseases, including respiratory and cardiovascular diseases (17). Fine particles can reach and be retained at the alveolar walls of the lungs, which results in allergies, asthma and lung emphysema. In previous studies, it has been demonstrated that $PM_{2.5}$ exposure caused significant formation of ROS and an inflammatory reaction. The mechanisms mainly involved $PM_{2.5}$ triggering systemic oxidative stress and inflammation (18-20).

Various studies have revealed that flavonoids are the main bioactive components in plants which possess health-promoting properties. Numerous flavonoids such as hesperidin, quercetin and apigenin have anti-nociceptive and anti-inflammatory properties (21,22). Nobiletin, as a classical polymethoxyflavone, has been reported to have an anticancer effect by inhibiting the migration ability of breast cancer cells (23). ISO is a compound of flavonoids, found in fruits and vegetables. In the present study, our results indicated that ISO could alleviate the injury of human bronchial epithelial cells and had a protective effect on 16-HBE by reducing the release of inflammatory cytokines and relieving the oxidative stress under $PM_{2.5}$ exposure.

It has been reported that the activators of Nrf2 and NF-κB proteins can act with strong antioxidative and anti-inflammatory activities after harmful chemical stimulation in a cell culture model (24,25). Nrf2 is a transcription factor which plays a vital role in activating antioxidant response by decreasing ROS production (26). NF-κB plays an important role in regulating the inflammatory response. The increase of

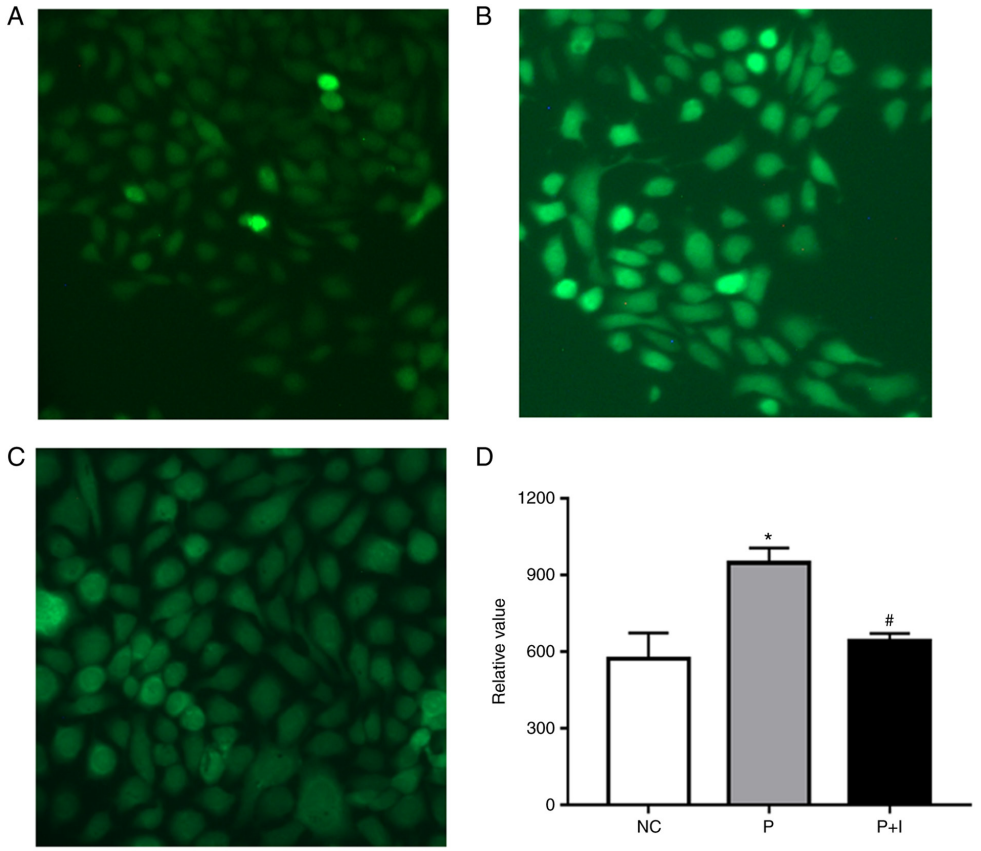

Figure 5. ISO treatment inhibits the increase in production of ROS following $PM_{2.5}$ exposure. (A) 16-HBE cells were cultured for 24 h and the ROS level was examined by DCFH-DA probe. (B) 16-HBE cells were exposed to $PM_{2.5}$ (25 μg/ml) for 24 h, and the ROS level was examined by DCFH-DA probe. (C) 16-HBE cells were exposed to $PM_{2.5}$ (25 μg/ml) for 24 h, then treated with ISO (80 μg/ml) for 24 h, and the ROS level was examined by DCFH-DA probe. (D) Quantification of 16-HBE cells exposed to $PM_{2.5}$ (25 μg/ml) or $PM_{2.5}$ (25 μg/ml) plus ISO (80 μg/ml) for 24 h. P indicates 25 μg/ml $PM_{2.5}$ solution treatment. P + I indicates 25 μg/ml $PM_{2.5}$ solution + 80 μg/ml ISO treatment. *P<0.05 compared with the NC group; $^{\#}$P<0.05 compared with the P group. ISO, isosinensetin; ROS, reactive oxygen species; $PM_{2.5}$, fine particular matter ≤2.5 μm in diameter; DCFH-DA, 2',7'-dicholorofluorescein-diacetate; NC, negative control.

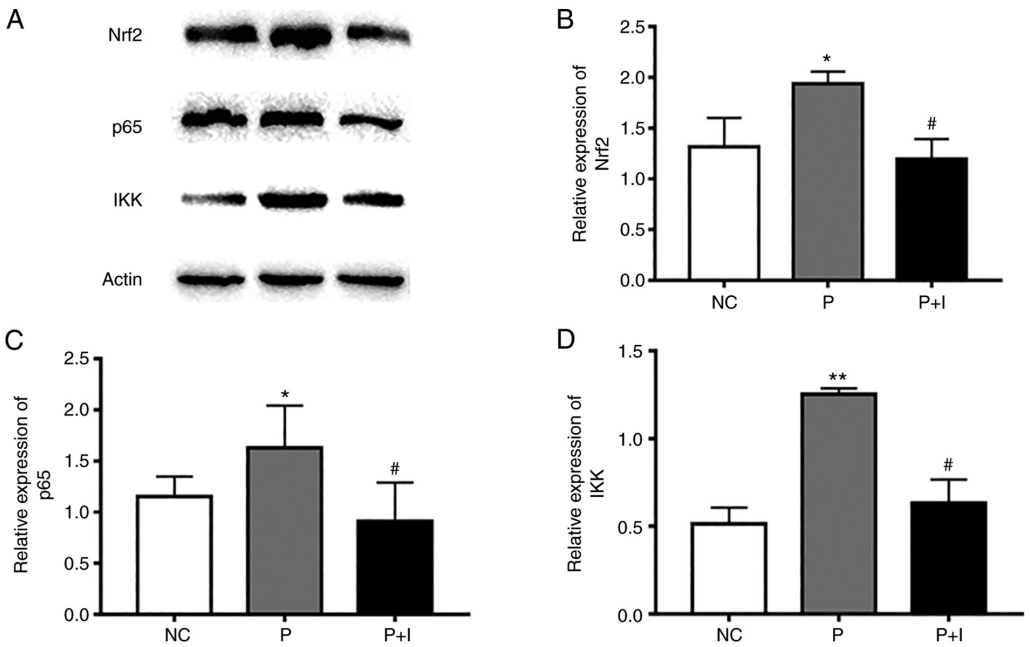

Figure 6. ISO treatment inhibits increase in production of related proteins induced by $PM_{2.5}$ exposure. (A) 16-HBE cells were cultured, exposed to $PM_{2.5}$ (25 μg/ml) or exposed to $PM_{2.5}$ (25 μg/ml, 24 h) and treated with ISO (80 μg/ml) for 24 h, and the expression levels of Nrf2, p65 and IKK proteins were detected by western blotting. Quantification of (B) Nrf2, (C) p65 and (D) IKK protein expression in 16-HBE cells. P indicates 25 μg/ml $PM_{2.5}$ solution treatment. P + I indicates 25 μg/ml $PM_{2.5}$ solution + 80 μg/ml ISO treatment. *P<0.05 and **P<0.01 compared with the NC group; $^{\#}$P<0.05 compared with the P group. ISO, isosinensetin; $PM_{2.5}$, fine particular matter ≤2.5 μm in diameter; Nrf2, nuclear factor erythroid 2-related factor 2; IKK, IκB kinase.

ROS can lead to continuous activation of NF-κB and promote the release of cytokines (27). Our data revealed that ISO could downregulate the expression of Nrf2 and NF-κB in 16-HBE cells induced by $PM_{2.5}$. Moreover, after inhibiting the release of ROS induced by $PM_{2.5}$, the expression levels of Nrf2, p65 and IKK proteins exhibited no obvious differences between the $PM_{2.5}$ group and $PM_{2.5}$ + ISO group. These results indicated that ISO could alleviate the injury of 16-HBE cells induced by $PM_{2.5}$ through the ROS-Nrf2/NF-κB signaling pathway. However, a limitation of the present study was that further investigation of the mechanism of Nrf2/NF-κB after $PM_{2.5}$ or ISO treatment was not performed in 16-HBE cells. It will be conducted in subsequent experiments.

The apparent effect of ISO has been revealed to be presumably dependent on the concentration. Nonetheless, the anti-inflammatory and oxidative mechanisms of ISO have not yet been clearly determined. Among the different molecules produced intracellularly, the transcription factor Nrf2 plays a vital role in the prevention of cell dysfunction in response to oxidative stress and in protection against exposure to toxins and carcinogens through the ARE-mediated expression of a battery of cytoprotective genes (28). It is anticipated that in future studies, ISO may be used to evaluate the therapeutic interventions on the cellular injury induced by $PM_{2.5}$.

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Availability of data and materials

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

Authors' contributions

YZ, SL, XL and CX conceptualized and designed the present study. YZ and SL performed all of the experiments and confirmed the authenticity of all the raw data. YZ, SL, YS, MM, HT, NW and JY were responsible for the data collection and analysis. XL and YS interpreted the data and drafted the manuscript. YZ and CX revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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