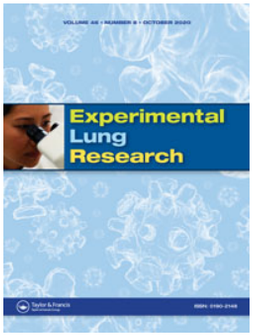

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Thymoquinone ameliorates the PM2.5-induced lung injury in rats

Mingqing Mao[a]*, Jing Li[b]*, Aiping Bi[a], Hui Jia[a], Qiong Li[a], Yang Liu[a], Xiaochuan Jiang[a], Desheng Huang[c] and Shuyue Xia[a]

[a]Department of Respiratory and Critical Care Medicine, Central Hospital Affiliated to Shenyang Medical College, Shenyang, People's Republic of China; [b]Shenyang Environment Monitor Central Station, Key Laboratory of Atmospheric Organic Compound Monitoring and Analysis, Ministry of Environmental Protection, Shenyang, People's Republic of China; [c]Department of Mathematics, College of Basic Medical Sciences, China Medical University, Shenyang, People's Republic of China

ABSTRACT

Background: This study aims to explore the effect of thymoquinone (TQ) on particulate matter 2.5 (PM2.5)-induced lung injury.

Methods: The PM2.5 sample was provided by Shenyang Environment Monitor Central Station. Lung injury was established by intratracheal instillation PM2.5 (7.5 mg/kg) followed by TQ treatment (20 and 40 mg/kg) for 14 d in rats. Hematoxylin and eosin (HE) and Evans blue dye (EBD) staining were detected on lung tissues. ELISA, real-time PCR, western blotting and TUNEL assays were also performed.

Results: The data showed that TQ diminished lung injury and EBD accumulation. The number of macrophages, neutrophils, eosinophils, and lymphocytes was ameliorated after TQ treatment. In addition, TQ suppressed the inflammation reaction parameters (interleukin-1β and -6, IL-1β and IL-6; tumor necrosis factor-α, TNF-α) and oxidative stress in PM2.5-induced lung injury. The levels of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase (HO-1) were increased due to the treatment of TQ. The number of TUNEL-positive cells was prominently reduced in TQ-treated rats compared with that in PM2.5 group. Intratracheal instillation PM2.5 activated autophagy, whilst TQ blocked it in lung.

Conclusions: Taken together, this study provides the first *in vivo* evidence that TQ suppresses inflammation, oxidative stress, apoptosis, and autophagy in PM2.5-induced lung injury.

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KEYWORDS
Thymoquinone; PM2.5; lung injury; inflammation; oxidative stress; apoptosis; autophagy

Introduction

With the development of heavy industry, air pollution has become increasingly worse, especially in developing countries.[1] The particulate matter 2.5 (PM2.5), less than 2.5 μm in diameter, accounts for a large proportion of air pollution.[2] Epidemiological evidence indicates that PM2.5 increases the risk of premature death for people in the world.[3] There are about 103 million lost lives after suffered from PM2.5 pollution in 2015, mainly in east and south Asia.[4] It has been confirmed that people who exposure to high concentrations of PM2.5 for a long time can cause a series of respiratory diseases including asthma,[5] chronic obstructive pulmonary disease (COPD),[6] acute lung injury (ALI)[7] and lung cancer.[8] Therefore, it is necessary to find new strategies to treat PM2.5-induced lung injury.

Researchers notice that inflammation and oxidative stress play critical roles in lung diseases caused by PM2.5. For instance, PM2.5 stimulates the production of reactive oxygen species (ROS) and pro-inflammatory mediators leading to lung injury.[9] Following inhalation of PM2.5, the induction of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-1β impairs endothelial cell function.[10] Under stress conditions, the level of malondialdehyde (MDA, a typical product of lipid peroxidation) was elevated, whereas activities of anti-oxidative enzymes were inhibited. The nuclear factor erythroid-2-related factor (Nrf2), a vital regulator against oxidative stress, is activated *in vivo* and *in vitro* to defend against

CONTACT Shuyue Xia syx262@126.com Department of Respiratory and Critical Care Medicine, Central Hospital Affiliated to Shenyang Medical College, 5 West Nanqi Road, Shenyang 110024, People's Republic of China.
*Mingqing Mao and Jing Li contributed equally to this study.

lung injury.[11,12] Thus, compounds with both anti-inflammatory and antioxidant activities may be beneficial for the treatment of PM2.5-induced lung injury.

Thymoquinone (TQ) is a natural product and is isolated from *Nigella sativa*. It has many biological activities such as anti-inflammation, antioxidant, neuroprotective and anticancer.[13–16] TQ attracts researchers' attention due to its protective effect on lung injury. For example, studies reveal that TQ attenuates lung injury induced by cyclophosphamide and chronic toluene exposure, respectively.[17,18] However, its effect on PM2.5-induced lung injury has not been reported. Based on its outstanding biological activities, we speculate that TQ may become a therapeutic candidate to improve the PM2.5-induced lung injury.

This study aims to explore a new method for preventing and treating lung injury caused by PM2.5. We investigate the pathological changes in the lungs of rats after intratracheal instillation PM2.5. Following, the protective effect of TQ against PM2.5-induced inflammatory responses, oxidative stress, apoptosis, and autophagy in rats is revealed.

Materials and methods

PM2.5 characterization

The PM2.5 sample was provided by Shenyang Environment Monitor Central Station which was located in Shenyang, Liaoning Province of China. The PM2.5 sample was collected from November 2016 to February 2017. It was collected continuously on the quartz fiber filter membrane with a diameter of 9 cm for 24 h. The filter membrane was replaced every 24 h during the whole sampling process. The collected PM2.5 was eluted with Milli-Q ultrapure water and centrifuged as 12000 r/min. The extracted PM2.5 liquid was vacuum freeze dried and stored at −80 °C. Before treatment for animal, the PM2.5 sample was mixed with saline and ultrasonic oscillation for 5 min.

In addition, the data of PM2.5 main components were obtained from Shenyang Environment Monitor Central Station. The chemical composition of PM2.5 mainly included anion, organic carbon (OC), cation, elemental carbon (EC), metals and polycyclic aromatic hydrocarbons (PAHs), whose concentrations were 23.0, 14.6, 9.42, 6.30, 0.67, and 71.5 ng/m^3, respectively. $SO4^{2-}$, $NO3^-$ and $NH4^+$ were the three kinds of ions with higher content (10.9 μg/m^3, 9.20 μg/m^3 and 6.97 μg/m^3). A total of 14 major metals were detected, including Al, Zn, Pb, Mn, Ti, As, Ba, Cu, Fe, Se, V, Cr, Ni and Cd.

Animals

The joint ethics committee of Shenyang Medical College approved the study protocol for animal welfare. The healthy male Wistar rats, aged 8 weeks with mean weighing of 280 g, were freely given food and water, and adapted to the environment for a week. The PM2.5-induced lung injury was performed by intratracheal instillation PM2.5 (7.5 mg/kg body weight/d) for 14 d. The TQ treatment referred to that the rats were given TQ (20 or 40 mg/kg body weight/d) by intragastric administration for 14 d. The rats were randomly divided into Control, Saline control (intratracheal instillation saline), TQ control (intratracheal instillation saline + 40 mg/kg body weight TQ), PM2.5, PM2.5 + Solvent (intratracheal instillation PM2.5 + equal volume of TQ solvent), PM2.5 + TQ20, PM2.5 + TQ40 groups.

Bronchoalveolar lavage fluid (BALF)

Rats were anesthetized by intraperitoneal injection pentobarbital sodium (50 mg/kg). Expose the rat trachea, open the thoracic cavity and ligate the right lung bronchus. Following, a thin plastic tube was inserted from the trachea. The lung lavage was performed with 4 °C physiological saline (1.5 ml per lavage), which was repeated three times. The BALF was collected.

Lung vascular permeability assay

Lung vascular permeability was evaluated by measuring Evans blue dye (EBD) leakage. In brief, rats were injected into the tail vein with 1% EBD (2 ml/kg) 1 h before tissue collection. After the left ventricle was perfused with saline, the

lungs (100 mg) were removed and homogenized with 1 ml formamide. The homogenate was incubated for 24 h at 60 °C followed centrifugation for 30 min at 10,000 *g*. The supernatant was collected and the absorbance was detected at 630 nm. The EBD content (μg/g of lung tissue) was calculated.

Cytokine measurement and oxidative stress index

Concentrations of IL-1β, IL-6 and TNF-α in BALF were measured according to the instruction of ELISA kits (Wanleibio, China). The lactic dehydrogenase (LDH) activity and total protein (TP) content in BALF were determined by commercial kits (Wanleibio, China; Nanjing Jiancheng Bioengineering Institute, China). Activities of superoxide dismutase (SOD) and glutathion peroxidase (GSH-PX) and concentrations of MDA in lung tissues were determined via commercial kits (Wanleibio).

Histopathology

After the lavage, the lung tissue was fixed with 10% formaldehyde, embedded in paraffin and sectioned at 5 μm. The sections were stained with hematoxylin and eosin (HE) (Wanleibio) and observed under a light microscope (OLUMPUS, Japan). The images were captured at 100× and 400×.

TUNEL assay

The TUNEL assay (Wanleibio) was used to evaluate the degree of apoptosis in lung tissues. The 5 μm sections were added 50 μl 0.1% Triton X-100 (Beyotime, China) to permeabilization and blocked with 3% H_2O_2. They were incubated with TUNEL reagent at 37 °C for 1 h in dark. They were added Converter-POD followed by DAB (Solarbio, China). After hematoxylin redyeing, the sections were observed under a light microscope (OLUMPUS, Japan).

Real-time PCR

Total RNA was isolated from the lung tissues using TRIpure (BioTeke, China). The cDNA was synthesized according to the manufacturer's instruction. Real-time PCR was performed using Exicycler^{TM} 96 Real-Time Quantitative Thermal Block (Bioneer, Korea). The primer pairs were as follows (forward and reverse, respectively): IL-1β, 5′-TTCAAATCTCACAGCAGCAT-3′ and 5′-CACGGGCAAGACATAGGTAG-3′; IL-6, 5′-AACTCCATCTGCCCTTCA-3′ and 5′-CTGTTGTGGGTGGTATCCTC-3′; TNF-α, 5′-TGGCGTGTTCATCCGTTCT-3′ and 5′-CCACTACTTCAGCGTCTCGT-3′. The expression level of each gene was normalized to that of β-actin, and was calculated by using the $2^{-\Delta\Delta Ct}$ method.

Western blot

The lung tissues were lysed by lysis buffer (Wanleibio). The lysate was homogenized, and the supernatant was obtained by centrifugation at 12,000 rpm for 10 min. The protein concentration was measured by the BCA assay (Wanleibio). The protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Then, the membranes were blocked with 5% nonfat milk for 1 h at room temperature. They were incubated with antibodies (Wanleibio) at 4 °C overnight. The corresponding antibodies as follows: nuclear factor erythroid 2-related factor 2 (Nrf2) (1: 400); heme oxygenase (HO-1) (1: 1000); LC3II/I (1: 400); Beclin1 (1: 500). Then, they were incubated with horseradish peroxidase-conjugated goat anti-rat antibodies (1: 5000). The bands were detected using enhanced chemiluminescence (ECL, Wanleibio). The density values of bands were analyzed by Gel-Pro-Analyzer software.

Statistical analysis

Data were expressed as the mean ± SD and analyzed with GraphPad Prism 7.0 software. Differences between the groups were performed using one-way analysis of variance (ANOVA) with Bonferroni's post hoc test. Significance was accepted when P values < 0.05.

Results

Pathologic changes in PM2.5-induced lung injury in rats

In this study, lung injury was established by intratracheal instillation PM2.5. HE staining was used to estimate the different degrees of lung injury (Figure 1A). By observing the lung tissues of the control, Saline control and TQ control groups, we found that the alveolar distribution was uniform, and alveolar space was no increase. There were few inflammatory cell infiltrations in the interstitial lung. However, the lung tissue appeared particulate accumulation and lots of inflammatory cell infiltration after intratracheal instillation PM2.5. TQ reversed this injury. Compared with those of PM2.5 group, alveolar structure was relatively complete and a small amount of alveolus were ruptured in the PM2.5 + TQ40 group. Meanwhile, the extent of inflammatory cell infiltration was also decreased. In addition, the effect of TQ on PM2.5-induced lung vascular permeability was evaluated by measuring EBD leakage. As shown in Figure 1B, PM2.5 led to an obvious increase in lung vascular leakage, whilst TQ inhibited PM2.5-induced EBD accumulation in rat lung tissues.

Effect of TQ on the inflammation reaction parameters in PM2.5-induced lung injury

To evaluate the effect of TQ on PM2.5-induced inflammation reaction, inflammatory cells in BALF were counted. As shown in Figure 2A, the number of macrophages, neutrophils, eosinophils, and lymphocytes in PM2.5 group were obviously increased compared with that in control (control, Saline control and TQ control) ($P < 0.01$). The above four kinds of inflammatory cells were decreased while the rats were treated with TQ. Especially, the lung injury was significantly improved under the concentration of 40 mg/kg body weight ($P < 0.01$). ELISA kits and real-time PCR were used to measure the levels of IL-1β, IL-6 and TNF-α (Figure 2B). It was found that TQ counteracted the increase of PM2.5-induced pro-inflammation factor levels (IL-1β, IL-6, and TNF-α). The finding indicated that TQ can repress the inflammation in PM2.5-induced lung injury.

Effect of TQ on the oxidative stress parameters in PM2.5-induced lung injury

On the other hand, oxidative stress parameters were monitored to further verify the protective effect of TQ. The TP and LDH contents were examined to estimate alveolar epithelial-capillary barrier damage. The elevated LDH and TP levels in PM2.5 rats illustrated that intratracheal instillation PM2.5 triggered lung tissue damage. As the same time, intratracheal instillation PM2.5 notably enhanced the concentration of predominant by-product of lipid peroxidation (MDA) and alleviated the activities of essential antioxidant (SOD and GSH-PX) in lung tissues of rats (Figure 3A, $P < 0.01$). On the contrary, PM2.5-induced damage of oxidative stress was partially reversed due to the treatment of TQ. Response to oxidative stress, the body first started self-protection measures. The protein levels of Nrf2 and HO-1 were detected by western blot. It was found that Nrf2 transferred into nucleus and HO-1 was increased after intratracheal instillation PM2.5. The role of TQ was that it strengthened this protective effect (Figure 3B, $P < 0.01$). Consequently, the Nrf2 protein in cell nucleus and HO-1 were ascendant whether in the PM2.5, PM2.5 + Solvent or PM2.5 + TQ20/40 groups. Taken together, these data showed a potent protective effect of TQ in lung under intratracheal instillation PM2.5.

TQ inhibited the apoptosis and autophagy in PM2.5-induced lung injury

TUNEL assay was used to estimate cell apoptosis in lung tissues. Apoptosis had been observed in lung tissue in the PM2.5 group (Figure 4A). There was no apoptosis in the three groups, including Control, Saline control, and TQ control. We discovered that the number of TUNEL-positive cells in the lung tissue was prominently reduced in the TQ-treated rats than that in the PM2.5. After treating rats with increasing concentrations of TQ, a dose dependent decrease in TUNEL-positive cells was observed. The results

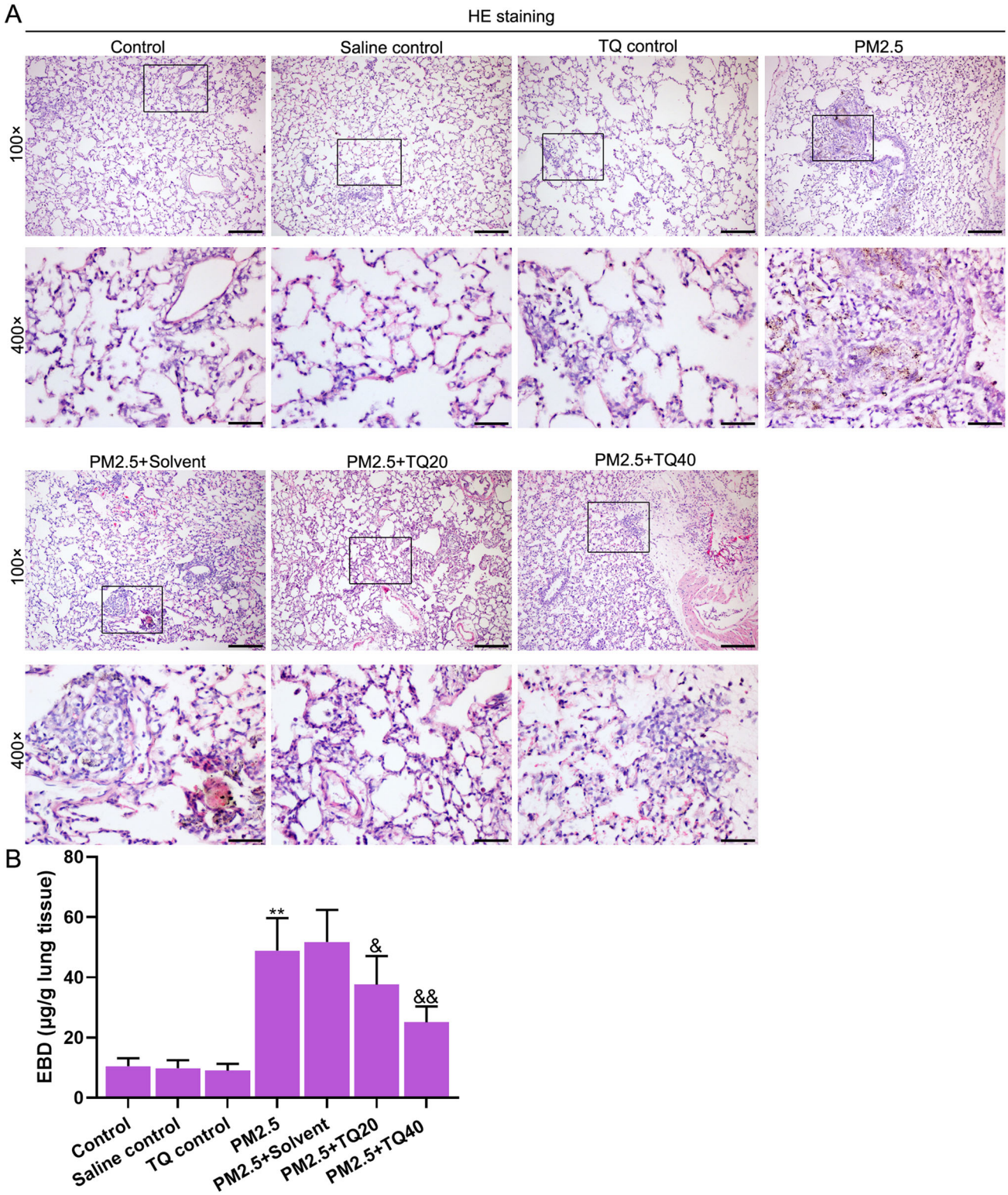

Figure 1. Histopathology of PM2.5-treated rats. (A) Representative image exhibited the morphological and pathological changes of rat lung tissues in Control, Saline control, TQ control, PM2.5, PM2.5 + Solvent, PM2.5 + TQ20, and PM2.5 + TQ40 groups (HE, 100×: scale bar = 200 μm; 400×: scale bar = 50 μm). (B) Lung vascular Evans blue leakage. $^{**}P < 0.01$ as compared to the Saline control group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ as compared to the PM2.5 + Solvent group.

implied that the treatment of TQ inhibited apoptosis in the lung of rats. Moreover, autophagy-related proteins (LC3-II and beclin-1) were examined by western blot. The data demonstrated that intratracheal instillation PM2.5 activated autophagy, nevertheless, treatment TQ blocked it

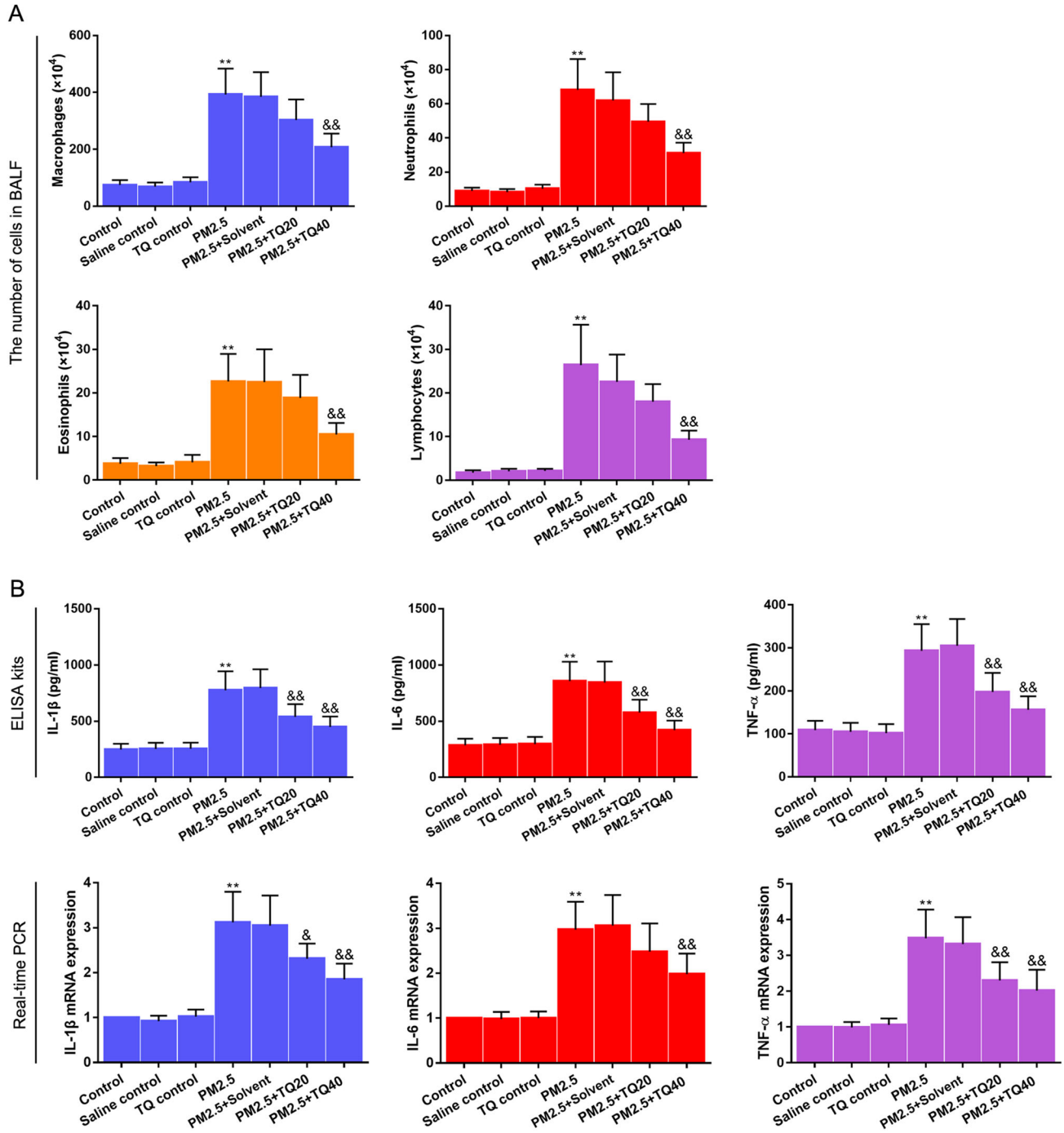

Figure 2. Effect of TQ on PM2.5-induced inflammatory response. (A) The number of inflammatory cells (macrophages, neutrophils, eosinophils, and lymphocytes) in the BALF was counted. (B) The contents and mRNA levels of IL-1β, IL-6, TNF-α were detected by ELISA and real-time PCR. $^{**}P < 0.01$ as compared to the Saline control group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ as compared to the PM2.5 + Solvent group.

in lung and the high concentration of TQ exhibited the stronger inhibition (Figure 4B).

Discussion

Although people around the world are working hard on environmental protection and our living environment is getting better and better, the connection between air pollution and lung diseases still deserves our attention. After PM2.5 is inhaled through the respiratory tract, it is able to infiltrate into the alveolar region of the lung to exert their toxicity.[19] However, inflammation and oxidative stress are often involved

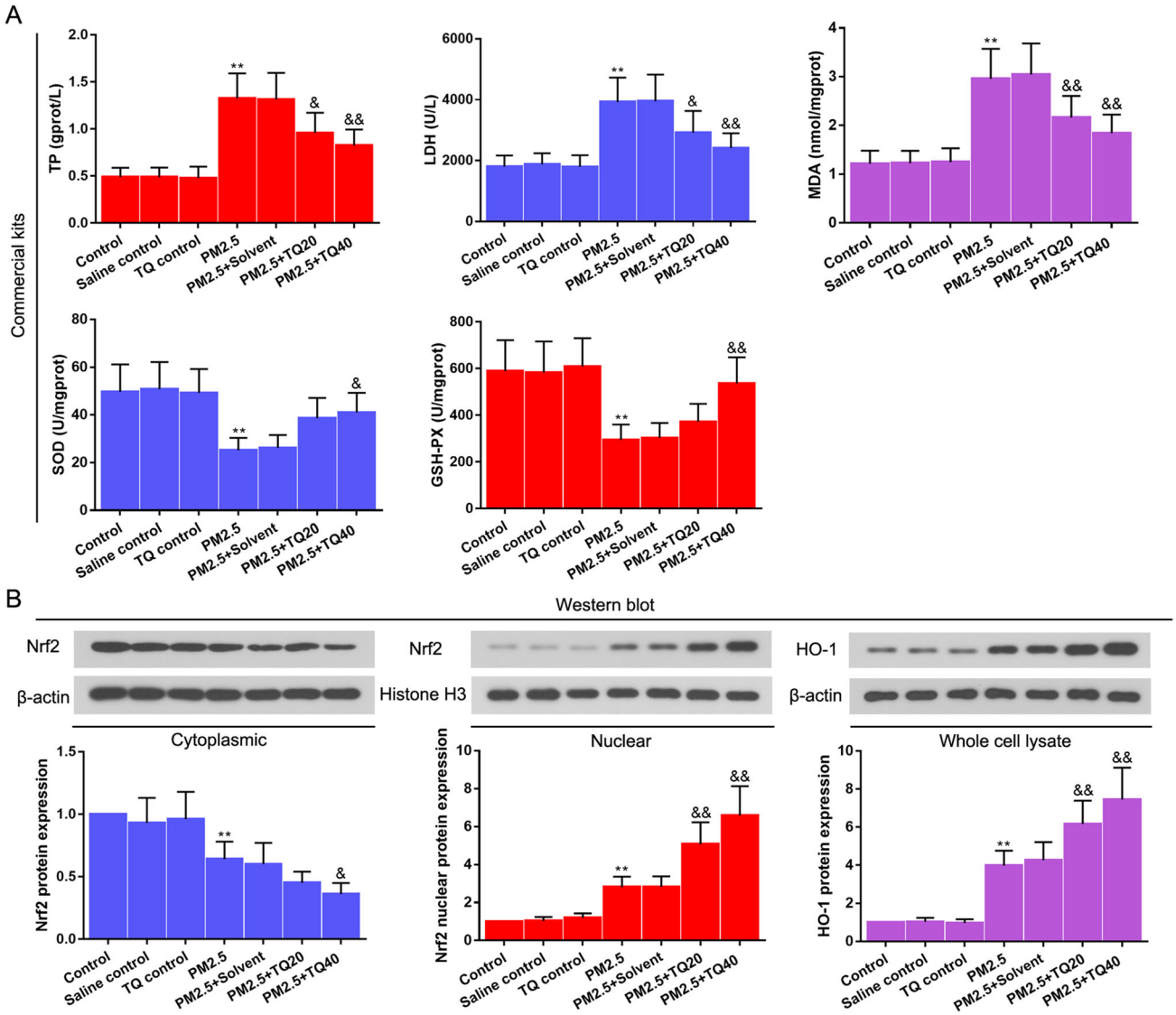

Figure 3. Effect of TQ on PM2.5-induced oxidative stress. (A) The TP, LDH, MDA, SOD and GSH-PX levels were monitored by commercial kits. The TP and LDH contents were used to assess alveolar epithelial-capillary barrier damage. Intratracheal instillation PM2.5 notably enhanced the concentration of predominant by-product of lipid peroxidation (MDA) and alleviated the activities of essential antioxidant (SOD and GSH-PX) in lung tissues of rats. Treatment with TQ significantly reversed this change. (B) The protein levels of Nrf2 (cytoplasm protein or nucleus) and HO-1 (whole cell lysate) were detected via western blot. Response to oxidative stress, the body first started self-protection measures. The Nrf2 transferred into nucleus and HO-1 was increased after intratracheal instillation PM2.5. The role of TQ was that it strengthened this protective effect with dose dependent. $^{**}P < 0.01$ as compared to the Saline control group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ as compared to the PM2.5 + Solvent group.

in response to the injury induced by particulate matter.[20,21]

The host's main defense against tissue damage is inflammation, however, excessive inflammation may lead to disease. A large number of studies demonstrated that exposure to air pollution triggers the release of pro-inflammatory factors. For example, zhao *et al* showed that the expression levels of pro-inflammatory cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, IL-1β and TNF-α in macrophages, were significantly increased after exposure to PM2.5.[22] Zhao *et al* reported that PM2.5 induced rat lung inflammatory via upregulating the expression of pro-inflammatory mediators like TNF-α and IL-1β.[23] IL-1β can induce the secretion and release of other inflammatory mediators such as IL-6 in various cells.[24] Zhang *et al* also showed that the production of TNF-α and IL-6 was increased in a mice model of PM2.5-caused lung injury.[25] These findings were similar with ours. Our data suggested that TQ reduced the

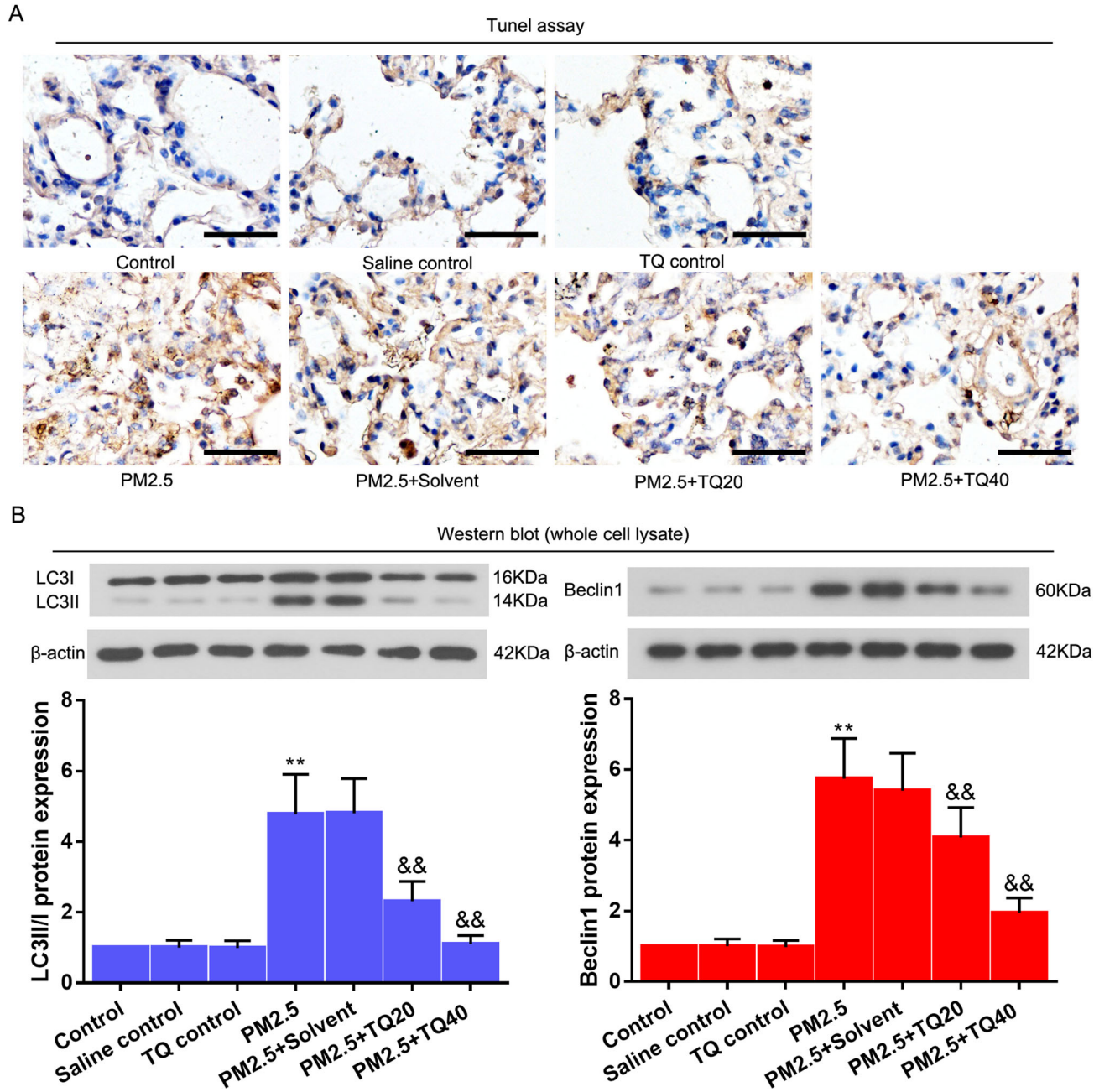

Figure 4. Effect of TQ on PM2.5-induced cell apoptosis and autophagy. (A) The cell apoptosis of lung tissues was measured via TUNEL assay, scale bar = 50 μm. (B) TQ blocked autophagy. The LC3I, LC3II, and beclin1 levels were normalized to actin. $^{**}P < 0.01$ as compared to the Saline control group; $^{\&\&}P < 0.01$ as compared to the PM2.5 + Solvent group.

elevation of inflammatory cell numbers (macrophages, neutrophils, eosinophils, and lymphocytes) induced by PM2.5 in BALF. Further, TQ inhibited PM2.5-triggered inflammation via diminishing pro-inflammatory factor levels (IL-1β, IL-6 and TNF-α).

Oxidative stress is common in the lung inflammation caused by particles exposure, which lead to damage to proteins, lipids and DNA in cells, even apoptosis.[26] TP is a significant marker to characterize the extent of alveolar epithelial-capillary barrier damage. Similarly, LDH is also an important indicator of cell membrane damage. The data demonstrated that PM2.5 indeed brought about lung injury due to the upgrade of TP, LDH and MDA. The active metals and organic components in PM2.5 made body produce a large amount of active oxygen radicals and reactive nitrogen radicals, which consumed the major antioxidant enzymes, SOD and GSH-

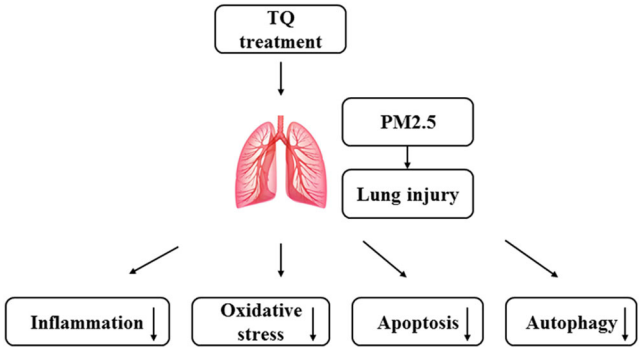

Figure 5. The proposed mechanism of actions. TQ suppressed inflammation, oxidative stress, apoptosis, and autophagy in PM2.5-induced lung injury.

PX. Interestingly, treatment TQ restored the antioxidant enzyme activities. It was reported that TQ also increased SOD enzyme activity in CCl_4-induced liver injury.[27] Cells activated Nrf2 signals to response to oxidative stress and repair the damage.[28] Zhang *et al* also showed Nrf2 activation could promote corneal epithelial wound healing and restoration of mechanical sensation in diabetic mice.[29] Our studies revealed that the activated Nrf2 by TQ facilitated the epithelial cells' tissue repair not only through attenuating the levels of LDH and MDA, but also by partially upregulating the HO-1 expression.

In addition, oxidative stress often initiates apoptosis. Research showed that TQ exerted anticancer activity by inducing apoptosis.[30] In this study, we found TQ alleviated cell apoptosis in PM2.5-induced lung injury. Many studies have illustrated that autophagy may be either cytoprotective or deleterious in pulmonary diseases.[31–33] For instance, autophagy was activated in LPS-induced ALI models, whilst inhibition of autophagy by 4-phenyl butyric acid (4-PBA) may play a protective role in ALI models through the classical AKT/mTOR signaling pathway.[34] Li *et al* confirmed that PM2.5 triggered autophagy formation, and PM2.5-induced oxidative injury was partially abolished through the suppression of autophagy.[35] TQ inhibited autophagy and induced cathepsin-mediated, caspase-independent cell death in glioblastoma cells.[36] Our results revealed that PM2.5 significantly up-regulated the levels of autophagy-related genes LC3II and Beclin1. Synthetically, these data demonstrated that PM2.5 activated autophagy in rat lung tissues, whilst TQ suppressed autophagy with a dose-dependent manner.

Conclusion

Taken together, the present study provides the first *in vivo* evidence that TQ suppresses inflammation, oxidative stress, apoptosis, and autophagy in PM2.5-induced lung injury (Fig. 5). Findings from this study could shed new light on the molecular mechanisms of pulmonary intervention for PM2.5 exposure. This study affords a new treatment strategy for PM2.5-induced lung injury.

Author contributions

Mingqing Mao and Jing Li performed the experiments and wrote this manuscript. Aiping Bi, Hui Jia, Qiong Li, Yang Liu, Xiaochuan Jiang, and Desheng Huang performed the experiments. Shuyue Xia designed this study and polished the manuscript.

Conflict of interest

All authors declare that they have no any conflict of interests.

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