Melatonin reverted apoptosis induced by chicken house $PM_{2.5}$ in A549 cell via oxidation resistance

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Research Article

Keywords: Particulate matter 2.5, Broiler breeding house, Cell survival, Endoplasmic reticulum stress, Oxidative stress

Posted Date: June 14th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1722882/v1

Abstract

The particulate matter 2.5 ($PM_{2.5}$) from the chicken production system attaching large amounts of harmful substances and microbes, can cause lung injury and reduce productivity through prolonged breath. Here, the basal epithelial cell of human lung adenocarcinoma (A549 cell) were subjected to $PM_{2.5}$ from the broiler breeding house to investigate the apoptosis induced by $PM_{2.5}$ as well as the anti-apoptotic effect of melatonin. The data showed that $PM_{2.5}$ (6.25, 12.5, and 25 μg/ml) increased the number of apoptotic cells substantially. The relative mRNA expression of *Bcl-2*, *Bad*, *Bax*, *PERK* and *CHOP* increased dramatically in $PM_{2.5}$ treated groups. Furthermore, $PM_{2.5}$ exposure upregulated the level of cleaved caspase-9 and cleaved caspase-3 as well as the endoplasmic reticulum stress (ERS) related protein including ATF6 and CHOP. Melatonin increased the viability of A549 cells and declined the relative mRNA expression of *Bcl-2*, *Bax*, *Bad*, cleaved caspase-3, cleaved caspase-9 in comparison with that exposed to $PM_{2.5}$. Importantly, melatonin decreased the level of Nrf2, MDA, and enhanced the intracellular content of T-SOD and CAT after treatment by $PM_{2.5}$. Collectively, our study proved that $PM_{2.5}$ from chicken house induced A549 cell apoptosis, and melatonin exerted an anti-apoptotic role on A549 cells by enhancing their antioxidative ability.

Introduction

During animal production, a large scale of particulate matters (PM) was generated, the source of which included feed, feces, animal dander, skin feathers, urine, and padding (Honey and McQuitty, 1979, Liu et al., 2015, Qi, Manbeck and Maghirang, 1992). The components of PM from the animal houses included organic compounds, elements, and ions including NH_4^+, NO_3^-, SO_4^{2-} etc. (Cambra-Lopez et al., 2011, Shen et al., 2018). Moreover, bacteria, fungi, viruses as well as endotoxin, ammonia, and hydrogen sulfide were also attached to the surface of PM (Dai et al., 2020, Donham et al., 1986, Mostafa et al., 2016). The exposure in poultry houses with high PM concentration for a long time was prone to suffer from respiratory diseases, asthma, and chronic obstructive pulmonary diseases, and seriously affected the health of animals and people as well as reduced the production efficiency of poultry (Al Homidan and Robertson, 2003, Cambra-Lopez et al., 2010, Sabino et al., 2012). Our latest research highlighted the systemic exposure of PM induced lung inflammation, microbial and metabolic disorder in broilers(Shen et al., 2022).

Studies have demonstrated that the particle size less than 2.5 μm ($PM_{2.5}$) can cause oxidative stress, inflammatory response as well as genotoxicity in the lung (Cho et al., 2018). Epidemiological investigations have shown that particle size less than 2.5 μm ($PM_{2.5}$) increased the susceptibility to pulmonary infection, chronic obstructive pulmonary disease, and bronchial asthma (Atkinson et al., 2015, Davidson, Phalen and Solomon, 2005). Researches have investigated the concentration proportion of $PM_{2.5}$ in the chicken house. Kilmont and Amann estimated that the proportion of particle size less than 10 μm (PM_{10}) and $PM_{2.5}$ from poultry production systems are 57% and 50% of emissions to the atmosphere from animal housing in the UK, which indirectly confirmed that more inhalable particles were

released from poultry house compare with other animal house (Klimont and Amann, 2002). Our previous study also indicated that the proportion of $PM_{2.5}$/total suspended particles (TSP) and $PM_{2.5}/PM_{10}$ were 20% and 50% respectively (Shen, et al., 2018). The accumulated evidence indicated that $PM_{2.5}$ accounted for a certain proportion of particulate matter in the chicken house, and its potential impact on the respiratory tract caused by long-term exposure cannot be ignored.

Excessive apoptosis can be caused by mechanical force, ray, ischemia, hypoxia, cholesterol, drugs, biological toxins such as endotoxin(MacFarlane and Williams, 2004). The accumulated evidence indicated that abnormal apoptosis was related to lung diseases closely. The patients with diffuse alveolar damage exhibited upregulation of Bax and Bcl-2 in alveolar pneumocytes(Matute-Bello and Martin, 2016). Another study indicated that the apoptosis of type II pneumocytes is partly responsible for the disappearance of these cells in patients with acute lung injury in the early phase(Li et al., 2004). Basal epithelial cells of human lung adenocarcinoma (A549 cell) widely served as the model of type II alveolar epithelial cell which mainly functioned in maintaining alveolar integrity and resisting the invasion of pathogens (Barkauskas et al., 2013). Our previous research has demonstrated that $PM_{2.5}$ from chicken house induced inflammation in A549 cells. Furthermore, few studies have shown that $PM_{2.5}$ from the atmosphere triggered apoptosis in A549 cells, importantly, the sources and the composition of $PM_{2.5}$ in the chicken houses were different from that in the atmosphere, therefore, the discrepant biological effects imposed by $PM_{2.5}$ was indicated.

N-acetyl-5-methoxytryptamine (melatonin) functioned as a regulator in the sleep/wake cycle and other circadian and seasonal rhythms (Siah, Wong and Ho, 2014). Additionally, melatonin played a role in anti-oxidation, anti-inflammation, and anti-apoptosis (Chen et al., 2015, Li et al., 2014, Zhang and Zhang, 2014). Melatonin resisted oxidation not only by scavenging reactive oxygen species (ROS) directly but also upregulating several antioxidant enzymes (Carmen et al., 2010). Melatonin inhibited cell apoptosis primarily through activating the expression of genes related to anti-apoptosis, as well as PI3K/AKT and ERK1/2 signal pathway, and declining cellular ROS content (Luchetti et al., 2009, Vriend and Reiter, 2015, Wang, 2009). In addition, some studies listed as follows have shown that melatonin contributed to animal production. Melatonin can promote ewe in estrus earlier(Sawalha et al., 2011); increase the laying quantity of layers (Jia et al., 2016); improve the daily gain of broilers and pigs (Akbarian et al., 2014, Ayles et al., 1999) and facilitate cashmere growth via Wnt signaling pathways(Zhang et al., 2021). However, the issue on whether melatonin relieves lung damage in animal production remains elusive. Our earlier research indicated the concentration of $PM_{2.5}$ from chicken houses exceeded the annual average concentration of $PM_{2.5}$ from the atmosphere. The composition of $PM_{2.5}$ in the chicken houses included organic carbons, ions, and endotoxin, as well as a large number of harmful bacteria and fungi (Dai, et al., 2020). In this study, we focused on the apoptosis induced by chicken house $PM_{2.5}$ in A549 cells and further investigated the alleviating effect of melatonin on apoptosis in A549 cells.

Materials And Methods

$PM_{2.5}$ sampling and extraction

The chicken house for $PM_{2.5}$ sampling, the season, the chicken ages in house, and the $PM_{2.5}$ extraction methods were consistent with our previous studies (Dai, et al., 2020, Dai et al., 2019). Briefly, the $PM_{2.5}$ in the chicken house was collected on filter membrane (Whatman USA), by BTPM-HS1 atmosphere particles sampler (Dandong Baxter Co., Ltd. Dandong Liaoning China). The $PM_{2.5}$ on filter membranes were oscillated into deionized water and then filtered with six layers of gauze before centrifugation. After drying, the $PM_{2.5}$ was dissolved into saline and then sterilized before being stocked at − 20℃.

Cell culture and treatment

A549 cell line (ATCC, USA) was employed in this experiment and was cultured in F12 medium (Gibco, USA) supplemented with 10% fetal serum (BI, Israel) and 0.5% penicillin-streptomycin-amphotericin B solution (Sigma, USA). Cells were kept at 37 ℃ constantly with 5% CO_2 and the humidity of the incubator was maintained by the water tray in the bottom. The primary components of $PM_{2.5}$ included organic carbon, element carbon, NO_3^-, NH_4^+, SO_4^{2-}, Cl^-, Ca^{2+}, K^+, Na^+, F^- and endotoxin (Dai, et al., 2020). The $PM_{2.5}$ (1mg/ml) and the stocked melatonin (10mmol/ml) (MedchemExpress, NJ, USA) were diluted by the medium at different concentrations (6.25 μg/ml, 12.5 μg/ml, 25 μg/ml for $PM_{2.5}$, and 0, 12.5 μmol, 25 μmol, 50 μmol, 100 μmol, 200 μmol, 400 μmol, 800 μmol, 1600 μmol, and 3200 μmol for melatonin) and the cells were treated after the confluence reached 80%. In cell cycle, cell apoptosis, transmission electron microscope (TEM), RT-PCR, and Western blot assays, the cells were cultured into a 6-cell plate, and then exposed to $PM_{2.5}$ or melatonin; as for MTT assay, the cells were detached into a 96-cell plate and then treated by $PM_{2.5}$ or melatonin.

The detection of cell cycle and cell apoptosis

For cell cycle, A549 cells were washed by ice-cold PBS three times after being digested by trypsin (Gibco, USA). Then the cells were fixed with 70% ethanol for 8 hours and stained with PI (Vazyme Biotech) for 10 min in dark. In cell apoptosis, cells were detached with trypsin excluded EDTA (Gibco, USA), and washed with ice-cold PBS for three times. Cells were centrifuged at 300 g, 4 ℃ for 5 min, and then stained with 5 μl Annexin V-FITC and 5 μl PI (Vazyme Biotech Co. Ltd., Nanjing, China) for 10 min in dark. Each sample was added with 400 μl 1 × binding buffer before detection. Cell cycle and cell apoptosis were determined by flow cytometry (BD, USA).

Transmission electron microscopy (TEM)

The cells were collected and then washed by PBS three times. A549 cells were fixed with 4% glutaraldehyde at 4°C for 72 h, then the cells were rinsed again by PBS and refixed with 1% osmium tetroxide for 30 min at room temperature. The following steps proceeding based on the procedure described by Winey et al(Winey et al., 2014).

The total RNA extraction and real-time PCR

A549 cells were lysed with TRIzol® reagent (Takara, Japan). RNA was extracted by chloroform (Jian Cheng) and isopropanol (Jian Cheng), and the RNA was washed with 75% ethanol (Jian Cheng) two times. The purity and concentration of RNA were detected by NanoDrop® ND-1000 (Thermo). RNA integrity was detected by 0.4% agarose gel electrophoresis. The reverse transcription was performed by the SuperScript (SS) First-Strand Synthesis System (Takara). The real-time PCR (RT-PCR) was conducted on the Applied Biosystem StepOneM Real-Time PCR system (ABI, USA) using SYBR Green Master Mix (Takara). The RT-PCR procedure was as follows: 95 °C for 30 s, 95 °C for 5 s and 34 s at 60°C for 40 cycles, and then 95°C for 15 s, 60°C for 1min, and 95°C for 15 s. The product specificity was determined by melting curve analysis. Every gene in each sample was detected three times. The data processing was performed by the ΔΔCt method (Pfaffl, 2001). The gene primers are shown in Table 1.

Table 1
The sequences of gene primers

Gene	The Sequence of Primers	Gene	The Sequence of Primers
Bad	F: GATCGGGCTTGGGGTGAGAC	CHOP	F: TTCACCACTCTTGACCCTGC
	R: TCATCTGTCTGCCGGGTCTG		R: TTCCTGCTTGAGCCGTTCAT
Bax	F: AGAAGCTGAGCGAGTGTCTC	β-actin	F: GATCTTCATTGTGCTGGGTG
	R: CGGAAAAAGACCTCTCGGGG		R: GGGAAATCGTGCGTGACATT
Bcl-2	F: CTTTGAGTTCGGTGGGGTCA		
	R: GGGCCGTACAGTTCCACAAA		
PERK	F: GCCAATGAGAGAGCAAACGC		
	R: ATCTCGGACATCGCCCATTG		

Western blot assays

Cells were lysed with cell lysis buffer containing 1% PMSF (Solarbio, Co., Ltd, Beijing, China). The protein concentration was detected by the BCA protein concentration detection commercial kit (Sigma). The protein was degenerated by boiling for 5 min after mixing with loading buffer (Beyotime Biotech, Co., Ltd, Shanghai, China). 12% agarose gels were used for protein electrophoresis at 140 V for 55 min, and then the transferring process was performed at 90 v for 70 min. The protein was blocked by 5% bovine serum albumin for 2 h, then incubated with antibody overnight at 4°C. The primary antibody included glucose regulating protein (GRP78) (CST, MA, Danvers, USA), C/EBP-homologous protein (CHOP) (CST), activating transcription factor 6 (ATF6) (CST), cleaved caspase-3 (CST), cleaved caspase-9 (CST) and NF-E2-related factor 2 (Nrf2) (CST). The second antibody includes HRP-conjugated anti-rabbit or mouse IgG (Biosharp, Suzhou, China). β-actin (CST) was used as the internal control.

The detection of cell viability

The A549 cells were cultured in the 96-cell plate for 24 h and then treated with melatonin at different concentrations (0, 12.5 μmol, 25 μmol, 50 μmol, 100 μmol, 200 μmol, 400 μmol, 800 μmol, 1600 μmol, and 3200 μmol). After 12 h, the supernatant was discarded and added 100 μl methyl thiazolyl tetrazolium (MTT) (Jian Cheng Co., Ltd, Nanjing, China) for another 4 h. Then, discarding MTT, the cells were incubated with 150 μl dimethyl sulfoxide (DMSO) per well for 15 min at room temperature. The absorbance was detected by microplate reader (Thermo Scientific, USA).

The detection of T- SOD, CAT, and MDA

The cellular supernatant was collected and centrifuged at 3000 rpm for 10 min to detect the oxidative stress indicators. The activity of total superoxide dismutase (T-SOD), catalase (CAT), and malondialdehyde (MDA) were detected by the xanthine oxidase method, ammonium molybdate colorimetry method, and thiobarbituric acid method respectively, using the commercial kit (Jian Cheng) and basing on the manufacturer guidelines.

Statistical analysis

The data were analyzed by GraphPad Prism 7.0 (GraphPad Software, CA, USA), and the data were shown as means ± standard deviation (SD) depending on at least three independent experiments. The difference between the two groups was performed by t-test (in Fig. 5B, 6B and 7B. One-way ANOVA performed with Tukey test was adopted in comparisons more than two groups either between the control group and treated group or between any two column means. $p < 0.05$ represents a significant difference between the two groups.

Results

The effects of $PM_{2.5}$ on the cell cycle distribution and cell apoptosis of A549 cell

In cell cycle and cell apoptosis, A549 cells were treated with $PM_{2.5}$ at concentrations of 6.25 μg/ml, 12.5 μg/ml, and 25 μg/ml for 12 h. The treatment concentration and time of $PM_{2.5}$ were based on our previous study (Dai, et al., 2019). The results showed that the proportion of G0/G1 phase cells gradually increased while the S phase cells decreased after treatment by $PM_{2.5}$. The proportion of A549 cell in G0/G1 phase cells were 50.2% ± 5.3, 52.4% ± 3.8, 53.9% ± 3.2 and 55.0% ± 2.8, and cells in S phase is 27.1% ± 4.9, 24.4% ± 2.6, 24.1% ± 3.2 and 22.3% ± 4.0, in control group, 6.25 μg/ml, 12.5 μg/ml and 25 μg/ml treated groups respectively (Fig. 1A and B). The results indicated that the cell cycle was disrupted after $PM_{2.5}$ exposure. As shown in Fig. 1C and D, the apoptotic cells increased significantly after exposure to $PM_{2.5}$ at 6.25 μg/ml, 12.5 μg/ml, and 25 μg/ml (Fig. 1D). The results indicated that $PM_{2.5}$ from chicken house induced apoptosis in A549 cells.

The subcellular structure of A549 cells was observed by transmission electron microscope (TEM). As shown in Fig. 2, $PM_{2.5}$ exposure induced nuclear enlargement, chromatin condensation, cytoplasm

shrinkage, and cell surface microvilli fracture (the arrows in the bottom left panel of Fig. 2 indicated) which suggested that cells were going to be apoptosis. Notably, many organelles in the cytoplasm were swelled into vacuoles, including mitochondria and endoplasmic reticulum (the arrows in the bottom right panel of Fig. 2 indicated). We speculated that $PM_{2.5}$ not only induced cell apoptosis but also caused endoplasmic reticulum stress (ERS) in A549 cells. Moreover, the expression of *Bcl-2*, *Bax*, and *Bad* increased significantly at 25 μg/ml in A549 cell ($p < 0.05$) (Fig. 3A). Consistently, the level of cleaved caspase-3 and cleaved caspase-9 increased at 12.5 μg/ml, while showed a declining level at 25 μg/ml after exposure to $PM_{2.5}$ (Fig. 3B).

$PM_{2.5}$ caused ERS in A549 cell

The protein kinase R-like ER kinase (*PERK*) increased considerably in 12.5 μg/ml and 25 μg/ml of $PM_{2.5}$ treated group ($p < 0.01$). The expression of *CHOP* increased significantly in all exposed groups in the A549 cell ($p < 0.05$). There was no change in the expression of GRP78 among all groups. The level of ATF6 and CHOP expressed in an accordant way, which rose gradually while declined at 25 μg/ml of $PM_{2.5}$ in A549 cells (Fig. 4B).

Melatonin alleviated apoptosis of A549 cell caused by $PM_{2.5}$ from chicken house

In this assay, the A549 cells were exposed to melatonin at the concentration of 12.5 μmol, 25 μmol, 50 μmol, 100 μmol, 200 μmol, 400 μmol, 800 μmol, 1600 μmol and 3200 μmol for 12 h. As shown in Fig. 5A, melatonin at 50 μmol and 100 μmol resulted in an increasing tendency on A549 cell viability. Further assays showed that melatonin at 100 μmol improved the proliferation of A549 cells ($p < 0.01$) (Fig. 5B). Consistent with the above results, the melatonin at 100 μmol prevented A549 cell from apoptosis significantly after exposure by $PM_{2.5}$ as shown in Fig. 5C and D. The further results indicated that the release of cytochrome C in the $PM_{2.5}$ treated group presented an upward trend, and melatonin declined the cytochrome C level although there were no substantial changes (data not shown).

Figure 6 showed that melatonin reduced the expression of *Bcl-2*, *Bad*, and *Bax* dramatically in comparison with that in $PM_{2.5}$ exposed group. There were a lower expression of cleaved caspase-9 and cleaved caspase-3 upon the combined treatment of melatonin with $PM_{2.5}$ compared to that in $PM_{2.5}$ treated group.

The effect of melatonin on antioxidant capacity in A549 cell exposed to $PM_{2.5}$

Our earlier research has indicated that $PM_{2.5}$ from chicken house induced oxidative stress proved by increasing the intracellular reactive oxygen species (ROS) content in A549 cell (Dai, et al., 2019). It's similar to what we had before, $PM_{2.5}$ stimulation increased MDA concentration and Nrf2 level in A549 cell (Fig. 7A and B). The melatonin stimulation reduced the level of MDA ($p < 0.05$) and Nrf2 after exposure to

$PM_{2.5}$. Importantly, melatonin improved the release of T-SOD and CAT considerably in $PM_{2.5}$ treated cells (Fig. 7A and B).

Discussion

The mitochondria plays a vital role in regulating apoptosis (Li and Dewson, 2015). Bad and Bid are activated after receiving intracellular death signals, and then changed the conformation of Bax. The Bax inserts into the outer membrane of mitochondria and changes the mitochondrial membrane permeability, leading to the release of apoptotic factors such as cytochrome C which can activate caspase-9 and caspase-3, triggering a series of downstream apoptotic reactions. Bcl-2 as an anti-apoptotic gene, acts on the outer membrane of mitochondrial to maintain its integrity(Hector and Prehn, 2009). Our earlier research has demonstrated that $PM_{2.5}$ from chicken houses contained endotoxin and a large number of organic compounds (Dai, et al., 2020). Emerged evidence has demonstrated endotoxin induced apoptosis in many cell lines (Han et al., 2018, Xie et al., 2019, Zhang et al., 2019). In this study, chicken house $PM_{2.5}$ induced A549 cell apoptosis through enhancing the expression of *Bad*, *Bax*, and activating cleaved caspase-9 and cleaved caspase-3. The current study is consistent with previous results showing that particles from the atmosphere and cooking oil fumes lead to human epithelial lung cell apoptosis (Che et al., 2014, Dagher et al., 2006).

Herein, $PM_{2.5}$ from the chicken house caused ERS in the A549 cell. ERS is well characterized by protein misfolding, accumulation of unfolded proteins, internal calcium loss, or calcium overload, during which many sensors could be activated, including PERK and ATF6 that are released by GRP78, aiming at recovering homeostasis (Xu, Bailly-Maitre and Reed, 2005). PERK and ATF6 can activate CHOP, the function of which promotes the expression Bad and Bax and suppresses Bcl-2 and Bcl-xl, further inducing apoptosis (Iurlaro and Munoz-Pinedo, 2016, Szegezdi et al., 2006). In this study, chicken house $PM_{2.5}$ caused ERS and promoted the expression of *PERK* and *CHOP*. Furthermore, immunoblotting assays indicated that ATF6 and CHOP levels increased after stimulation by $PM_{2.5}$ in A549 cells. In summary, $PM_{2.5}$ from chicken house induced ERS and then caused apoptosis through the PERK/ATF6-CHOP-caspase-3 signal pathway. It's worth noting that the expression of ATF6 and CHOP decreased on the concentration of $PM_{2.5}$ at 25 μg/ml in A549 cell which was consistent with the expression pattern of cleaved-caspase3 and cleaved-caspase9, further demonstrating that the expression of them is in a dose-dependent way.

The increase of the Bcl-2 and the decrease of Bax indicates the resistance of the cells to apoptosis (Pena-Blanco and Garcia-Saez, 2018). Studies shows melatonin had anti-apoptotic properties by improving the Bcl-2 expression and resisting the Bax level. In human monocytic U937 cells irradiated by ultraviolet, melatonin prevented apoptosis by improving Bcl-2 level and declining the release of cytochrome C (Luchetti, et al., 2009). Melatonin exerted protection on neurodegenerative diseases including experimental stroke, Parkinson disease, Alzheimer disease through resisting apoptosis by elevating the Bcl-2 and Bcl-xl expression and inhibiting Bax level (Culmsee and Landshamer, 2006, Lukiw and Bazan,

2006, Mattson, Culmsee and Yu, 2000, Seung-Yun and Seol-Heui, 2010). In this study, melatonin promoted cell proliferation by downregulating the expression of *Bad* and *Bax*, as well as the level of cleaved caspase-9 and cleaved caspase-3. Notably, the expression of *Bcl-2* also declined in A549 cells by melatonin exposure. We speculated that melatonin stimulation increased other anti-apoptotic members of the *Bcl-2* family such as *Bcl-xl*, *Mcl-1*. Moreover, Zhou et. al has demonstrated that melatonin in high concentrations can inhibit the viability and migration of A549 cell (Zhou et al., 2014). Another study indicated that melatonin showed anti-tumor action by resisting the expression of vascular endothelial growth factor (VEGF) which is involved in angiogenesis (Goradel et al., 2017). Herein, melatonin decreased the *Bcl-2* levels, which may prevent the excessive proliferation of A549 cells.

Previous studies have well established that oxidative stress can induce signal transduction pathways involved in apoptosis initiated by mitochondrial (Kujoth et al., 2005, Siah, Wong and Ho, 2014). Melatonin, as an antioxidant, has been employed in many assays demonstrating its anti-oxidative capacity (Reiter et al., 2016, Rodriguez et al., 2004). Furthermore, the study indicated that melatonin as a targeting molecule to mitochondrial exerted a protecting role on mitochondrial by scavenging ROS and inhibiting the mitochondrial permeability transition pore (MPTP) (Tan et al., 2016). Currently, melatonin showed an anti-apoptotic effect by decreasing the expression of cleaved caspase-3 and cleaved caspase-9 as well as increasing the level of SOD and CAT in A549 cells. In addition, ERS can also be initiated by oxidative stress (Malhotra and Kaufman, 2007), therefore, melatonin alleviated the apoptosis of A549 cells induced by $PM_{2.5}$ by inhibiting oxidative stress and ERS. Nrf2, as the central regulator of cellular oxidative stress, initiated the expression of detoxifying enzymes and antioxidant enzyme genes and increased the cell resistance to electrophilic chemicals (Loboda et al., 2016). In our results, melatonin down-regulated the Nrf2 expression in A549 cells exposed to $PM_{2.5}$, which is a conflict with previous studies indicating that melatonin protected cells from oxidation by increasing Nrf2 level (Ding et al., 2014, Wang et al., 2012). Here, two hypotheses have been made as follows. Cancer cells, including the A549 cell, have a strong antioxidant capacity to maintain rapid division and proliferation (Harris et al., 2015). Moreover, melatonin has been shown an anticancer effect in some studies(Di Bella et al., 2013, Goradel, et al., 2017, Zhou, et al., 2014), which may lead to decrease the expression of Nrf2. In addition, our results indicated melatonin enhanced antioxidant enzyme expression, and other studies also revealed that melatonin can scavenge free radicals directly(Rodriguez, et al., 2004), which was contributed to maintain cell homeostasis and further inhibit the Nrf2 expression.

The PM concentration in poultry houses is higher in comparison with that from pig or cattle feeding systems, and imposed a heavier burden on animal respiratory health, further causing low productivity and rising mortality by the incurred lung injury(Cambra-Lopez, et al., 2010). Apoptosis is related to acute respiratory distress syndrome, pulmonary fibrosis and promoted emphysema development when apoptosis occurs in lung epithelial and endothelial cells (Lu, Harrington and Rounds, 2005). This study revealed that $PM_{2.5}$ from the chicken house led to the apoptosis of A549 cells, and melatonin played a positive action in preventing cell apoptosis. Our study provided new insight into melatonin in alleviating

lung damage caused by chicken house $PM_{2.5}$, and the findings further expanded the potential applications of melatonin as feed additives in chicken production.

Declarations

Conflict of Interest

All authors declared that they had no competing interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No.31772648 and No. 32072781).

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Figures

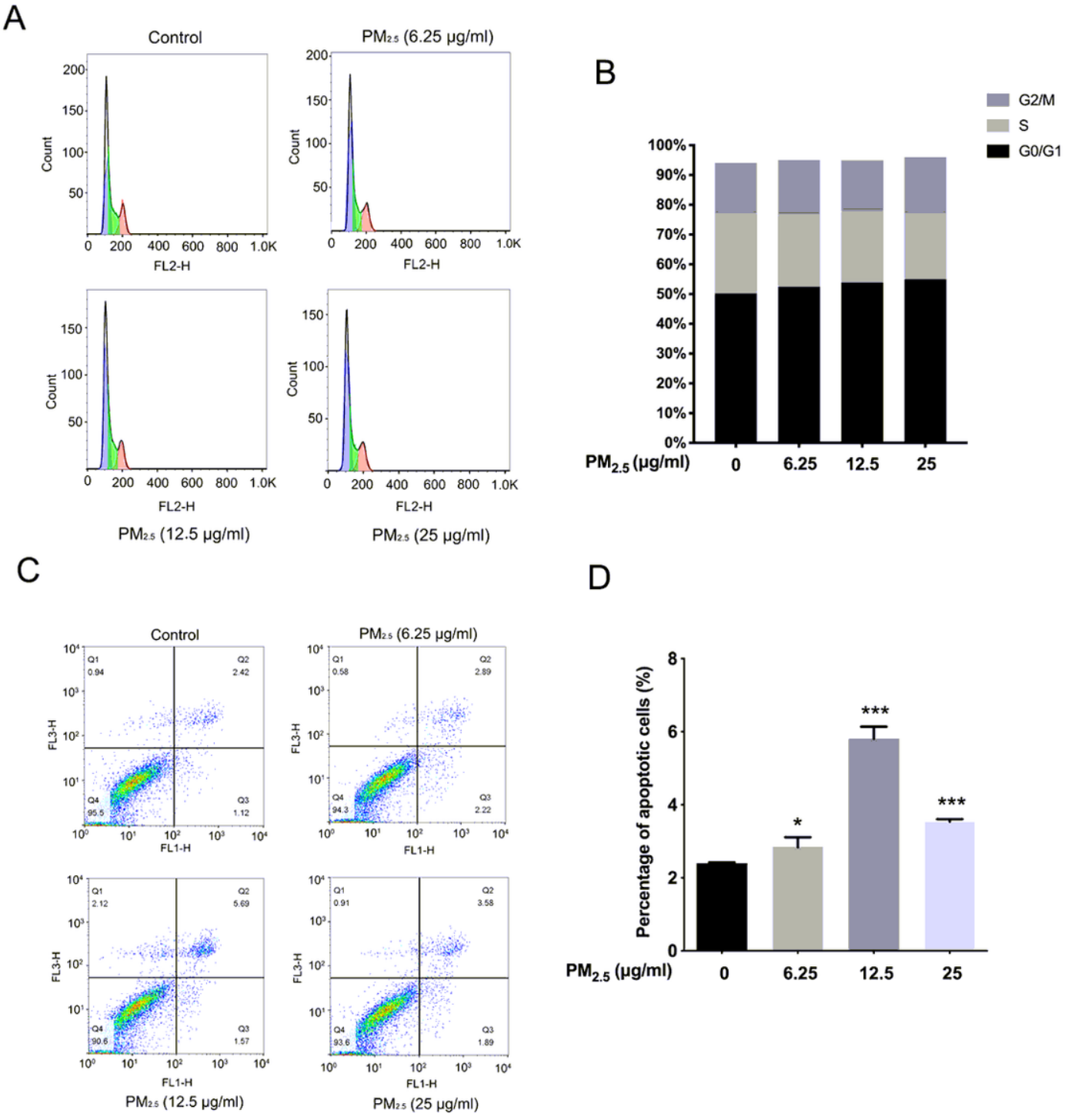

Figure 1

The effect of $PM_{2.5}$ on the distribution of cell cycle and apoptosis of A549 cell.

(A) Cell cycle was determined by flow cytometry after $PM_{2.5}$ treatment on the concentration of 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml for 12 h. (B) The quantification of cell cycle distribution after $PM_{2.5}$ treatment, n = 3. (C) The apoptosis was detected by flow cytometry after exposure by $PM_{2.5}$ at different

concentrations (0, 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml) for 12 h, n=3. (D) The quantification of percentage of apoptotic cells after $PM_{2.5}$ treatment. *$p < 0.05$, ***$p < 0.001$, n = 3.

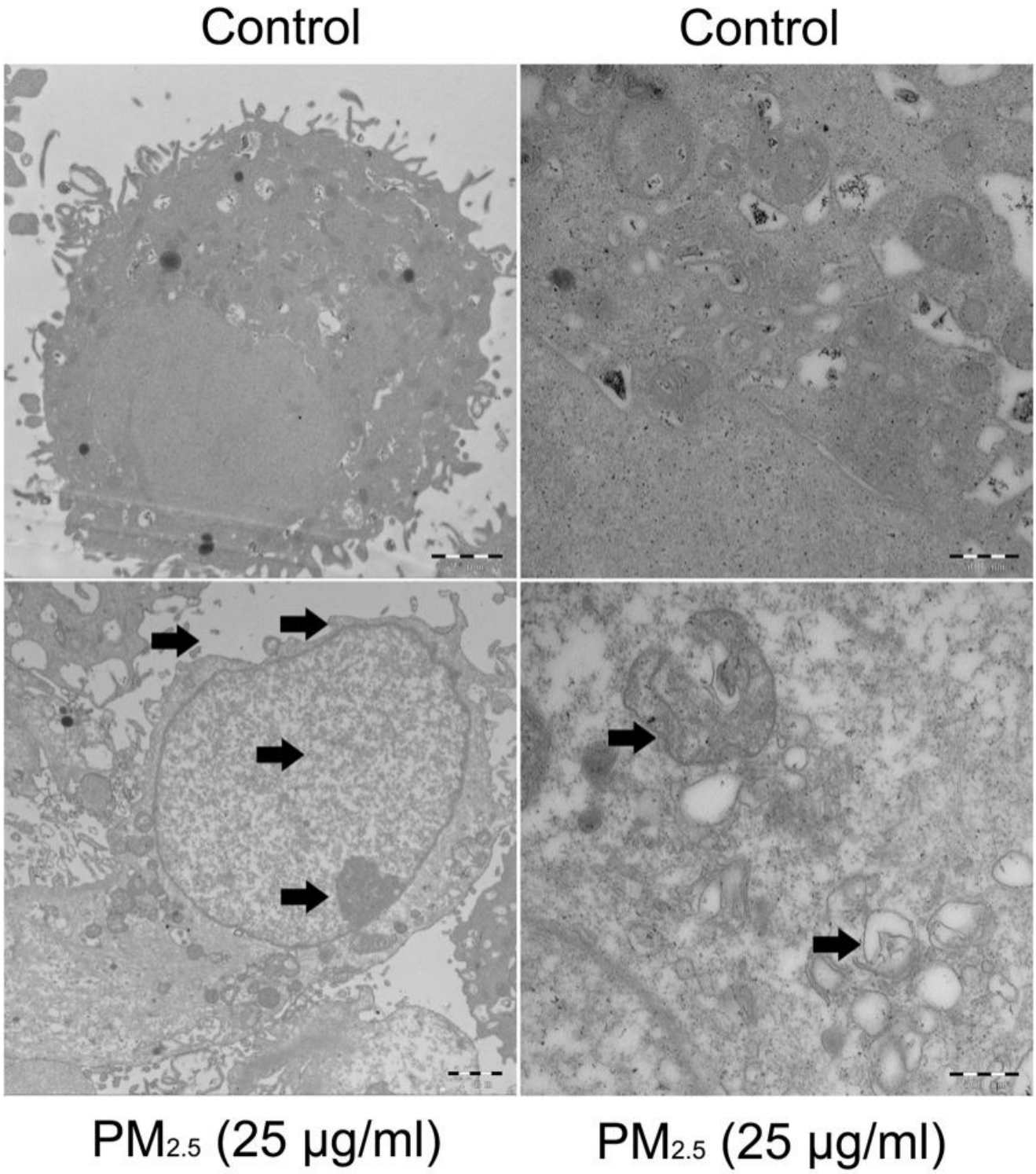

Figure 2

The ultrastructure observation of A549 cell exposed to $PM_{2.5}$.

The subcellular structure observation of A549 cell exposed to $PM_{2.5}$ (25 μg/ml) for 12 h by transmission electron microscope (TEM). Black arrows indicated the apoptosis characteristics of A549 cell.

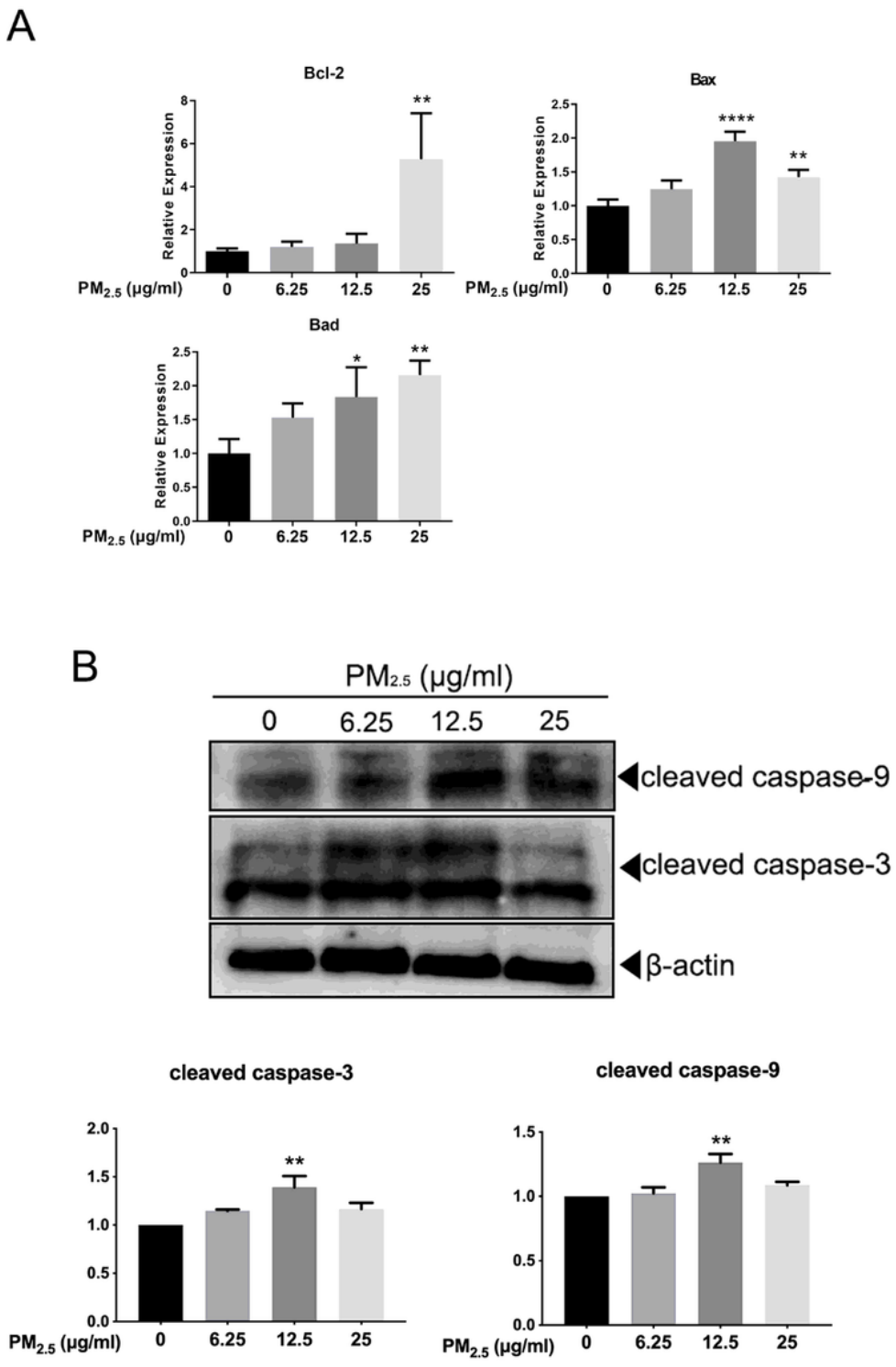

Figure 3

$PM_{2.5}$ increased the expression of apoptosis related genes and proteins in A549 cell.

(A) The analysis on genes related to apoptosis, including *Bcl-2*, *Bax*, and *Bad*, in A549 cell exposed to $PM_{2.5}$ at 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml for 12 h. (B) Immunoblotting analysis detected the level of cleaved-caspase3 and cleaved-caspase9 in A549 cell with same treatment as shown above. The data shown is the representative of three independently experiments. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, n = 3.

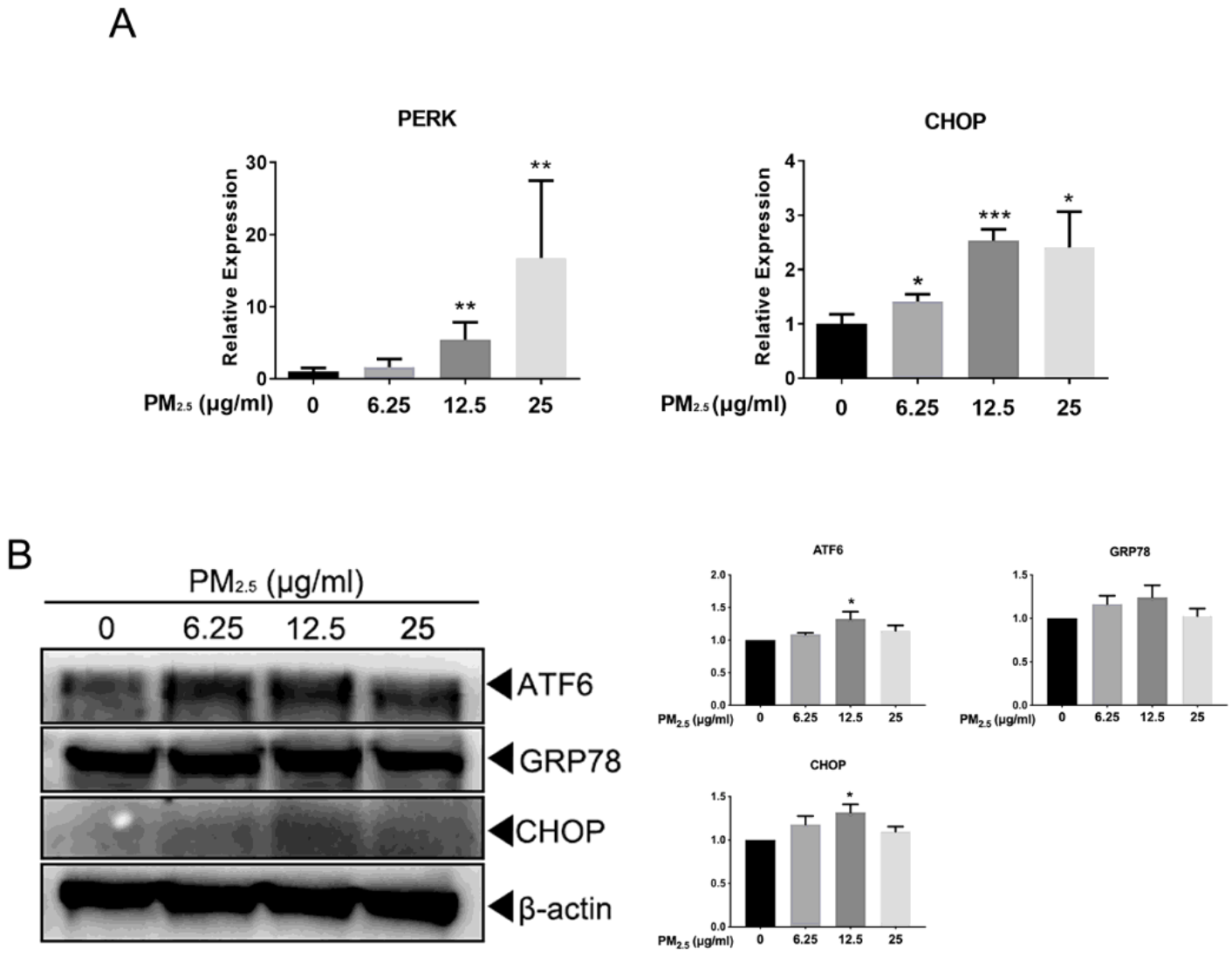

Figure 4

$PM_{2.5}$ induced endoplasmic reticulum stress in A549 cell.

(A) The expression of *PERK* and *CHOP* were detected in A549 cell exposed to $PM_{2.5}$ at 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml for 12 h. (B) The level of ATF6, GRP78 and CHOP were determined by immunoblotting analysis in A549 cell with same treatment as shown above. The data shown is the representative of three independently experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n = 3.

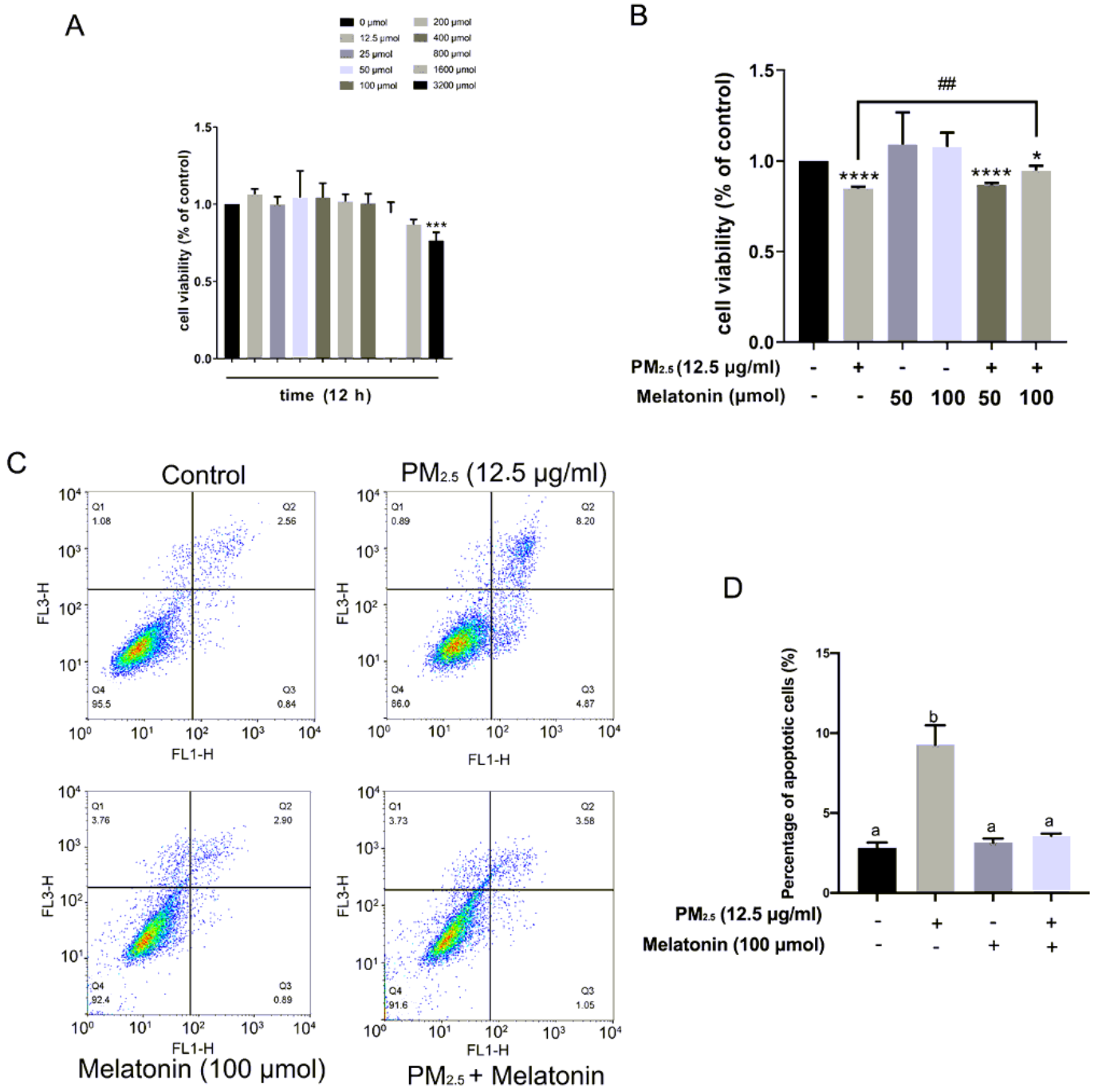

Figure 5

Melatonin resisted apoptosis caused by $PM_{2.5}$ in A549 cell.

(A) and (B) the effect of melatonin and cotreatment of melatonin with $PM_{2.5}$ on A549 cell viability respectively. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, $^{\#\#}p < 0.01$, n = 4 (A), n = 3 (B). (C) The effect of melatonin on apoptosis of A549 cell stimulated by $PM_{2.5}$ via flow cytometry. (D) The quantification of

percentage of apoptotic cells after treatment. The different letters of columns represent significant difference, n = 3.

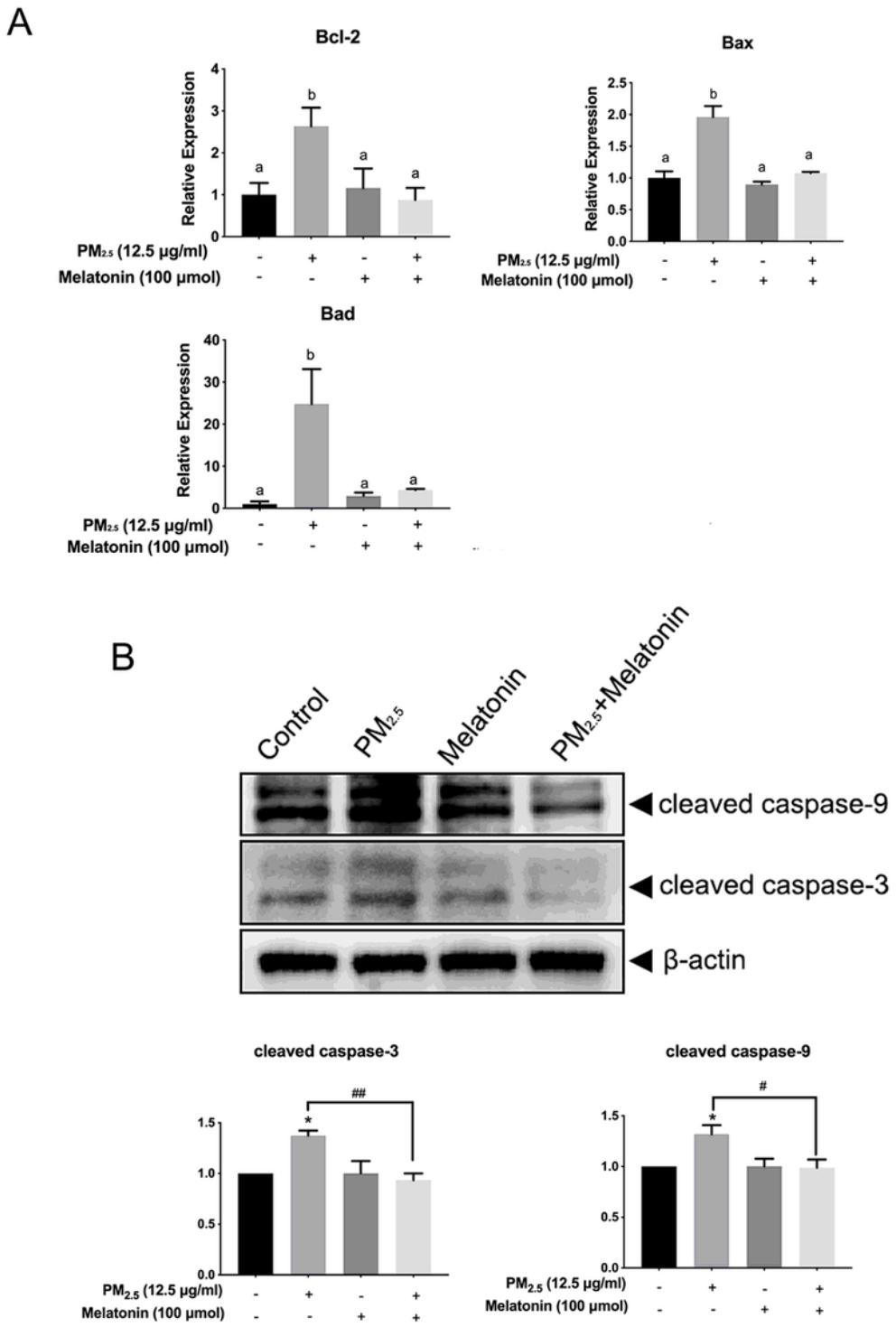

Figure 6

The effect of melatonin on the expression of apoptosis related genes and proteins.

(A) The expression of *Bcl-2*, *Bad* and *Bax* in A549 cell exposed to $PM_{2.5}$ (12.5 μg/ml), melatonin (100 μmol) or cotreatment with $PM_{2.5}$ and melatonin for 12 h. The different letters among columns indicate significant difference, n = 3. (B) The detection of cleaved caspase-9 and cleaved caspase-3 by immunoblotting assay in A549 cell with same treatment as shown above. The data shown is the representative of four independently experiments. * or $^{\#}p < 0.05$ $^{\#\#}p < 0.01$.

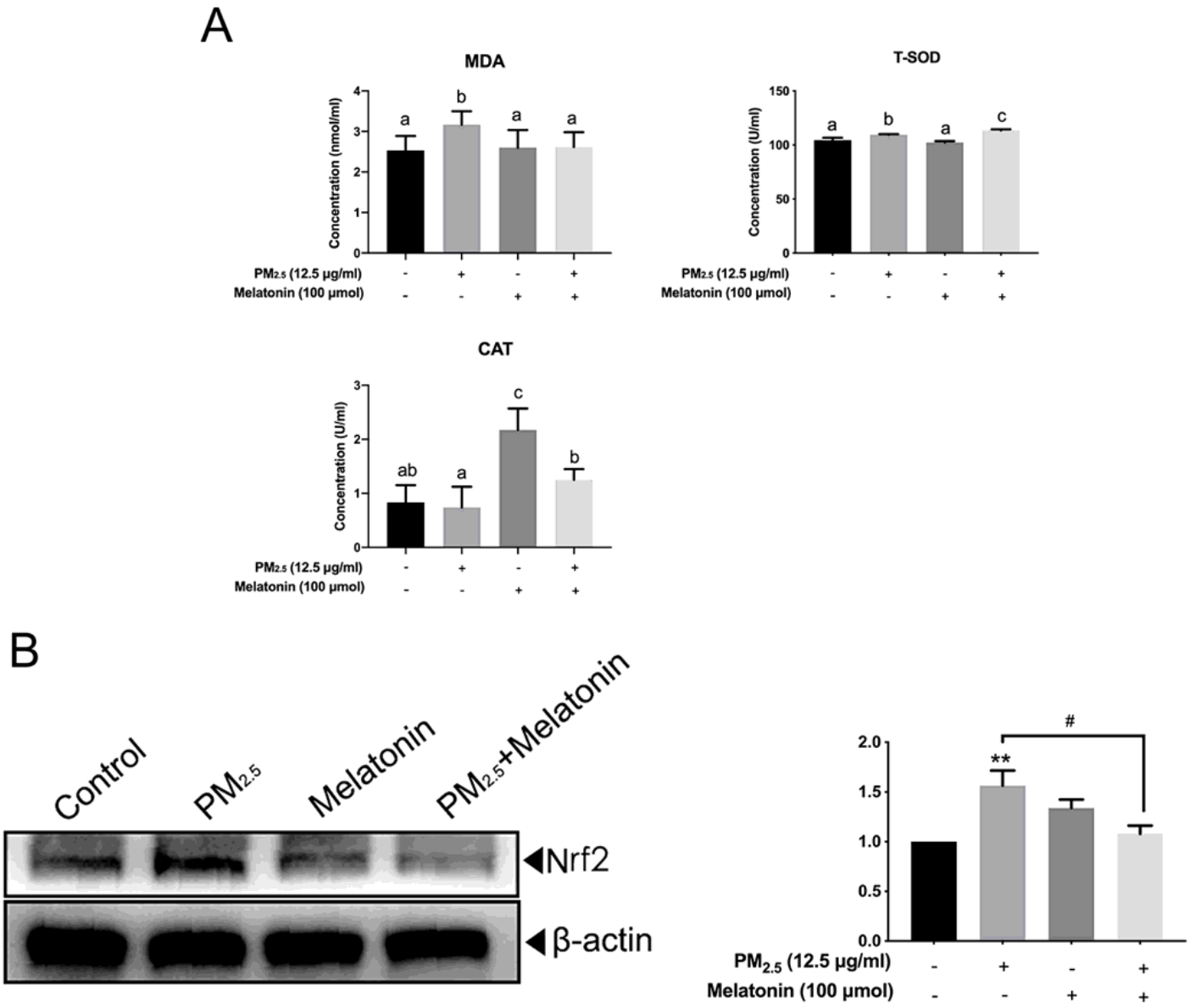

Figure 7

The effect of melatonin on the antioxidant capacity of A549 cell treated by $PM_{2.5}$.

(A) The level of MDA (n=5), T-SOD (n=3) and CAT (n=7) were detected in supernatant from groups treated by $PM_{2.5}$ (12.5 μg/ml), melatonin (100 μmol) and $PM_{2.5}$ with melatonin. The different letters among columns indicate significant difference, n = 3. (B) The expression of Nrf2 determined by immunoblotting

assay in A549 cell with same treatment as shown above. The data shown is the representative of five independently experiments. $^{\#}p < 0.05$, $^{**}p < 0.01$, n=3.

Supplementary Files

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- floatimage1.png