Iran J Public Health, Vol. 53, No.3, Mar 2024, pp.614-624

Original Article

MitoQ Alleviated $PM_{2.5}$ Induced Pulmonary Epithelial Cells Injury by Inhibiting Mitochondrial-Mediated Apoptosis

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(Received 06 Jul 2023; accepted 11 Sep 2023)

Abstract

Background: Fine particulate matter ($PM_{2.5}$), an important component of ambient air pollution, induces significant adverse health effects. MitoQuinone (MitoQ), a mitochondria-targeted antioxidant, has been reported to play a protective role in various diseases. However, the roles of MitoQ in $PM_{2.5}$ induced pulmonary toxicity remains to be elucidated.

Methods: All the experiments were performed at Higher Educational Key Laboratory for Translational Oncology of Fujian Province, Putian City, China in 2023. Pulmonary epithelial cells (A549) were pretreated with 4 μM MitoQ for 2 h and exposed to $PM_{2.5}$ for 24 h. Cell viability was tested through CCK8 assay. Oxidative stress state and active mitochondria was used to study MitoQ's effect on $PM_{2.5}$ induced injury, and cell apoptosis was measured using a flow cytometer and analyzed by Bcl-2 family.

Results: MitoQ pretreatment significantly relieved a decreased cell viability, subsequently, MitoQ alleviated ROS production and prevented the reduction of T-AOC and GSH and increased the expression of NF-E2-related factor 2 (Nrf2) and p62 in A549 cells exposed to $PM_{2.5}$. MitoQ restored the decreased mitochondrial dysfunction and dynamics disorder and inhibited activated mitochondrial-mediated apoptosis induced by $PM_{2.5}$. Furthermore, the decreased ratio of Bcl-2/Bax and expression of Mcl-1 and the enhanced expression of Caspase-3 were reversed by MitoQ pretreatment.

Conclusion: MitoQ might be regarded as a potential drug to relieve $PM_{2.5}$ induced pulmonary epithelial cells damage.

Keywords: MitoQuinone; Mitochondrial dynamics; Mitochondrial-mediated apoptosis

Introduction

With the rapid industrialization and urbanization, ambient air pollution has become a major public health issue over past decades (1-3). There is a clear link between development and occurrence of ambient air pollution and chronic disease, such as chronic obstructive pulmonary disease (COPD), asthma and stroke (4-6). Among the different types of ambient air pollution, fine par-

ticulate matter ($PM_{2.5}$) is a silent killer and kills the most people worldwide (2,4,7). $PM_{2.5}$, which is consists of particles smaller than approximately 2.5 microns, can be easy to be inhale to the smallest airway, even exert into blood. Nevertheless, the mechanisms involved in $PM_{2.5}$ induced cell death still need to be addressed.

Physically, Mitochondria is thought to a sensitive target of $PM_{2.5}$. $PM_{2.5}$ can impair mitochondrial structural and functional integrity (4,8,9), represented as mitochondrial membrane depolarization and fragmentation accompanied by an excessive generation of mitochondrial reactive oxygen species (mtROS) (9,10). A high level of mtROS can activate intrinsic apoptosis and cause the release of mitochondrial apoptosis-inducing factors into the cytoplasm, suggesting that inhibiting mtROS may act as a potential therapeutic strategy to lessen $PM_{2.5}$ induced toxicological effects.

MitoQuinone (MitoQ) is a mitochondria-targeted antioxidant which complex formed by the respiratory complex II coenzyme Q10 covalently with a lipophilic group (11,12). MitoQ could play a protective role in various diseases, such as neurodegenerative disease, cardiovascular disease (13), and idiopathic pulmonary fibrosis. MitoQ protected lung epithelial cell from paraquat and benefit in mitochondria protection (14).

We aimed to investigate the effects of MitoQ on $PM_{2.5}$ induced pulmonary epithelial cells injury and determine its possible regulatory effects of the mitochondrial-mediated of apoptosis.

Materials and Methods

Cell culture and treatments

A pulmonary epithelial cells line (A549) was obtained from the Procell Life Science & Technology Co.,Ltd. (Wuhan, China), incubated RPMI 1640 (Hyclone, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and 1% streptomycin/penicillin (Gibco, USA) in an incubator (BB-150, Thermo Fisher, USA) with 5% CO_2 at 37 °C. The A549 were seeded in culture flasks and grown by 70%-80%. As our previous study (15), $PM_{2.5}$ samples were collected from Putian city, Fujian Province, China, then were suspended to the required concatenation (200 μg/mL) in in RPMI 1640 medium (Hyclone, USA). In the cell experiment, the A549 cells were pretreated 4 μM MitoQ (Glpbio, USA) for 2 h before exposure to 200 μg/mL $PM_{2.5}$ suspend for 24 h to establish the cell damage model.

These experiments were approved by The Ethics Committee of the Affiliated Hospital of Putian University (No. 2019024).

Cell morphology and viability

The cells morphology was captured by using an optical microscope (DM1L, Leica, Germany). The cell viability after exposure to $PM_{2.5}$ was detected using cell counting kit-8 (CCK8) assay (Biosharp, China) according to the instructions. The absorbance (OD) was measured by Thermo Multiskan FC enzyme reader (Thermo Fisher, USA). Simultaneously, the treated cells were stained with 0.4% trypan blue solution (Meilune, Dalian, China) for 5 min. We calculated the cell count under microscope (Nikon, Japan).

Measurement of ROS production

The ROS level was performed using a fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA, Zomanbio, China). According to the manufacturer's instructions, 10 μM DCFH-DA was added to every group after treatments. Then, the cells were incubated for 20 min, after washing with RPMI 1640 three times, the fluorescence intensity was detected using FacsCalib flow cytometer (BD Biosciences, USA).

Detection of ATP, total antioxidant capacity colorimetric (T-AOC) and glutathione (GSH)

The cellular ATP levels were measured using ATP assay kit (Solarbio, China) using the UV-

1800 spectrophotometer (Mapada, China). Thereafter, the cells supernatant was collected and detected by Thermo Multiskan FC enzyme reader (Thermo Fisher, USA) to assess the activities of T-AOC (Elabsciense, USA) and GSH (Elabsciense, USA) accordance with the manufacturer's instructions.

Assessment of active mitochondria

For detecting active mitochondria, the treated cells were incubated with 100 nM MitoTracker Green FM (Yeasen, China) for 30 min, then analyzed using FacsCalib flow cytometer (BD Biosciences, USA). Next, to measure the changes in mitochondrial membrane potential (MMP), JC-1 (Solarbio, China) staining was performed, the red/green fluorescence intensity ratio, considered as a direct assessment of mitochondrial depolarization, was analyzed using FacsCalib flow cytometer (BD Biosciences, USA).

Cell apoptosis assay

Detection of cell apoptosis was carried out according to the manufacturer's protocols using an Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (Zomanbio, China) following the instructions. And cell apoptosis was analyzed with FacsCalib flow cytometer (BD Biosciences, USA).

Western blot analysis

As previous studies(13,15), expressions of proteins involved in regulating mitochondrial morphology and mitochondrial-mediated apoptosis were determined by Western blot analysis, including *Nrf2* (Biodragon, China), *p62* (Proteintech, China), *Bcl-2* (Beyotime, China), *Bax* (Beyotime, China), *Drp1* (Proteintech, China), *Fis1* (Beyotime, China), *Mfn2* (Beyotime, China), *Mcl-1* (Beyotime, China), and *Caspase-3* (Beyotime, China). As an internal control, *β-actin* (Proteintech, China) and *GAPDH* (Proteintech, China) was detected.

Statistical analysis

All experiments were repeated at least three times. Data were presented as means ± standard error (SE). Statistical analysis was performed using R programming, Comparisons and correlations were by one-way analysis of variance (ANOVA) and Pearson's correlation coefficient, respectively. A value of $P<0.05$ was accepted as being statistically significant.

Results

MitoQ improved the viability of A549 induced by $PM_{2.5}$

To detect the adverse role of $PM_{2.5}$ on human alveolar basal epithelial cells A549, we treated A549 with $PM_{2.5}$ suspend as our previous study (15). As shown in Fig. 1A, morphological characteristic of cell death was clearly observed. While the CCK8 assay revealed that the cell viability was significantly decreased after $PM_{2.5}$ treatments ($P<0.05$). Simultaneously, we further demonstrated the cell death using 0.4% trypan blue staining, the result showed that $PM_{2.5}$ should induce cell death significantly ($P<0.05$), as show in Fig. 1B-C. Notable prevention ability were observed in A549 after MitoQ treatment ($P<0.05$).

MitoQ alleviated the redox damage of A549 induced by $PM_{2.5}$

In order to confirm the redox damage of A549 exposed to $PM_{2.5}$, we detected the ROS generation in A549 cells treated with $PM_{2.5}$ after 4 μM MitoQ administration (2 h) by flow cytometry (Fig. 2A-B), $PM_{2.5}$ increased the ROS generation significantly ($P<0.05$), MitoQ could reduce the excessive ROS production induced $PM_{2.5}$ significantly ($P<0.05$). The level of T-AOC and GSH in A549 cells were both downregulated after $PM_{2.5}$ exposure (Fig. 2C-D), MitoQ elevated the level of T-AOC and GSH and lessened the reduction of the level of T-AOC and GSH caused by $PM_{2.5}$

(P<0.05). We also detected both *Nrf2* and *p62* expression via WB analysis, the protein level of *Nrf2* and *p62* were downregulated by $PM_{2.5}$ exposure and upregulated by MitoQ treatment, meanwhile, the expression of *Nrf2* and *p62* in A549 pretreated by MitoQ (4 μM, 2 h) and exposed by $PM_{2.5}$ (200 μg/mL, 24 h) were increased significantly relative to $PM_{2.5}$ exposure (P<0.05).

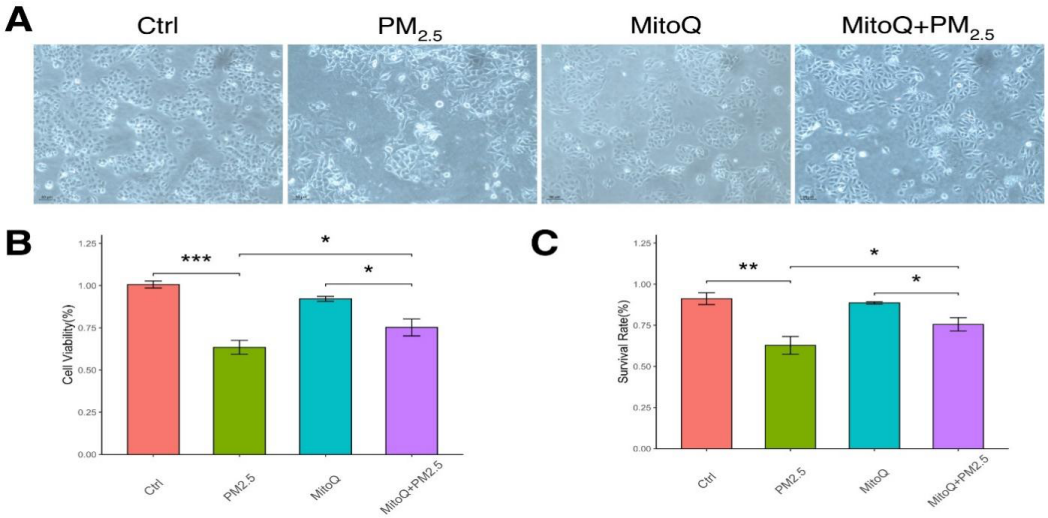

Fig. 1: $PM_{2.5}$ induced cell death, MitoQ can lessen cell death caused by $PM_{2.5}$. A. Representative pictures showed the morphological cell death after 200 μg/mL $PM_{2.5}$ exposure for 24 h and 4 μM MitoQ treatment for 2 h. B-C. The CCK8 assay and The 0.4% trypan staining of A549 cells after 200 μg/mL $PM_{2.5}$ exposure for 24 h and pretreated MitoQ (4 μM, 2 h) vs. controls (Ctrl). Data was calculated from 3 independent experiments (Error bar, mean ± SE) and analyzed by ANOVA and Tukey post-hoc test. * P<0.05, ** P<0.01, *** P<0.001

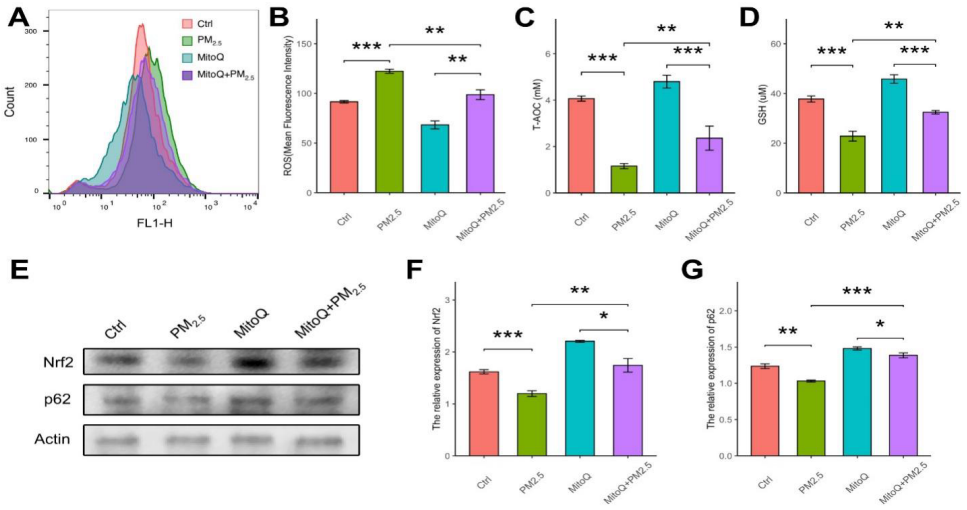

Fig. 2: $PM_{2.5}$ induced excessive ROS generation in A549 cells, MitoQ could inhibit ROS generation through Nrf2/p62 pathway activation. **A.** ROS generation of A549 cells were measured by flow cytometry. **B.** Quantitative analysis of mean fluorescence intensity of ROS. **C.** The concentrations of T-AOC in A549 after $PM_{2.5}$ exposure (200 μg/mL, 24 h) and MitoQ treatment (4 μM, 2 h) were detected. **D.** The levels of GSH in A549 after $PM_{2.5}$ exposure (200 μg/mL, 24 h) and MitoQ treatment (4 μM, 2 h) were tested. **E.** The expression of *Nrf2* and *p62* in A549 cells after $PM_{2.5}$ exposure and MitoQ treatment were measured by WB analysis, *β-actin* was used as a loading control. F-G. Quantification of average WB band intensities of *Nrf2* and *p62*. Data was calculated from 3 independent experiments (Error bar, mean ± SE) and analyzed by ANOVA and Tukey post-hoc test. * P<0.05, ** P<0.01, *** P<0.001

MitoQ eliminated mitochondrial dysfunction in A549 treated by $PM_{2.5}$

The excessive ROS production would involve dysfunction of mitochondria, which increases in mitochondrial fragmentation and induces the insufficient generation of ATP. First, the decreased ATP activity were observed in A549 cells exposed by 200 μg/mL $PM_{2.5}$ for 24 h, and MitoQ treatment could reverse these effects (Fig. 3A).

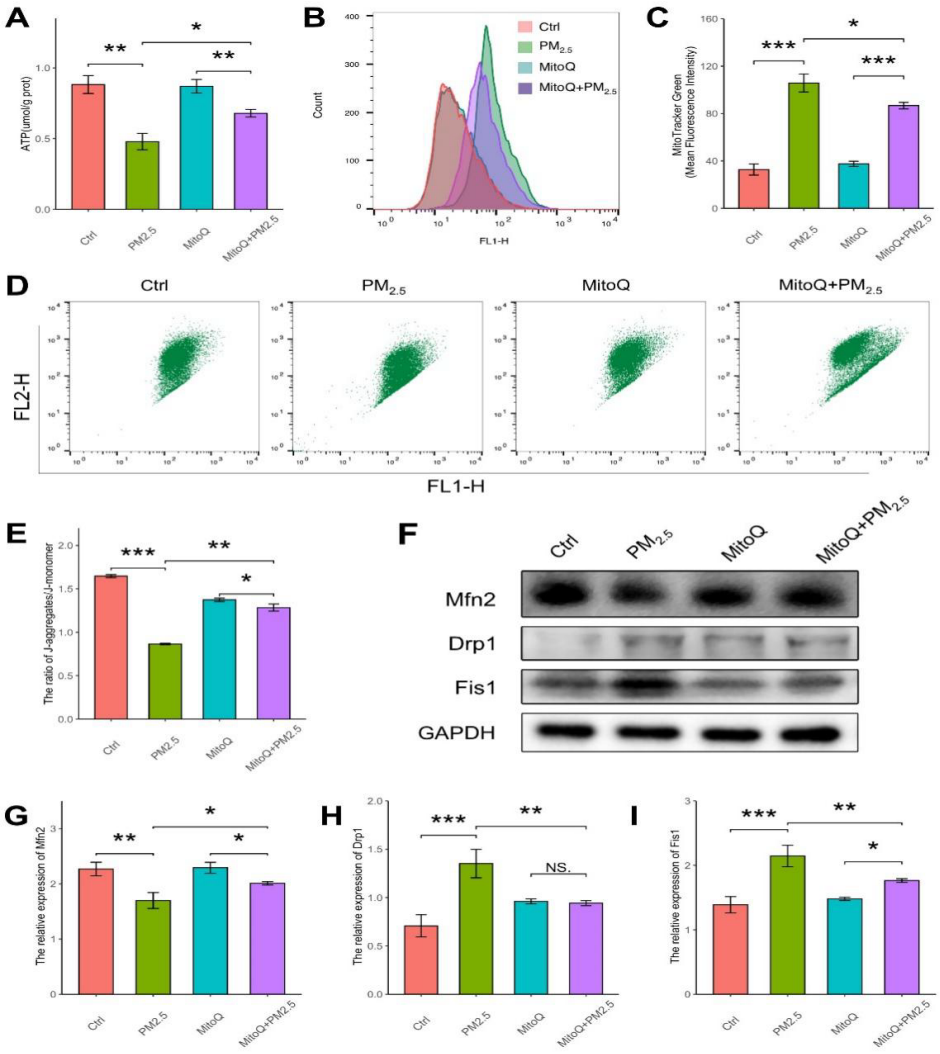

Fig. 3: MitoQ could attenuate mitochondrial damage in A549 cells subjected to $PM_{2.5}$ exposure. **A.** Cellular ATP activities in A549 after $PM_{2.5}$ exposure (200 μg/mL, 24 h) and MitoQ treatment (4 μM, 2 h) were measured. **B.** Active mitochondria was detected using MitoTracker Green FM by flow cytometry. **C.** Quantitative analysis of mean fluorescence intensity of active mitochondrial staining by MitoTracker Green FM. **D.** The change of MMP was detected using JC-1 by flow cytometry. **E.** Quantitative analysis of the ratio of J-agaregates and J-monomer by JC-1. **F.** The expression of *Mfn2, Drp1* and *Fis1* in A549 cells a after $PM_{2.5}$ exposure and MitoQ treatment were measured by WB analysis, *GAPDH* was used as a loading control. G-I. Quantification of average WB band intensities of *Mfn2*, *Drp1* and *Fis1*. Data was calculated from 3 independent experiments (Error bar, mean ± SE) and analyzed by ANOVA and Tukey post-hoc test. * P<0.05, ** P<0.01, *** P<0.001, NS, non-significant

Next, we assessed whether MitoQ can protect mitochondrial dysfunction induced by PM2.5. As shown in Fig. 3B-C, the fluorescence intensity results showed that MitoQ treatment significantly alleviated PM2.5 induced mitochondrial damage (P<0.05). Meanwhile, we monitored the changes of MMP in A549 cells using JC-1 staining (Fig. 3D-E), the results showed that the loss of MMP caused by PM2.5 (200 μg/mL, 24 h) was attenuated by MitoQ pretreatment (4 μM, 2 h).

MitoQ regulated the dynamics of mitochondria of A549 induced by $PM_{2.5}$

We measured the protein expression of *Fis1*, *Drp1* and *Mfn2* via WB analysis (Fig. 3F-I), the increased *Fis1* expression in A549 after $PM_{2.5}$ exposure accompanied by up-regulated *Drp1* expression, simultaneously, decreased *Mfn2* expression was noted. However, these changes were significantly lessened by MitoQ treatment (P<0.05).

MitoQ reduced the mitochondrial-mediated apoptosis of A549 expossed by $PM_{2.5}$

To confirm the pivotal role of mitochondria-mediated apoptosis of A549 exposed by $PM_{2.5}$ and treated by MitoQ. Firstly, the ratio of apotopsis increased after $PM_{2.5}$ exposure, and MitoQ remarkably alleviated upregulated apotopsis (P<0.05) in A549 exposed by $PM_{2.5}$ (Fig. 4A-B). In our study, (Fig. 4C-F) decreased *Bcl-2/Bax* ratio as well as upregulated Caspase-3 in A549 cells were exposed by $PM_{2.5}$, while decreased expression of *Mcl-1*. Pretreated MitoQ could eliminated the promoting apoptosis effect of $PM_{2.5}$ (P<0.05), indicating MitoQ exerts protective effect on $PM_{2.5}$ induced A549 cells injury via the activation of apoptosis.

Discussion

The WHO reported that ambient air pollution is one of the greatest environmental risk to health with increasing economic and social pressures (5,6). As known as $PM_{2.5}$, which is main component of ambient air pollution, it causes cardiovascular and respiratory disease (5,7). A growing number of studies have showed that $PM_{2.5}$ can trigger pulmonary inflammatory responses and impair lung function (16,17). $PM_{2.5}$ could induce cell death via a variety of mechanisms, such as apoptosis, autophagy, and ferroptosis (7,18). This study also showed that $PM_{2.5}$ exposure led to a change in the cell morphology and a decrease in cell viability, which has been consistent with recent studies (10,19,20). Mitochondria has been suggested the highly sensitive target of $PM_{2.5}$ toxicity (21-23). $PM_{2.5}$ has been shown to accumulate within mitochondria (10,21,22), decrease the MMP and induce mitochondrial dynamics disorder (10), which manifested as enhanced mitochondrial fission and weaken fussion. As some recent studies (8,18,24), redox damage could occur through $PM_{2.5}$ induced ROS generation and decreased antioxidant enzyme activity in this study. An excessive generation of ROS could be generally associated with apoptosis activation (8,24), the ratio of *Bcl-2/Bax* was decreased and Caspase-3 expression was increased as a reaction to $PM_{2.5}$ exposure this study. Therefore, we concluded from these cumulative results that excessive generation of ROS and mitochondrial-mediated apoptosis were key point in $PM_{2.5}$ triggered cytotoxicity.

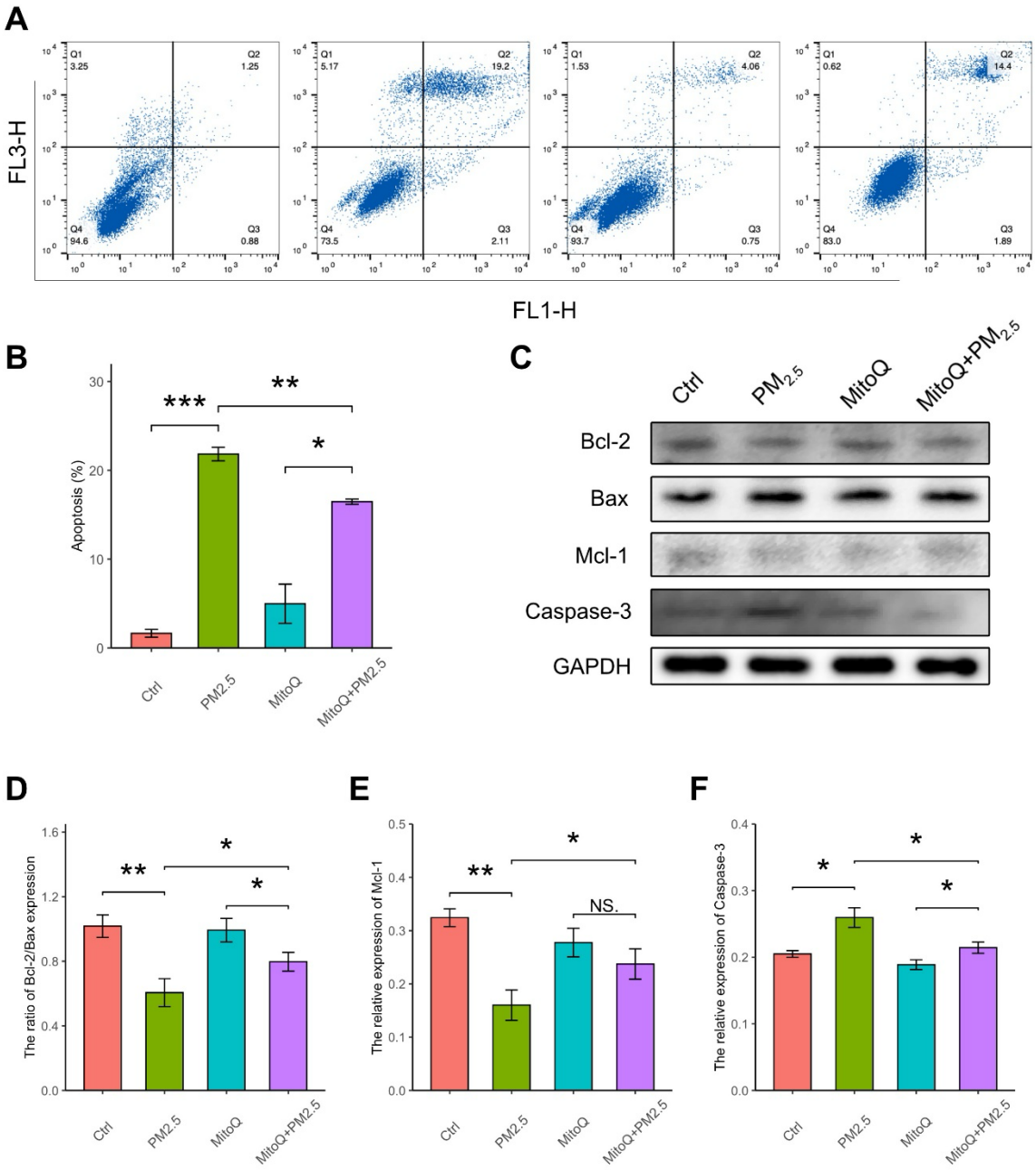

Fig. 4: MitoQ lessened $PM_{2.5}$ induced activation of mitochondrial-mediated apoptosis in A549 cells. **A.** Apoptosis of A549 cells were measured by flow cytometry. **B.** Quantitative analysis of ratio of apoptosis. **C.** The expression of *Bcl-2* family proteins, which included *Bcl-2, Bax, Mcl-1* and *Caspase-3*, in A549 cells after $PM_{2.5}$ exposure and MitoQ treatment were measured by WB analysis, *GAPDH* was used as a loading control. **D.** Quantitative analysis of the ratio of *Bcl-2/Bax*. **E-F**. Quantification of average WB band intensities of *Mcl-1* and *Caspase-3*. Data was calculated from 3 independent experiments (Error bar, mean ± SE) and analyzed by ANOVA and Tukey post-hoc test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, NS, non-significant

Increasing studies indicated that inhibition of oxidative stress and mitochondrial damage should be an effective method to relieve pulmonary diseases caused to $PM_{2.5}$ and prevent pulmonary in-

jury. MitoQ, which reduces mitochondrial ROS, blocks lipid peroxidation and attenuates mitochondrial damage (12), was implicated in this study. As reported that MitoQ alleviated hypoxic pulmonary vasoconstriction, chronic hypoxia-induced pulmonary hypertension and right ventricular remodelling in mouse (25). Sepsis-induced acute lung injury has been shown that could be attenuated through the activation of the *PI3K/Akt/mTOR* pathway by MitoQ in rats (26). In vitro studies, lipopolysaccharide (LPS) had activated alveolar epithelial cell (AEC) (27) and human pulmonary microvascular endothelial cells (HPMECs) (28) apoptosis, which caused acute lung injury (ALI) and respiratory distress syndrome (27,28). In this study, we first observed the protective effect of MitoQ on A549 cells injury caused to $PM_{2.5}$ exposure, manifested as improving the generation of ROS, the decrease in MMP and activation apotopsis.

Here, we also investigated the molecular mechanisms by which MitoQ regulates A549 cells apoptosis under $PM_{2.5}$ exposure. *Nrf2* is an emerging regulator of cellular resistance to oxidants, *p62* is a target gene for Nrf2 and creates a positive feedback loop by inducing antioxidant responses. MitoQ could attenuate cell apoptosis via Nrf2-dependent mechanism (27,28), our study had shown that MitoQ pretreatment could upregulate *Nrf2* and *p62* expression and activity and restore downregulated *Nrf2* and *p62* expression induced by $PM_{2.5}$ exposure, suggesting MitoQ could activate *Nrf2/p62* pathway to reduce redox damage. We specifically focused on and analyzed the correlation between mitochondrial damage and the expression patterns of mitochondrial fusion and fission proteins. The protective effects of MitoQ for ALI was attenuated with *Drp1* overexpression induced by LPS and regulated the *Drp1*-mediated mitochondrial fission (27). In this study, MitoQ partially rectified mitochondrial dysfunctions and reduced mitochondrial fragmentation in A549 cells. In addition, dysfunction mitochondria play key roles in activating apoptosis (29). It suggested that this process could be controlled by the *Bcl-2* family. We found the ratio of apoptosis increased after $PM_{2.5}$ exposure, MitoQ could inhibit $PM_{2.5}$ induced apoptosis. *MCL-1*, which is a widely recognized pro-survival member of the *Bcl-2* family, is a promising target for much disease therapy (30). *Mcl-1* plays important role of mitochondrial quality control and homeostasis regulation, our study suggested that downregulated *Mcl-1* expression induced by $PM_{2.5}$ could be restored by MitoQ. All these findings suggested that MitoQ reversed the increase in ROS and the mitochondrial damage caused by PM2.5, thereby inhibiting mitochondrial-mediated apoptosis. Recent studies had indicated that non coding RNA (ncRNA) participated in variety of physiological and pathological processes after $PM_{2.5}$ expression, and the expression of ncRNA change during pulmonary diseases (31,32). MitoQ can maintain mitochondrial metabolism and dynamic stabilization through affecting the expression of ncRNAs (34). ncRNA can interactive *Nrf2* or *Bcl-2* signaling pathways (34,35). In the future, the regulation of ncRNAs induced by MitoQ would be explored.

In recent years, there has been increasing attention paid to the pulmonary injury induced by mitochondrial damage. In order to overcome this problem, MitoQ, as a mitochondria-targeted antioxidant, would be considered to play an important role in protecting the lung from damage, as proved in vitro and in vivo (26-29). Meanwhile, some clinical studies had been in progress. In a double-blind randomized clinical trial, combined MitoQ and cycle ergometer (ET) had more prominent improvements in cardiac health and amelioration of blood pressure in patients with Hypertension (36). After administration of oral supplementation with MitoQ the patients with impaired endothelial function improved brachial artery flow-mediated dilation, aortic stiffness, and plasma oxidized low-density lipoprotein (37,38). A preprint study showed MitoQ and mitoquinol

mesylate could play an anti-inflammatory and antiviral role through its mitochondrial antioxidant properties (39). Although MitoQ can reduce pulmonary damage in vitro and in vivo, but the clinical trials showing that MitoQ treats or protects the pulmonary disease are still lacking (26-29). Future studies can develop these aspects to determine whether MitoQ treatment reduces the pulmonary injury in patients.

There were some limitations in this study. ROS were generated by multiple cellular processes, mainly including imbalance reduced nicotinamide adenine dinucleotide phosphate (NADPH) and mitochondrial ROS production (40). Our study only detected total ROS production; it cannot be fully stated mitochondrial ROS (mtROS). In spite of the dreased ATP activity and MMP are indirectly demonstrated the generation of mtROS, mitoSOX assay should be used to measure mtROS directly (41). The potential molecular mechanism, which the change of *Nrf2* and *Bcl-2* family induce by MitoQ treatment, is still unclear, and further experiments are needed to indicate the regulatory mechanisms of *Nrf2* expression in mediating the regulation of *Bcl-2* family. Moreover, we did not perform an animal trial due to our limited experimental condition, the preventive and therapeutic effects of MitoQ cannot be observed directly.

Conclusion

Taken together, our study confirmed the generation of ROS, mitochondrial damage and activation mitochondrial-mediated apoptosis induced by $PM_{2.5}$ exposure. MitoQ could improve the adverse effects caused to $PM_{2.5}$. The underlying mechanisms may involve into reducing mitochondrial ROS, maintaining mitochondrial dynamic balance, inhibiting apoptosis via Bcl-2 family. These findings identify a beneficial role of MitoQ as a potential substance for relieving the adverse effects of $PM_{2.5}$ exposure. For further clinical investigation, we will focus on solving this important question and design the animal or clinical study to determine the effect of MitoQ on protecting the pulmonary injury caused by $PM_{2.5}$

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Funding

This work was supported by the General Program of the Natural Science Foundation of Fujian Province, China (No. 2020J011251), the Training Project for Young and Middle-aged Key Talents of Fujian Provincial Health Commission, China (2020GGA078).

Conflict of interest

The authors declare that there is no conflict of interests.

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