MnTMPyP inhibits paraquat-induced pulmonary epithelial-like cell injury by inhibiting oxidative stress

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ABSTRACT

Objective: To investigate the protective effect and underlying mechanism of the superoxide dismutase mimic, manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), on paraquat (PQ)-induced lung alveolar epithelial-like cell injury. Methods: Lung alveolar epithelial-like cells (A549) were pretreated with 10 μM MnTMPyP for 1.5 hr and then cultured with or without PQ (750 uM) for 24 hr. Cell survival was determined using the MTT assay. Apoptosis, mitochondrial transmembrane potential, reactive oxygen species (ROS) production, and Ca2+ levels were measured using flow cytometry. Glutathione reductase activity (GR activity) and caspase-3 activation were determined using spectrophotometry. Expression of the apoptosis proteins, Bcl-2 and Bax, and the endoplasmic reticulum (ER) stress proteins, glucose regulatory protein 78 (Grp78) and C/EBP homologous protein (CHOP), was measured using Western blot analysis. Results: Cell viability, mitochondrial membrane potential, GR activity, and Bcl-2 expression were decreased, but apoptosis, ROS production, caspase-3 activity, cytoplasmic Ca2+ levels, and Bax, Grp78 and CHOP expression were all increased in the PQ group compared to the control group. There were no statistically significant changes in the MnTMPyP group. Cell viability, GR activity, mitochondrial membrane potential, and Bcl-2 expression were all increased, while apoptosis, ROS production, cytoplasmic Ca2+ levels, caspase-3 activity, and Bax, Grp78 and CHOP expression were all significantly reduced in the MnTMPyP group compared to PQ group. Conclusion: MnTMPyP effectively reduced PQ-induced lung epithelial-like cell injury, and the underlying mechanism is related to antagonism of PQ-induced oxidative stress.

Key words: MnTMPyP, Paraquat, Lung alveolar epithelial cell, Oxidative stress, Apoptosis

INTRODUCTION

Paraquat (PQ; 1,1′-dimethyl-4,4′-bipyridinium dichloride) is a strong herbicide that has been widely used in the agricultural field since 1960 (Müller et al., 2018). However, PQ results in high toxicity after being absorbed via gastrointestinal ingestion, inhalation, or skin contact in both animals and humans. Due to a lack of antidotes, the mortality rate attributed to PQ poisoning remains extremely high. Accidental or intentional ingestion of PQ has led to thousands of deaths in Asia, with a mortality rate of 60-70% (Gil et al., 2014). The lung is the primary target organ of PQ poisoning. PQ can cause pulmonary edema, hemorrhage and exudation, as well as inflammatory cell infiltration of lung interstitial and alveoli, fibroblast proliferation, and excessive deposition of collagen, all of which results in lung and bronchi epithelial cell damage and subsequent pulmonary fibrosis (Ahmed, 2009). The typical clinical manifestation of PQ poisoning is termed “paraquat lung”. Early manifestation is acute respiratory distress syndrome, while irreversible pulmonary interstitial fibrosis manifests at later stages, at which point a majority of patients suffer respiratory failure and death.

The exact mechanism by which PQ poisoning causes lung injury remains unclear. However, many studies have confirmed that PQ-induced redox reactions produce large amounts of oxygen free radicals that cause lung injury. Reactive oxygen species (ROS) oxidize unsaturated fatty acids on various biofilms to trigger endoplasmic reticulum (ER) stress and apoptosis cascades, which exacerbate the lung injury. Currently, there is no available effective antidote for PQ poisoning, although many studies have focused on reducing ROS production, eliminating generated ROS, and reducing inflammatory responses against PQ-induced lung toxicity (Hasanuzzaman et al., 2018; Hu et al., 2017a; de Oliveira et al., 2018). How-
ever, these treatments have been disappointing and mortality still remains high. Thus, there is an urgent need to develop new effective treatments.

The role of oxidative stress and altered superoxide dismutase (SOD) levels in PQ-induced lung injury have been widely reported (Pourgholamhossein et al., 2018; Shen et al., 2017). Previous studies showed that a SOD mimic reduced PQ-induced oxidative stress (Cochemé and Murphy, 2008; Patki and Lau, 2011; Pourgholamhossein et al., 2018). SOD mimics can rescue an impaired antioxidant defense system as they are able to penetrate a cell and effectively eliminate superoxide ions. Superoxide ions are produced in the mitochondria due to electron transfer reactions. PQ has been reported to damage mitochondrial oxidation as evidenced by the following reports: Human peroxiredoxin 5 expression in mitochondrial showed effective protection against PQ poisoning in yeast (Tiên Nguyên-nhu and Knoops, 2003); manganese-SOD (MnSOD) heterozygous mice (MnSOD is the SOD isoform located in the mitochondrial matrix) are hypersensitive to PQ; RNA silencing of MnSOD increases sensitivity to PQ toxicity in flies (Kirby et al., 2002); and mitochondrial dysfunction-induced apoptosis is a primary mechanism underlying PQ toxicity in vitro (Zhao et al., 2017). Thus, the effect of PQ on the mitochondria plays a key role in mediating toxicity.

Assessing the effects of SOD mimics could provide new insight regarding the association between mitochondrial dysfunction and oxidative stress. Manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP) is a metalloporphyrin that can suppress lipopolysaccharide-induced generation of dopaminergic neurodegeneration and free radicals (Wang et al., 2004) and protect against peroxynitrite-induced glial cell death by suppressing mitochondrial transmembrane potential (Choi et al., 2000). Treatment of ischemia-reperfusion kidney injury with MnTMPyP effectively reduced expression of the pro-apoptotic protein Bax and inhibited caspase-3 activation by scavenging free radicals (Nilakantan et al., 2010). It has also been demonstrated that PQ-induced oxidative stress and increased intracellular mitochondrial permeability inactivate MnSOD and decrease cell viability (Cochemé and Murphy, 2008; Patki and Lau, 2011; Wang et al., 2004). However, the underlying mechanism of PQ-mediated ER toxicity in lung injury has not been well investigated.

To determine whether MnTMPyP can mitigate PQ toxicity in lung epithelial cells, we measured cell survival, apoptosis, mitochondrial transmembrane potential, ROS, Ca2+ levels, glutathione reductase activity (GR activity), caspase-3 activation, and protein expression of Bax, Bcl-2, C/EBP homologous protein (CHOP), and glucose regulatory protein 78 (Grp78) in lung alveolar epithelial-like cells (A549) pretreated with MnTMPyP prior to PQ exposure.

MATERIALS AND METHODS

Materials and reagents

Reagents used in this study were all purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. PQ was dissolved in phosphate buffer saline (PBS) to prepare a stock solution and kept at 4°C. MnTMPyP was purchased from Merck Millipore (Darmstadt, Germany) and was reconstituted with PBS and stored at −20°C. Antibodies were purchased from Proteintech Group (Rosemont, IL, USA) or Abcam (Cambridge, UK).

Cell culture and treatment

A549 cells (human alveolar type II epithelial cells) were obtained from the Experimental Center of China Medical University (Shenyang, China). Cells were cultured with RPMI 1640 medium (Hyclone; GE Healthcare Life Science, UT, USA) containing 10% fetal bovine serum (FBS) (Gemini Bio, West Sacramento, CA, USA) at 37°C in an incubator with 5% CO2. A549 cells were pretreated with 10 μM of MnTMPyP (Choi et al., 2000) for 1.5 hr, followed by incubation with PQ (750 μM) for 24 hr. Control cells only received PBS.

Cell viability assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay was conducted to evaluate cell viability. Briefly, following treatment, the cell culture media was replaced with media containing the MTT reagent (5 mg/mL), and incubated for 4 hr. A total of 150 μL dimethylsulfoxide (DMSO) was added into each well to solubilize the formazan crystals after carefully discarding the culture media. A Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to measure the absorbance at a wavelength of 490 nm. Relative viabilities of challenged cells to the control cells were calculated.

Apoptosis measured by flow cytometry

PQ-induced cell apoptosis was detected using the Annexin V-FITC/PI kit (Dojindo, Tokyo, Japan). Briefly, cells stained with Annexin V-FITC/PI were sorted using a flow cytometer (BD FACSCalibur, Becton Dickinson Co., Franklin Lakes, NJ, USA) after treatment.
Mitochondrial membrane potential (ΔΨm) assay

1) Detection of ΔΨm using flow cytometry

The mitochondrial membrane potential (ΔΨm) was measured using rhodamine 123 dye. Briefly, 2 × 10^5 cells/well in a 6-well plate were subjected to designated treatments and incubated at 37°C for 30 min in the dark with 5 μg/mL of rhodamine 123/well. After washing with PBS, the pellet was re-suspended in 1 mL of PBS and immediately analyzed using a flow cytometer.

2) Rh123 staining

A total of 2 × 10^5 cells/well in a 6-well plate were exposed to designated treatments. DCFH-DA was diluted with serum-free medium to produce a concentration of 10 μM for 20 min in the dark at 37°C after being washed with PBS three times, trypsinized, and re-suspended in 1 mL of serum-free medium. The 5 mM N-acetyl-L-cysteine was used to compare the efficiency of MnTMPyP. Intracellular ROS generation was measured using the Fluorometric Intracellular ROS Kit (Beyotime, Shanghai, China) using 2′,7’-dichlorofluorescein diacetate (DCFH-DA). Briefly, 2 × 10^5 cells/well in a 6-well plate were exposed to designated treatments. DCFH-DA was diluted with serum-free medium to produce a 10 μM working solution. Cells were incubated with DCFH-DA for 20 min in the dark at 37°C after being washed with serum-free media. The cells were then washed with serum-free medium three times, trypsinized, and re-suspended in 1 mL of serum-free medium. The 5 mM N-acetyl-L-cysteine was used to compare the efficacy of MnTMPyP. ROS was measured using a flow cytometer with a 488 nm laser for excitation and 535 nm for detection.

Detection of intracellular calcium levels

Fura-3/AM (Beyotime), a membrane permeable derivative of the ratiometric calcium indicator, was used to measure intracellular calcium levels. Fura-3 specifically binds to cytosolic calcium after the acetoxyethyl group of fura-3/AM is removed by cellular esterases. Briefly, 2 × 10^5 cells/well in a 6-well plate were treated as above, followed by incubation with 5 mM of fura-3/AM/well at 37°C for 30 min in a CO₂ incubator. Intracellular calcium levels were immediately analyzed using a flow cytometer following three washes with PBS.

Measurement of caspase-3 activity

The Caspase-3 Colorimetric Assay Kit (Nanjing KeyGEN Biotech Institute, Nanjing, China) was used to determine caspase-3 activity. Caspase-3 pNA cleaved Ac-DEVD-pNA substrate was spectrophotometrically quantified by an ELISA reader at 405 nm. Relative caspase-3 activity was calculated by the change in optical density.

Measurement of glutathione reductase (GR) activity

The bicinechonic acid (BCA) protein assay kit was used to measure protein concentration. GR activity was evaluated using the glutathione reductase assay kit (Beyotime) by monitoring glutathione-dependent oxidation of NADPH at 340 nm. NADPH oxidation was corrected without GSSG and expressed as mU/mg protein; one unit indicated the nmol of NADPH oxidized/min at 2°C.

Western blot assay

Protein expression of Bax and Bcl2 (apoptosis markers), and C/EBP homologous protein (CHOP) and Grp78 (ER stress markers) were determined using Western blot analysis. In brief, harvested cells were washed with ice-cold PBS and lysed in 100 μL of lysis buffer with 1% phenylmethane sulfonyl fluoride (PMSF) on ice for 30 min, followed by centrifugation at 12,000 rpm at 4°C for 30 min. The BCA kit was used to measure protein concentration. A total of 30 μg of protein were separated by 10% sodium dodecylsulfate-polyacrylamide electrophoresis gel and transferred onto a polyvinylidenefluoride membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline with 0.1% (v/v) Tween-20 (TBST) at room temperature for 2 hr, and incubated with anti-Bcl2 (1:1,000), anti-Bax (1:4,000), anti-CHOP (1:1,000), anti-Grp78 (1:1,000), or anti-β-actin (1:1,000) antibodies overnight at 4°C, and further incubated with a secondary goat anti-mouse IgG or horseradish peroxidase conjugated goat anti rabbit IgG antibody (Beijing Dingguo...
Changsheng Biotechnology Co. LTD, Beijing, China) for 2 hr at room temperature. The membranes were exposed to X-ray film following brief incubation with an enhanced chemiluminescence (ECL) solution. Protein bands were quantified using the Image Pro Plus 6.0 software (Media Cybernetics Inc., Sarasota, FL, USA).

Statistical analysis
Three independent experiments were performed and results are presented as the mean ± standard deviation (SD). All statistical analyses were conducted by GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). A two-tailed Student’s t-test was used to determine the differences between two groups, one-way analysis of variance (ANOVA) was used for Multiple comparisons, and Dunnett’s test was used to analyze group difference. P-value ≤ 0.05 was considered statistically significant.

RESULTS

MnTMPyP reduces PQ-induced apoptosis
PQ significantly reduced A549 cell viability, and MnTMPyP inhibited PQ-mediated cytotoxicity without any side effects. These data indicate that MnTMPyP protects against PQ-induced cytotoxicity and that MnTMPyP is not cytotoxic at the concentrations tested (Fig. 1A). There were low levels of apoptosis in the control group (Fig. 1B); however, PQ-induced apoptosis (27.6%) was significantly reversed by MnTMPyP (18.3%) (P < 0.05), indicating that MnTMPyP effectively inhibits PQ-induced A549 cell apoptosis.

MnTMPyP reduces PQ-mediated ΔΨm depolarization
Since PQ can interfere with mitochondrial complex activity, increase free-radical formation (Cochemé

![Fig. 1.](image-url) Lung alveolar epithelial-like cells (A549) were pretreated with 10 μM of the superoxide dismutase mimic MnTMPyP for 1.5 hr and then cultured with or without PQ (750 μM) for 24 hr. A. Cell viability was determined using the MTT assay. B. Cell apoptosis was determined using flow cytometry. ** P < 0.01 vs control group; # P < 0.05 vs PQ group; ## P < 0.01 vs PQ group. a. percentage of apoptotic A549 cells; b. representative graphs of apoptotic A549 cells.
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and Murphy 2008) and decrease mitochondrial membrane potential ($\Delta \Psi_m$), we investigated the effect of PQ on $\Delta \Psi_m$. PQ decreased rhodamine 123 fluorescence and increased DCFH-DA fluorescence. These data suggest that PQ decreases $\Delta \Psi_m$ and increases mitochondrial ROS, further supporting our hypothesis that the mitochondria is the target site of PQ. Pretreatment with MnTMPyP significantly repolarized the $\Delta \Psi_m$ and reduced ROS production, as shown in the Fig. 2 (P < 0.01). This indicates that MnTMPyP effectively inhibits PQ-induced decreases in $\Delta \Psi_m$ in A549 cells. To confirm the ROS scavenging effect of MnTMPyP, we also treated the cells with N-acetyl-L-cysteine (NAC), which is a well-known ROS scavenger, as a positive control. These results suggest that MnTMPyP acts as an efficient ROS scavenger under PQ-exposed conditions.

**MnTMPyP preserves GR activity in PQ-induced A549 cells**

GR catalyzes NADPH to reduce oxidized glutathione

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**Fig. 2.** Lung alveolar epithelial-like cells (A549) were pretreated with 10 $\mu$M of the superoxide dismutase mimic MnTMPyP for 1.5 hr and then cultured with or without PQ (750 $\mu$M) for 24 hr. After 24 hr treatment, mitochondrial membrane potential and intracellular ROS levels were measured. A (representative graphs) and B (the value), analysis of mitochondrial membrane potential of A549 cells by flow cytometry. C. Inverted fluorescence microscope observation of mitochondrial membrane potential fluorescence intensity (original magnification 400 x). D (representative graphs) and E (the value), analysis of ROS fluorescence intensity of A549 cells by flow cytometry. We added N-Acetyl-L-cysteine (ROS inhibitor) which used to compare the efficacy of MnTMPyP. F. Inverted fluorescence microscope observation of ROS fluorescence intensity (original magnification 400 x). ** $P < 0.01$ vs control group; *** $P < 0.01$ vs PQ group.
Fig. 2. (Continued).
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(GSSG) into glutathione (GSH) and helps maintain the GSH/GSSG ratio in vivo. GR plays a key role in removal of ROS in redox reactions. To observe the anti-oxidative stress effect of MnTMPyP, we measured intracellular GR activity in A549 cells subjected to different treatments. As shown in Fig. 3, GR activity was significantly decreased when cells were treated with PQ, and MnTMPyP pretreatment restored GR activity, indicating that MnTMPyP effectively increases GR activity and promotes ROS scavenging.

**MnTMPyP reduces PQ-induced caspase-dependent apoptosis**

Our data showed that MnTMPyP attenuates PQ-induced increases in mitochondrial ROS and decreases in \( \Delta \Psi_m \) (Fig. 2) and GR activity (Fig. 3), indicating that mitochondria are the target site for MnTMPyP to prevent PQ entry and counteract subsequent cytotoxicity. In order to functionally demonstrate this, we measured expression of key anti-apoptotic and pro-apoptotic proteins to further evaluate the signaling mechanisms involved in the caspase-dependent apoptotic protection observed above. Significant differences were observed in Bcl-2 and Bax protein expression and caspase-3 activity across treatment groups (Fig. 4), indicating that MnTMPyP effectively reduces PQ-induced caspase-dependent apoptosis in A549 cells.

**MnTMPyP reduces PQ-induced ER stress**

Ca\(^{2+}\) homeostasis dysfunction in ER causes unfolded protein accumulation and activation of the ER stress-induced apoptosis pathway (Mengesdorf et al., 2001). Therefore, we measured intracellular Ca\(^{2+}\) levels using fura-3/AM. We also investigated the effect of MnTMPyP on activation of the crucial ER stress marker CHOP and expression of the ER stress initiator Grp78 following PQ exposure using Western blot analysis. We found that PQ significantly increased Ca\(^{2+}\) levels and expression of Grp78 and CHOP. Pretreatment with MnTMPyP decreased the PQ-induced elevation of Ca\(^{2+}\) levels and expression of Grp78 and CHOP (Fig. 5). These findings suggest that MnTMPyP prevents PQ-induced ER stress.

**DISCUSSION**

In this study, we showed that MnTMPyP plays a significant protective role against PQ-induced lung epithelial-like cell damage. Furthermore, we showed that MnTMPyP reduces ROS production and inhibits oxidative stress-related mitochondrial damage, intracellular Ca\(^{2+}\) overload, and apoptotic pathway protein activation. Previous studies demonstrated that MnTMPyP antagonizes oxidative stress-induced mitochondrial protein oxidation and depolarization of mitochondrial membrane potential to protect against liver, kidney, and brain ischemia-reperfusion-induced injury (Choi et al., 2000; Nilakantan et al., 2010; Moon et al., 2008). However, there have been relatively few studies focused on the role of MnTMPyP in PQ-induced lung injury.

ROS, as a natural byproduct of normal oxygen metabolism, plays an important role in cell signaling. PQ mainly accumulates in the lungs, where its concentration is 6-10 times higher compared to plasma levels following exposure. During PQ poisoning, ROS levels increase dramatically, resulting in oxidative stress and significant cell structure damage. It has been reported that oxidative stress plays a key role in PQ-induced cell injury (Hu et al., 2017b). PQ causes damage to the alveolar epithelium and increases alveolar inflammation, leading to pulmonary fibrosis (Ahmed, 2009).

PQ induces apoptosis of A549 cells through the mitochondrial apoptotic pathway (He et al., 2016). ROS triggers endogenous apoptosis through interaction with mitochondrial permeability transition complex proteins. In response to ROS, the mitochondrial membrane undergoes hyperpolarization, collapsing the mitochondrial membrane potential (\( \Delta \Psi_m \)), and prompting translocation of mitochondrial pro-apoptotic proteins Bax and Bad and release of cytochrome c. The electron transport chain is also disrupted, further increasing ROS production due to the excessive loss of mitochondrial cytochrome c (Chen and Lesniewsky, 2006). Bcl-2 is an
anti-apoptotic protein that can bind to the pro-apoptotic protein Bax and prevent the increase in mitochondrial outer membrane permeability. In addition, Bax-mediated release of cytochrome c triggers caspase-3 activation during apoptosis (Gogada et al., 2011) and apoptotic cascades that reduce Bcl-2 expression but enhance Bax expression (Abe et al., 1988). Caspase-3 is the end product of the endogenous and exogenous apoptotic cascade and its protein level reflects the severity of apoptosis (Drakopanagiotakis et al., 2008). However, in this study, we found that MnTMPyP significantly increased the ΔΨm and Bcl-2 expression, while reducing Bax expression and PQ-induced caspase-3 activity. These findings indicate that MnTMPyP inhibits PQ-induced ROS production and promotes mitochondrial pathway-induced apoptosis.

To confirm the antioxidant effect of MnTMPyP, we measured intracellular ROS levels and GR activity. GSH is a non-enzymatic antioxidant and the natural ROS scavenger and main reducing agent in intracellular biochemical reactions. GSH is also the most abundant sulfhydryl material in the cytoplasm. PQ toxicity involves NADPH oxidation and decreases GR activity (Suntres, 2002). Our results showed that PQ increased ROS production and decreased GR activity. However, MnTMPyP successfully reduced ROS production and increased GR activity, indicating its antioxidative effect.

The ER is an important organelle that maintains protein synthesis, intracellular calcium homeostasis, post-translational modifications, and protein folding. The ER is also an important storage site for Ca2+ and plays a role in the cytoplasmic Ca2+ signaling pathway. Most intra-ER chaperones are Ca2+-binding proteins (Prell et al., 2013). Furthermore, the ER mediates chemical poison-induced apoptosis, which underlies the pathogenesis of many diseases (Li et al., 2015). ROS production and the change of Ca2+ homeostasis result in the accumulation of unfold-

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**Fig. 4.** Lung alveolar epithelial-like cells (A549) were pretreated with 10 μM of the superoxide dismutase mimic MnTMPyP for 1.5 hr and then cultured with or without PQ (750 μM) for 24 hr. After 24 hr treatment, caspase-3 activity and expression of mitochondrial apoptosis pathway proteins were measured. A. Quantitative analysis of caspase-3 activity. B. Representative immunoblots and quantitative analysis of Bcl-2 and Bax protein expression. **P < 0.01 vs control group; ***P < 0.01 vs PQ group.
ed proteins, which further enhances ER stress and apoptosis (Clarke et al., 2014). The unfolded protein response (UPR) can relieve ER stress by promoting protein degradation, reducing protein synthesis, and generating chaperone proteins to assist in the normal folding of proteins. A typical UPR is mediated by three ER transmembrane transduction proteins: phosphorylated extracellular regulated kinase (P-ERK), inositol requiring enzyme 1a (IRE1a), and activating transcription factor 6 (ATF6). The UPR is induced by the chaperone protein Grp78. Under normal conditions, PERK, ATF6 and IRE1 bind to Grp78, but the accumulation of unfolded proteins separates Grp78 from the UPR’s three transmembrane transduction proteins and promotes their activation. Thus, Grp78 is considered an indicator of ER stress, especially when ER stress is initiated by elevated Ca²⁺ in the ER cavity (Li et al., 2015).

CHOP is both an ER stress marker and a pro-apoptotic transcription factor. CHOP expression under physiological conditions is very low, but is significantly increased under severe or persistent ER stress. As a growth-inhibiting and DNA damage-inducing protein, CHOP is a central regulator of ER stress-induced apoptosis, and its activity is regulated by all three UPR transmembrane transduction proteins. When cells are unable to maintain homeostasis, all three UPR proteins collectively promote CHOP expression and further induce apoptosis in response to ER stress (Zhang et al., 2014).

Fig. 5. Lung alveolar epithelial-like cells (A549) were pretreated with 10 μM of the superoxide dismutase mimic MnTMPyP for 1.5 hr and then cultured with or without PQ (750 μM) for 24 hr. After 24 hr treatment, intracellular Ca²⁺ levels and expression of ER stress pathway proteins were measured. A. Intracellular Ca²⁺ levels were analyzed using flow cytometry. B. Western blot analysis was used to measure protein expression of Grp78 and CHOP. **P < 0.01 vs control group; ***P < 0.01 vs PQ group.
The results of this study confirmed that PQ increases ROS, cytoplasmic Ca\(^{2+}\) levels, and expression of ER stress proteins, Grp78 and CHOP. It is suggested that the production of ROS and outflow of Ca\(^{2+}\) induced by PQ may lead to the accumulation of unfolded or misfolded proteins in the ER cavity, disturbing the Ca\(^{2+}\) balance and activating the ER stress response. ER homeostasis is destroyed under sustained ER stress, which in turn activates apoptotic signaling pathways and induces apoptosis. MntTMPyP successfully reduced PQ-induced ROS production and enhanced GR activity, therefore successfully inhibiting ROS-induced ER stress responses by reducing Grp78 and CHOP expression.

In conclusion, we provide evidence to suggest that the ER is the first targeted organelle in PQ-induced lung epithelia cell toxicity. However, both the ER and mitochondria are important in PQ poisoning and need to be further explored. The close intracellular localization of these two organelles may allow for intra-organelle crosstalk, likely via Ca\(^{2+}\) (Malhotra and Kaufman 2011; Dorn and Scorrano, 2010). Therefore, further research is necessary to understand if Ca\(^{2+}\) mediates PQ toxicity between the ER and mitochondria. Overall, our study suggests that MntTMPyP prevents PQ-induced apoptosis by attenuating ER stress and mitochondrial dysfunction, and its mechanism is related to reducing ROS production and the related damage caused by oxidative stress. This study shows that effective anti-oxidant reagents may be key to treating PQ poisoning, which will provide a basis for the design of clinical trials. More work is necessary to completely define the signaling pathways that involve oxidant-mediated programmed cell death in PQ-induced lung injury.

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**Conflict of interest**——The authors declare that there is no conflict of interest.

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