TBHQ alleviates pyroptosis and necroptosis in chicken alveolar epithelial cells induced by fine particulate matter from broiler houses

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ABSTRACT Fine particulate matter (**$PM_{2.5}$**) from poultry houses has adverse effects on the health of animals and workers. Tert-butylhydroquinone (**TBHQ**), an antioxidant, is widely used in feed additives. The present study investigated the effects of TBHQ on broiler house $PM_{2.5}$-induced damage in chicken primary alveolar epithelial cells (**AECII**) extracted from 16-day-old chicken embryos using the method of differential adhesion. AECII were exposed to $PM_{2.5}$ and TBHQ alone or in combination, and then, cell membrane integrity, pyroptosis, and necroptosis were detected. Our results showed that $PM_{2.5}$ from broiler houses caused cell rupture and loss of cell membrane integrity. This result was confirmed by the obvious increases in lactate dehydrogenase (**LDH**) release and propidium iodide (**PI**)-positive cells compared to the control group. In addition, the intracellular reactive oxygen species (**ROS**) levels and the expression levels of pyroptosis-related genes (*NLRP3, IL-18, IL-1β*) and necroptosis-related genes (*RIPK3*) were also significantly enhanced. However, TBHQ significantly inhibited intracellular ROS, improved cell viability, and reduced the release of LDH and the number of PI-positive cells compared to those in the $PM_{2.5}$ group. The expression levels of pyroptosis-related genes (*Caspase-1, NLRP3, IL-18, IL-1β*) and necroptosis-related genes (*RIPK3*) were also significantly decreased in the co-treatment group. In summary, these results indicated that TBHQ can alleviate $PM_{2.5}$-mediated cell pyroptosis and necroptosis in chicken AECII and provide a basis for overcoming the danger that air pollutants from broiler houses pose to the health of chickens.

Key words: TBHQ, pyroptosis, necroptosis, fine particulate matter, chicken alveolar epithelial cell

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INTRODUCTION

Intensive livestock and poultry breeding can produce a large amount of particulate matter (**PM**), which increases the risk of respiratory diseases and seriously reduces the production performance of animals (Hamscher et al., 2003). Our previous research found that the range of $PM_{2.5}$ concentrations in the layer house was from 32.87 to 98.76 $\mu g/cm^3$ (Shen et al., 2018), which was far lower than the standards in NT/T 388−1999 "Standards of Agricultural Industry of the People's Republic of China" (1999). However, the PM concentration in broiler houses is higher than that in laying houses (Cambra-López et al., 2010). The primary sources of PM from poultry houses are feeds, feathers, manure, urine mineral crystals, and so on (Mostafa, 2012). Activities of workers and broilers, such as sweep, flapping of wings, and coughing, can cause the production of $PM_{2.5}$, which can stay in the air for a long time. Many endotoxins, pathogens, organic and inorganic ions adhere to the surface of PM (Dai et al., 2020). Compared with PM with a diameter greater than 2.5 μm, fine particulate matter 2.5 ($PM_{2.5}$) is receiving more attention. $PM_{2.5}$ can carry more adverse toxic substances than larger PM because of its larger specific surface area and can pass through the respiratory tract based on its small size, in turn attach to alveolar epithelial cells, and injure the respiratory system and even the whole body (Radon et al., 2001). Overwhelming evidence has indicated that $PM_{2.5}$ can induce many lung diseases, such as respiratory system inflammation, asthma and chronic obstructive pneumonia (Beker et al., 2004). However, the molecular mechanism of $PM_{2.5}$ from broiler houses in lung injury of broiler requires further exploration.

Severe inflammatory reactions can disrupt the body's homeostasis and accompany cell death. Our previous study found that $PM_{2.5}$ from a laying hen house could cause an inflammatory response in human alveolar

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type II epithelial cells (A549) (Dai et al., 2020). Another study found that $PM_{2.5}$ triggered corneal inflammation and pyroptosis (Niu et al., 2021). Pyroptosis is a new form of programmed cell death that can induce a variety of autoimmune and inflammatory diseases (Kepp et al., 2010). Pyroptosis is possibly dependent on the regulation of Caspase-1, with the maturation and release of the proinflammatory factors interleukin 1β (**IL-1β**) and interleukin 18 (**IL-18**) (Case and Craig, 2013). However, the activation of Caspase-1 is regulated by inflammasomes, including NLRP1, NLRP3, NLRC4 and AIM-2 (Martinon et al., 2002). Our previous study reported that $PM_{2.5}$ from pig houses induced an immune response via the NLRP3 inflammasome in alveolar macrophages (Tang et al., 2019). Thus, we hypothesize that $PM_{2.5}$ from broiler houses induces NLRP3 inflammasome activation and subsequent pyroptosis in chicken alveolar epithelial cells. Additionally, a recent study showed that $PM_{2.5}$ enhanced airway hyperresponsiveness by inducing necroptosis in BALB/c mice (Zhao et al., 2019). Necroptosis, a new type of cell death, has become a research hotspot in recent years (Wu et al., 2012). Receptor-interacting proteins 1 and 3 (**RIPK1** and **RIPK3**) and mixed-lineage kinase domain-like proteins (**MLKL**) are important functional proteins in the process of necroptosis (Holler et al., 2000; Galluzzi et al., 2009). These proteins have high proinflammatory properties and can activate the immune system (Dickens et al., 2012). An increasing number of studies have reported that necroptosis plays an important role in the pathogenesis of pulmonary diseases and is involved in the regulation of the occurrence and development of pulmonary diseases (Mizumura et al., 2016; Siempos et al., 2018). These observations led us to surmise that necroptosis might also take part in the inflammatory response induced by $PM_{2.5}$ from broiler houses in chicken alveolar epithelial cells.

Tert-butylhydroquinone (**TBHQ**) is a recognized antioxidant that can effectively delay oil oxidation and improve the stability of food. In recent years, TBHQ has been used as a feed additive in animal feed factories. Studies have shown that TBHQ can disrupt the metabolic processes of exogenous toxic compounds and reduce the synthesis of toxic metabolites in the body, thereby reducing oxidative stress damage (Hirose et al., 1999; Abdel-Wahab, 2003). It is well known that oxidative stress produces a large amount of reactive oxygen species (**ROS**), including superoxide anions, hydroxyl radicals, and nonfree radicals (Forman et al., 2015). Some studies have indicated that ROS contribute to the development of inflammatory responses and cell death, either directly or indirectly (Carlo and Loeser, 2010; Seon-Mi and Kim, 2014). However, few studies have demonstrated whether ROS and the resulting cell death are involved in lung injury induced by $PM_{2.5}$ from broiler houses. Moreover, the role of $PM_{2.5}$ from broiler houses in pyroptosis and necroptosis of alveolar epithelial cells has not yet been investigated. The available information on the mitigation of TBHQ on these cell damage is limited so far.

Alveolar type II epithelial cells (**AECII**) are important functional and structural cells in the alveoli (Ewald., 2015). AECII have functions such as immortal proliferation, pulmonary water transport, and secretion of surfactants (Bove et al., 2010). Therefore, AECII are the target of attack in the pathogenesis of several lung diseases (Liang et al., 2013). However, regrettably, a chicken AECII cell line has not yet been generated. It is well known that primary cells maintain their biological properties. Primary cells more closely resemble and reflect growth characteristics in vivo. Consequently, we extracted chicken primary AECII for isolation and culture and used them as a cell model to explore the alleviating effect of TBHQ on chicken AECII cell damage induced by $PM_{2.5}$ from broiler houses.

MATERIALS AND METHODS

Collection and Extraction of $PM_{2.5}$ From a Broiler House

Before sampling, quartz filter membranes (47 mm diameter, Whatman Inc., Clifton, NJ) were wrapped in foil paper (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), placed in a muffle furnace (Thermo Scientific, Waltham, MA), and then baked at 450°C for approximately 2 h to remove organic impurities. After baking, the filter membranes were dried in a desiccator for 6 h to a constant weight. Then, the filter membranes were kept in a plastic film box, which was sealed with sealing film. The $PM_{2.5}$ samples were collected with a BTPM-HS1 air particulate sampler (Dandong Baxter Co., Ltd., Dandong, China). When sampling, the sampler was placed in the centre of the chicken house, and the flow rate was 16.67 L/min. Broilers of 15,000 at the age of 30 d were reared in the broiler house where tunnel ventilation and floor beddings were provided. The sampling time was from 7:00 to 22:00 for 30 d in July 2019. The filter membranes with the collected $PM_{2.5}$ were cut into small pieces with plastic scissors and placed in a beaker containing deionized water. The beaker was placed into an ultrasonic oscillator (Thermo Scientific) and oscillated at amplitude of 70 Hz. The filter membranes were removed with small plastic tweezers, and the suspension was filtered with gauze and then centrifuged at 12,000 rpm for 30 min. The supernatant was discarded, and the sediment was evaporated in a vacuum rotary evaporator for approximately 12 h. Then, the precipitate was redissolved in physiological saline and placed at 4°C for use after sterilization.

Cell Extraction and Culture

Chicken AECII were obtained from 16-day-old specific pathogen-free chicken embryos provided by Jiangsu Academy of Agricultural Sciences, Nanjing, China. And the number of samples was 10. After the embryos were

killed, their lungs were transferred to a penicillin bottle containing D-Hank's balanced salt solution (Nanjing Jiancheng Bioengineering Institute). Three washes were performed with D-Hank's balanced salt solution, and then, the lungs were cut into pieces with scissors. They were moved to a centrifuge tube with 2 mL of 0.1% type I collagenase (Dingsi Bioengineering Institute, Nanjing, China) and incubated for 20 min in a thermostat water bath at 37°C. Next, the supernatant was collected and centrifuged at 1,000 r/min for 5 min at 4°C. The supernatant was discarded, and the cell pellet was resuspended in DMEM (Gibco, NY) containing 1% streptomycin/penicillin (Gibco, NY) and 10% foetal bovine serum (**FBS**, Gibco, NY). Cells were moved to 2 cell cultuvre dishes and incubated at 37°C with 5% CO_2 for 1 h. Then, the supernatant was collected and centrifuged at 800 r/min for 8 min at 4 °C. The steps from the cell pellet was resuspended in DMEM to the supernatant was collected and centrifuged at 800 r/min for 8 min at 4 °C mentioned above were repeated three times, and then, the cell culture dishes were covered with 1 mg/mL chicken immunoglobulin G (IgG, Nanjing Jiancheng Bioengineering Institute). The cells were filtered through a 400 mesh screen, and the filtered cells were grown in DMEM (20% FBS) at 37 °C and 5% CO_2.

The study and the use of chicken embryos were approved by the Animal Care and Use Committee of Nanjing Agricultural University (No. 2012CB120762).

Cell Identification

There are AECI, AECII, fibroblasts, macrophages, neutrophils, and other cells in chicken embryo lung tissue. Alkaline phosphatase can be used as a marker of AECII differentiation. Alkaline phosphatase attaches to the membranes of AECII and neutrophils, while membranes of other cells do not have alkaline phosphatase. Neutrophils are small and have lobulated nuclei, which are clearly distinguished from AECII. Therefore, AECII can be accurately identified by alkaline phosphatase method. The cells were fixed with a paraformaldehyde for approximately 3 min. The substrate application solution was prepared according to the instructions of the alkaline phosphatase staining kit (Nanjing Jiancheng Bioengineering Institute). The substrate application solution was added dropwise to the cells and incubated at 37°C for 15 min and protected from light, whereas a negative control was prepared by replacing the substrate application solution with PBS. After the incubation was complete, the cells were washed for 2 min and restained with the restaining solution for 30 s. Then, the cells were washed with distilled water for approximately 1 min, dried, and microscopically examined.

Cell Treatment

Chicken AECII were seeded in plastic plates containing DMEM supplemented with 20% FBS, 10 μg/mL streptomycin and 100 units/mL penicillin. The cells were cultured at 37°C in a 5% CO_2 incubator (Thermo Scientific) for 18 h. Subsequently, the cells were exposed to $PM_{2.5}$ or TBHQ (Yuanye Bioengineering Institute, Shanghai, China) for a certain period of time for subsequent experiments.

Cell Viability Assay

Chicken AECII were treated with different concentrations of $PM_{2.5}$ (0, 3.125, 6.25, 12.5, 25, 50, or 100 μg/mL) or TBHQ (0, 6.25, 12.5, 25 or 50 μM) for 6 h, 12 h, or 24 h. Then, 50 μL MTT (Nanjing Jiancheng Bioengineering Institute) was added to each well for another 4 h of culture. The supernatant was replaced with 150 μL dimethylsulfoxide (**DMSO**, Sinopharm Chemical Reagent Co., Ltd., Nanjing, China), and absorbance was measured at 570 nm after 10 min.

Lactate Dehydrogenase Release Assay

After 12 h of treatment, the cell culture supernatants were collected. Subsequently, the supernatants were centrifuged at 300 × g for 5 min at low temperature and stored at −20 °C. The lactate dehydrogenase (**LDH**) release levels were detected with an LDH assay kit (Nanjing Jiancheng Bioengineering Institute). Briefly, 20 μL cell culture supernatant with 5 μL coenzyme I or double distilled water was mixed with 25 μL substrate, then incubated at 37°C for 15 min. Then, 25 μL 2,4-dinitrophenylhydrazine was mixed with the specimens and incubated at 37°C for 15 min. Finally, 250 μL NaOH (0.4 mol/L) was added and kept at room temperature for 5 min. A spectrophotometric microplate reader (Thermo Scientific) was used to determine absorbance at 450 nm.

Hoechst 33342/Propidium Iodide Staining

Chicken AECII were seeded into 24-well plates (5×10^5 cells/well) and treated with test drugs for 12 h. After the cell culture supernatant was discarded, 500 μL Hoechst 33342 (Beijing Solarbio Science and Technology Co., Ltd, Beijing, China) was added to each well and incubated at 37°C for 20 min. Then, washes were performed with PBS, and 500 μL PI (Biosharp Science and Technology Co., Ltd, Hefei, China) was added and incubated at 37°C in the dark for another 20 min. A fluorescence microscope (Thermo Scientific) and ImageJ software were used to capture images of cells and quantify the number of propidium iodide (**PI**)-positive cells.

Detection of Reactive Oxygen Species Generation

After treatment with $PM_{2.5}$ or TBHQ, the cell culture supernatant was discarded. Then, 500 μL DCFH-DA (Beyotime, Shanghai, China) was added to each well and incubated at 37°C for 20 min in the dark. Then,

Table 1. Sequences and parameters of the primers used for qRT-PCR.

Gene (abbreviation)	Sequence (5'→ 3')	Length of DNA product (bp)
IL-1β	F:ACTGGGCATCAAGGGCTACA R:GCTGTCCAGGCGGTAGAAGA	142
IL-18	F:AGTTGCTTGTGGTTCGTCCA R:TCCACTGCCAGATTTCACCTC	80
NLRP3	F:AGCTACCACACATCTAGGAT R:GGTGTCCAAATCCTCAATCT	207
Caspase-1	F:TTCCTTCAACACCATCTACG R:GGTGAGCTTCTCTGGTTTTA	209
RIPK1	F:CTTCAACCAGCGCCATTAGC R:TTGAGTCTTCTGTATCCGTGTCT	120
RIPK3	F:ACATCCTTCGCTCACAGCAA R:ACCTGTGCTGCCTTCTCTCC	130
MLKL	F:AAGGTGGACTGGATGCAAGG R:TAGAGGTCGTAGCGCTCAGT	158
β-actin	F:TGTTACCAACACCCACACCC R:TCCTGAGTCAAGCGCCAAAA	110

Abbreviations: *IL-1β*, interleukin 1β; *IL-18*, interleukin 18; *NLRP3*, NOD-like receptor protein 3; *Caspase-1*, cysteine aspartate specific proteinase 1; *MLKL*, mixed-lineage kinase domain-like proteins; *RIPK1*, receptor interacting protein 1; *RIPK3*, receptor interacting protein 3.

washes were performed with DMEM. The expression of reactive oxygen species (**ROS**) was observed with laser confocal microscopy.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted using an RNA isolator (Vazyme Science and Technology Co., Ltd, Nanjing, China) according to the manufacturer's instructions. The quality and concentration of RNA were detected by a NanoDrop 2000 (Tsingke Science and Technology Co., Ltd, Beijing, China). Agarose gel electrophoresis was used to determine the integrity of RNA. Then, a 2 × TSINGKE Master Mix reagent kit (Tsingke Science and Technology Co., Ltd) was used to reverse-transcribe RNA into cDNA. The nucleic acid sequence of the target gene was obtained from NCBI. The designed primer sequences for each gene are shown in Table 1. Quantitative real-time PCR was carried out according to the manufacturer's instructions for 2 × T 5 Fast Qpcr Mix (SYBR Green I) Real-Time PCR (Tsingke Science and Technology Co., Ltd). Then, melting-curve analysis was performed to check the identity of the products. In addition, the obtained data were analyzed using the $2^{-\triangle\triangle Ct}$ method (Wang et al., 2020) and *β-actin* was used as an internal reference.

Statistical Analysis

The experimental results were analyzed by GraphPad Prism 6.0 (GraphPad, San Diego, CA). All data are presented as the mean ± standard error (mean ± SEM), and one-way ANOVA was used to determine the data. Tukey method was used for multiple comparisons. The values were considered to be significantly different when $P < 0.05$.

RESULTS

Identification of Chicken AECII

Alkaline phosphatase can be used as a marker of AECII differentiation, and it mainly exists in the cell membrane. The cells were observed under an inverted microscope after alkaline phosphatase staining. The nuclei were stained dark purple with diffuse red or reddish-brown granules at the alkaline phosphatase active

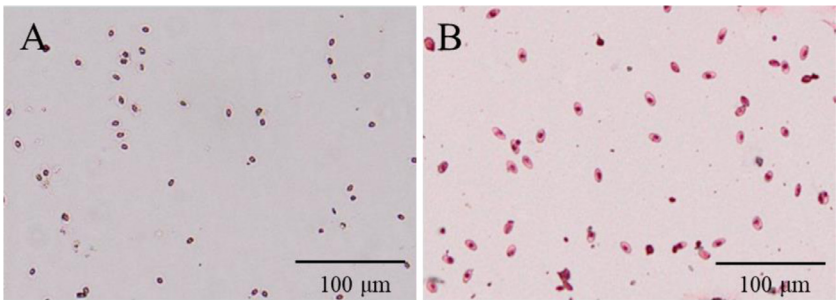

Figure 1. Alkaline phosphatase staining of chicken AECII. (A) Negative control group; (B) alkaline phosphatase-positive cells. The tests were performed under a light microscope. Scale bar = 100 μm. Abbreviation: AECII, alveolar epithelial cells.

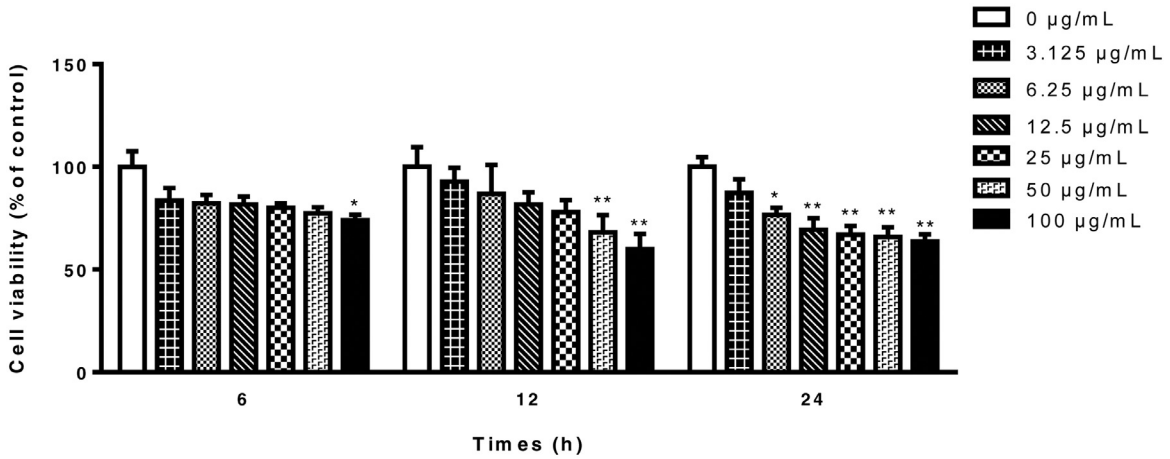

Figure 2. Effects of exposure to different concentrations of $PM_{2.5}$ for different times on chicken AECII viability. Cells were treated with different concentrations of $PM_{2.5}$ (0−100 μg/mL) for 6 h, 12 h, or 24 h. The effects of different concentrations of $PM_{2.5}$ on the viability of chicken AECII were detected by the MTT assay. The values are presented as the mean ± SEM of three independent experiments. Significance compared with control, $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviation: AECII, alveolar epithelial cells

site localized on the cytomembrane (Figure 1B). There were also cells in which the cytoplasm was not stained. In the negative control group, the nuclei were dark purple, and the cytoplasm was not stained (Figure 1A).

$PM_{2.5}$ Decreases the Viability of Chicken AECII

AECII were exposed to $PM_{2.5}$ at different concentrations (0, 3.125, 6.25, 12.5, 25, 50, or 100 μg/mL) for 6, 12, or 24 h. $PM_{2.5}$ treatment reduced chicken AECII viability in a dose-dependent and time-dependent manner. As shown in Figure 2, $PM_{2.5}$ treatment (100 μg/mL) for 6 h resulted in a marked decrease in cell viability ($P < 0.05$). At 12 h, $PM_{2.5}$ dramatically decreased chicken AECII viability at concentrations of 50 μg/mL and 100 μg/mL ($P < 0.01$), and cell viability was found to initially significantly decreased at a concentration of 6.25 μg/mL at 24 h ($P < 0.05$). Accordingly, concentrations of 25, 50, and 100 μg/mL for 12 h were chosen to perform subsequent assays.

$PM_{2.5}$ Destroys the Integrity of the Chicken AECII Cell Membrane

To detect the integrity of the chicken AECII cell membrane after $PM_{2.5}$ treatment, the cell morphology was observed, and LDH release and PI staining were examined. As shown in Figure 3A, with increasing concentrations of $PM_{2.5}$, chicken AECII swelled, ruptured, and even generated fragments. Compared with the control group, a large amount of cell debris was produced, which was significantly increased in groups treated with 50 or 100 μg/mL $PM_{2.5}$. The release of LDH (Figure 3B) and the number of PI-positive cells (Figures 3C and 3D) were both observably increased in chicken AECII treated with $PM_{2.5}$ (50 or 100 μg/mL) compared with those in the control group ($P < 0.05$, $P < 0.01$).

$PM_{2.5}$ Induces ROS Production, Pyroptosis and Necroptosis in Chicken AECII

As shown in Figures 4A and 4B, the levels of ROS increased in a concentration-dependent manner after treatment with $PM_{2.5}$. Compared with the control, the fluorescence intensity of ROS observably increased in the other groups. Pyroptosis-related genes were tested after treatment with $PM_{2.5}$ (Figures 4C and 4D). Compared with the control group, the gene expression of *NLRP3* and *IL-1β* dramatically increased in a dose-dependent manner ($P < 0.01$). *IL-18* gene expression was markedly increased in both the 25 and 100 μg/mL groups ($P < 0.05$, $P < 0.01$). The expression of *RIPK3*, a necroptosis-related gene, was observably increased after exposure of cells to $PM_{2.5}$. ($P < 0.01$).

Effects of TBHQ on the Viability of Chicken AECII

To explore the optimal concentration of TBHQ, the viability of chicken AECII exposed to gradient concentrations of TBHQ was examined. As shown in Figure 5, exposure to 50 μM TBHQ significantly enhanced cell viability compared with any other treatment group ($P < 0.01$). Compared with the $PM_{2.5}$ group, the cell viability of the co-treatment with $PM_{2.5}$ and TBHQ (12.5, 25 or 50 μM) group increased observably ($P < 0.01$). However, cell viability did not change significantly after co-treatment with $PM_{2.5}$ and TBHQ (25 or 50 μM) compared with that of the control group ($P > 0.05$). Therefore, 25 μM TBHQ was employed in subsequent experiments.

Effects of TBHQ on the Integrity of the Chicken AECII Cell Membrane

As shown in Figure 6, compared with that of the $PM_{2.5}$ group, the level of LDH release observably

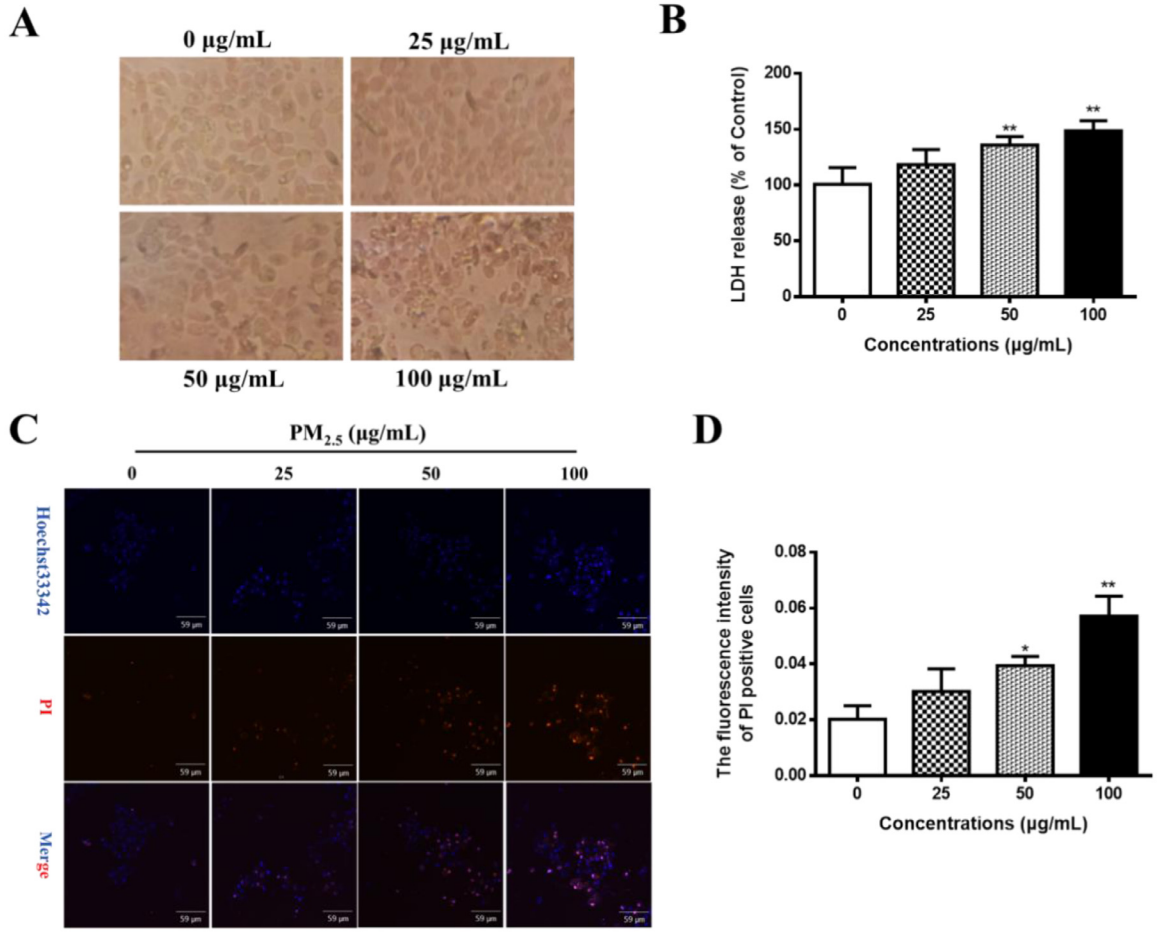

Figure 3. Morphological changes, LDH release and Hoechst 33342/PI staining of chicken AECII cells exposed to $PM_{2.5}$. Cells were treated with $PM_{2.5}$ at 0, 25, 50, or 100 μg/mL for 12 h. (A) Cell morphology images. After exposure to $PM_{2.5}$, cells were observed under an optical microscope ($\times$ 100). (B) Release of intracellular LDH. Cell culture supernatants were collected and detected by the LDH assay; (C) cell fluorescence images. Cells were treated with different concentrations of $PM_{2.5}$ and then stained with Hoechst 33342 and PI. PI-positive cells were analyzed for fluorescence intensity by ImageJ software. Scale bar = 59 μm; (D) quantification of (C). Data are presented as the mean ± SEM. Significance compared with control, $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: AECII, alveolar epithelial cells; LDH, lactate dehydrogenase.

decreased after co-treatment with $PM_{2.5}$ and TBHQ ($P < 0.01$, Figure 6A). The fluorescence intensity of PI-positive cells was dramatically reduced after co-treatment with $PM_{2.5}$ and TBHQ ($P < 0.01$) compared with that of the $PM_{2.5}$ group ($P < 0.05$, Figures 6B and 6C).

Effects of TBHQ on ROS Levels, Pyroptosis, and Necroptosis Induced by $PM_{2.5}$ in Chicken AECII

The levels of ROS in chicken AECII were examined by immunofluorescence after treatment with $PM_{2.5}$ or TBHQ for 12 h. Compared with those in $PM_{2.5}$-treated cells, the levels of ROS were significantly decreased in cells co-treated with TBHQ and $PM_{2.5}$ ($P < 0.01$, Figures 7A and 7B). In the TBHQ and $PM_{2.5}$ co-treatment groups, the pyroptosis-related genes *NLRP3* ($P < 0.01$), *Caspase-1* ($P < 0.05$), *IL-18* ($P < 0.01$), and *IL-1β* ($P < 0.01$) all had significantly decreased expression levels compared to that in the $PM_{2.5}$ treatment alone group. The expression of *RIPK3*, a necroptosis-related gene, was markedly reduced in cells co-treated with TBHQ and $PM_{2.5}$ compared with that in the $PM_{2.5}$ group ($P < 0.01$, Figures 7C and 7D).

DISCUSSION

As one of the air pollutants in livestock and poultry houses, $PM_{2.5}$ can cross the air-blood barrier and enter the bloodstream (Radon et al., 2001). $PM_{2.5}$ is carried to various organs and endangers the health of animals. AECII play an important role in maintaining the structure and function of the air-blood barrier (Bove et al., 2010). Alkaline phosphatase is a marker enzyme of AECII and is located on the cell membrane (Edelson et al., 1988). In our study, we found that the extracted cells were positive for alkaline phosphatase expression, suggesting that they were identified as AECII and could be used in subsequent experimental studies.

In the broiler houses, the whole body of broilers response to infestation of $PM_{2.5}$, which is a complicated process. And this is different from the way cells respond to $PM_{2.5}$. Therefore, we choose different concentrations

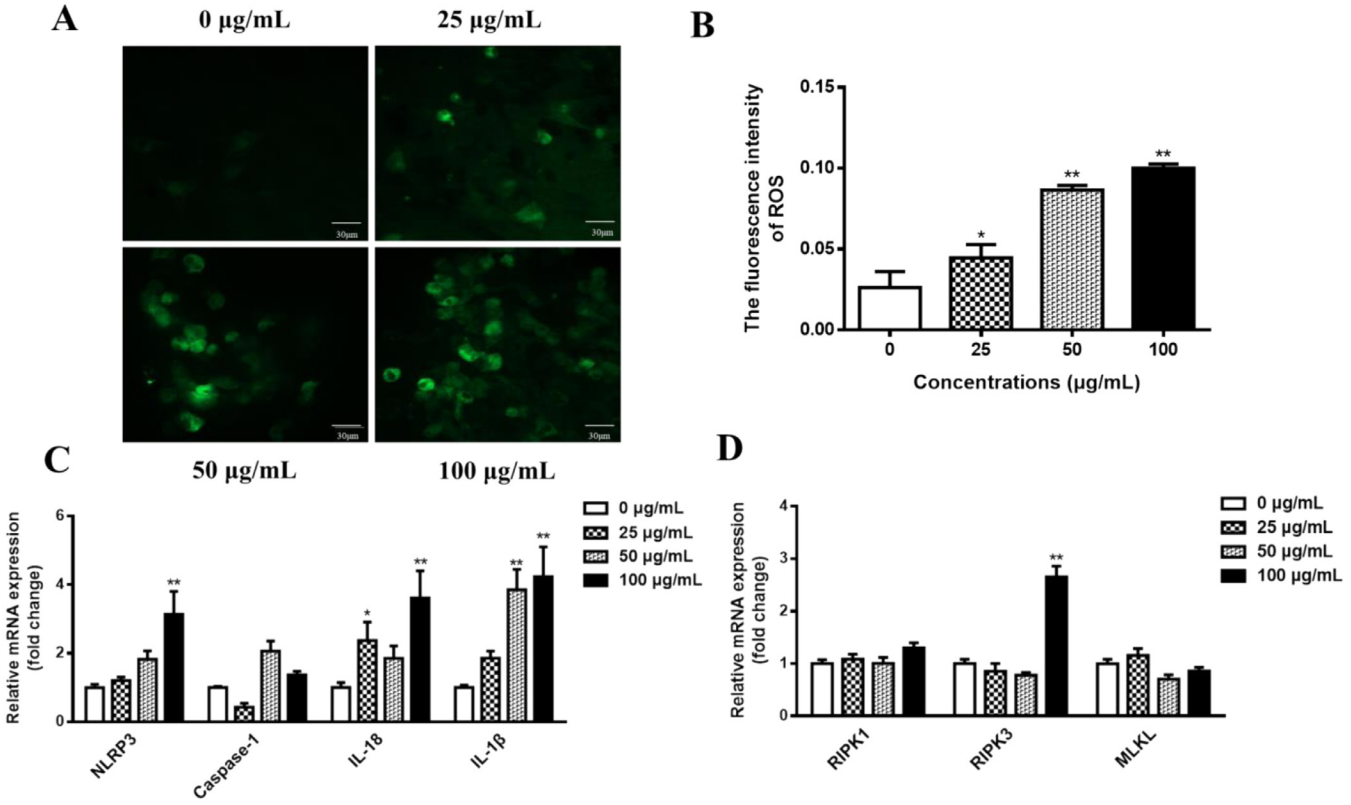

Figure 4. ROS generation and mRNA expression levels of pyroptosis-related and necroptosis-related genes in chicken AECII induced by $PM_{2.5}$. Cells were treated with $PM_{2.5}$ at 0, 25, 50, and 100 μg/mL for 12 h. (A) Cell fluorescence images. ROS generation was determined by immunofluorescence using DCFH-DA (green fluorescence, 5 mM). Scale bar = 30 μm; (B) quantification of (A); (C) pyroptosis-related gene expression levels. *IL-1β, IL-18, Caspase-1* and *NLRP3* expression is associated with pyroptosis. (D) Necroptosis-related gene expression levels. *RIPK1, RIPK3*, and *MLKL* gene expression is associated with necroptosis. Data are presented as the mean ± SEM. Significance compared with the control, $^{*}P < 0.05$ and $^{**}P < 0.01$. Abbreviation: ROS, reactive oxygen species.

of $PM_{2.5}$ to treat the cells instead of using the concentrations of $PM_{2.5}$ in the broiler houses. Cell viability in vitro plays a central role in predicting the toxicity of toxins. Some articles have reported that treating cells with different concentrations of $PM_{2.5}$ for different times can decrease cell viability in a concentration-dependent and time-dependent manner (Deng et al., 2013; Tang et al., 2019), which is consistent with our research results. These finding imply that $PM_{2.5}$ from broiler houses has a toxic effect on chicken AECII. Pyroptosis and necroptosis are 2 novel forms of programmed cell death, both of which can form pores in the cell membrane and disrupt it (Wu et al., 2012, 2018). LDH release and PI staining are common methods to detect the integrity of cell membranes (Miao et al., 2011). In the present study, $PM_{2.5}$ from broiler houses significantly increased LDH release

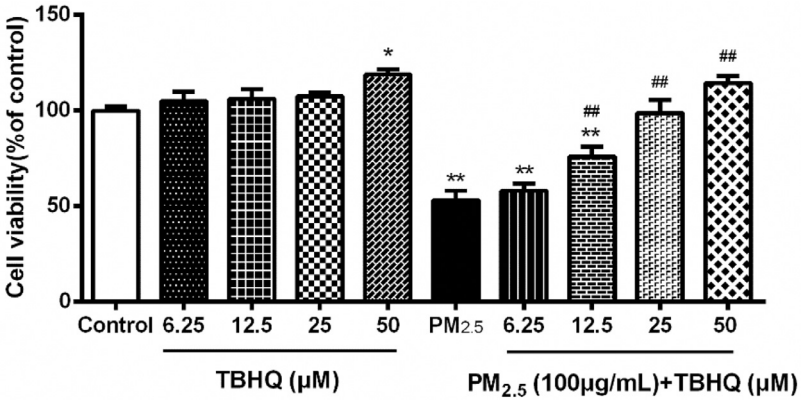

Figure 5. Cell viability of chicken AECII cells exposed to TBHQ or $PM_{2.5}$. (A) Cells were treated with different concentrations of TBHQ (0−50 μM) for 12 h; (B) cells were treated with 100 μg/mL $PM_{2.5}$ with or without TBHQ (0−50 μM) for 12 h except the control group. The viability of chicken AECII was detected by the MTT assay. Data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the $PM_{2.5}$ group. Abbreviations: AECII, alveolar epithelial cells; TBHQ, Tert-butylhydroquinone.

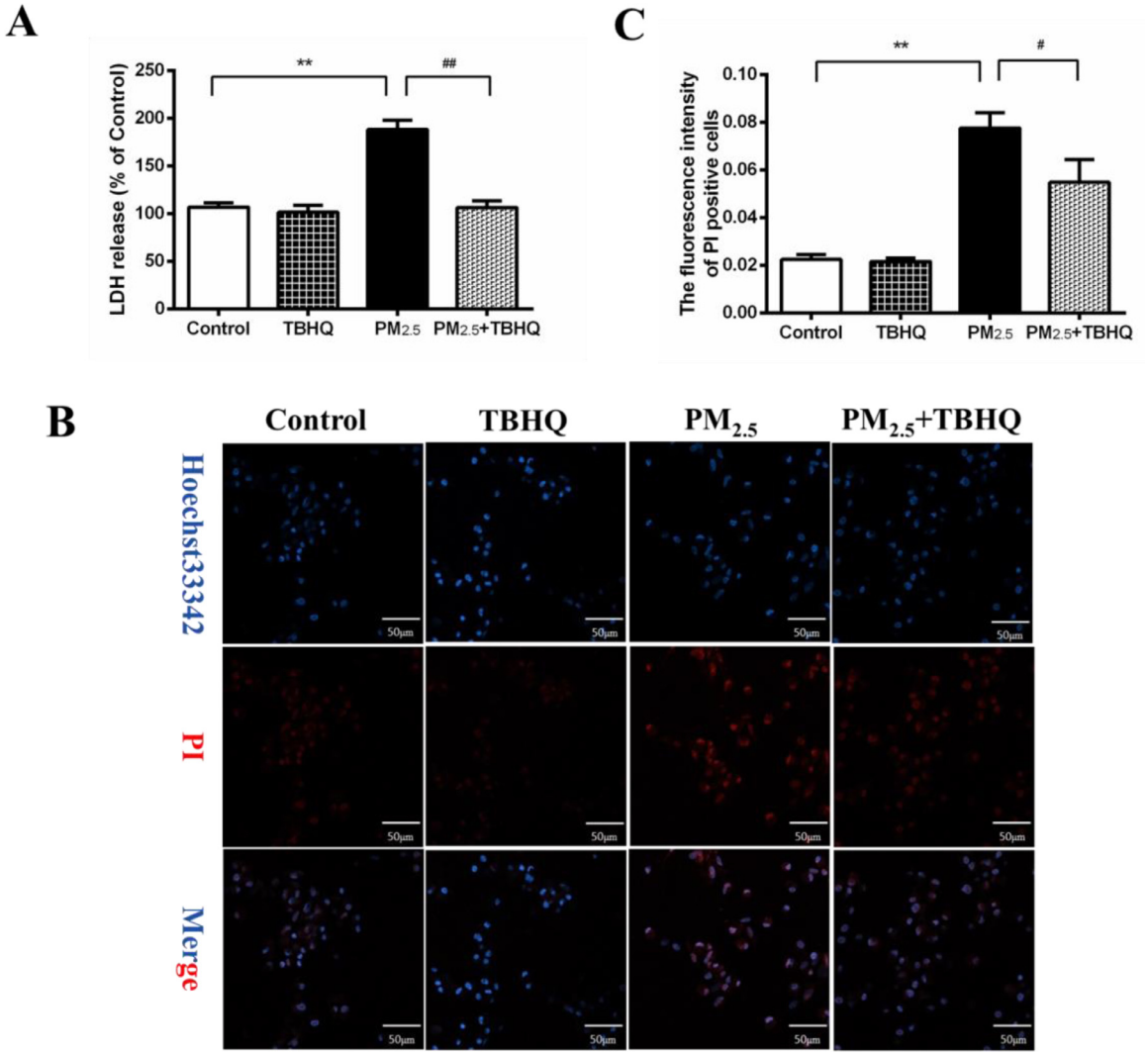

Figure 6. LDH release and Hoechst 33342/PI staining of chicken AECII exposed to $PM_{2.5}$ or TBHQ. Cells were treated with 100 μg/mL $PM_{2.5}$ with or without 25 μM TBHQ for 12 h. (A) Release of intracellular LDH. Cell culture supernatants were collected and detected by the LDH assay; (B) cell fluorescence images. Cells were stained with Hoechst 33342 and PI. PI-positive cells were analysed for fluorescence intensity by ImageJ software. Scale bar = 50 μm; (C) quantification of (B). Data are presented as the mean ± SEM. $^{**}P < 0.01$ vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the $PM_{2.5}$ group. Abbreviations: AECII, alveolar epithelial cells; LDH, lactate dehydrogenase; PI, propidium iodide; TBHQ, Tert-butylhydroquinone.

and PI-positive cells, indicating that $PM_{2.5}$ is capable of disrupting the integrity of the chicken AECII cell membrane. Subsequently, membrane disruption leads to cell death, possibly involving pyroptosis and necroptosis based on its characteristics. On the one hand, pyroptosis is dependent on the activity of caspases (Wallach et al., 2016). Activated caspases cleave related proteins to release the N-terminal domain, which displaces and perforates the cell membrane, further inducing water penetration and cell swelling (Lagrange et al., 2018). On the other hand, phosphorylated mixed lineage kinase domain-like proteins undergo oligomerization reactions and translocate to the cell membrane to cause cell rupture during necroptosis (Zhao et al., 2016).

It is well known that the innate immune defence system is able to fight against many $PM_{2.5}$-induced lung diseases. Inflammasomes are critical sensors/receptors in innate immunity (Duewell et al., 2010). Among the various inflammasomes, the activated NLRP3 inflammasome has been shown to be involved in the origin and development of multiple inflammation-mediated lung diseases. In addition, a recent study showed that cigarette smoke extracts could cause endothelial cell pyroptosis through NLRP3 inflammasome activation (Wang et al., 2019). A similar result was found in the present study; $PM_{2.5}$ treatment of chicken AECII significantly upregulated *NLRP3, IL-18*, and *IL-1β* gene expression. The expression of the *Caspase-1* gene also showed an increasing trend. This is because Caspase-1 is a key protease in the classical pyroptosic pathway and is activated by inflammasomes and promotes the maturation and release of the inflammatory factors IL-18 and IL-1β (Liu et al., 2018). Therefore, according to this study, the NLRP3 inflammasome plays an important role in the activation of Caspase-1. These results implied that $PM_{2.5}$-mediated cytotoxicity was partially due to the induction of pyroptosis. Furthermore, we found that the expression level of *RIPK3* was observably increased

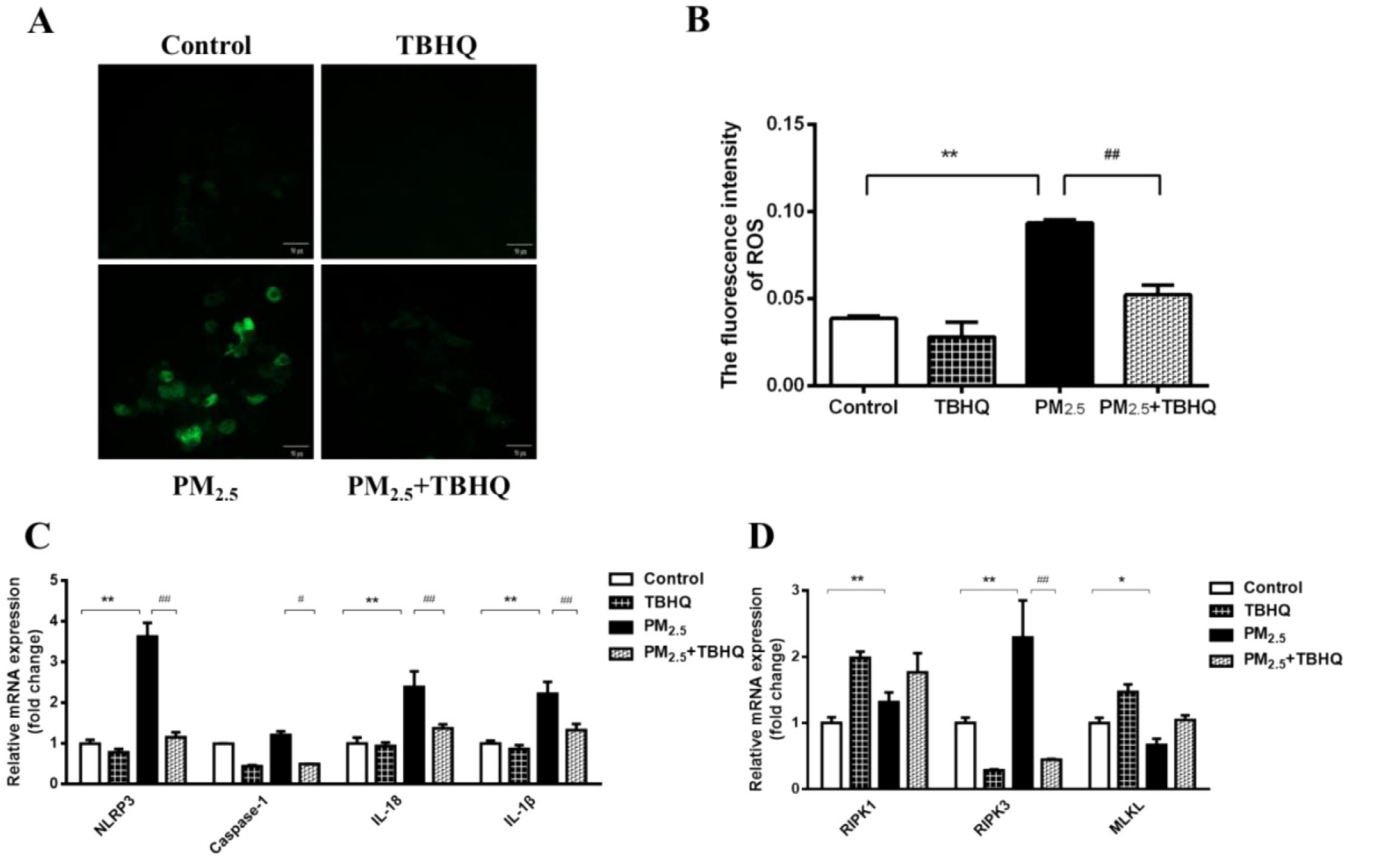

Figure 7. ROS generation and mRNA expression levels of pyroptosis-related and necroptosis-related genes in chicken AECII exposed to $PM_{2.5}$ or TBHQ. Cells were treated with 100 μg/mL $PM_{2.5}$ with or without 25 μM TBHQ for 12 h. (A) Cell fluorescence images. ROS generation was determined by immunofluorescence using DCFH-DA (green fluorescence, 5 mM), Scale bar = 30 μm; (B) quantification of (A); (C) pyroptosis-related gene expression levels. *IL-1β, IL-18, Caspase-1* and *NLRP3* expression is associated with pyroptosis. (D) necroptosis-related gene expression levels. *RIPK1, RIPK3*, and *MLKL* gene expression is associated with necroptosis. Data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the $PM_{2.5}$ group. Abbreviations: AECII, alveolar epithelial cells; ROS, reactive oxygen species; TBHQ, Tert-butylhydroquinone.

in chicken AECII after exposure to $PM_{2.5}$. RIPK3, a necroptosis-associated protein, belongs to the family of RIPs and is important in reducing the inflammatory response. RIPK3 can form necrosomes by autophosphorylation and independently induce necroptosis. An article reported that mouse cytomegalovirus infection induced a type of necrosis that required RIPK3 but not RIPK1 (Upton et al., 2010). Thus, $PM_{2.5}$-mediated cytotoxicity is also related to necroptosis. Based on these results, pyroptosis and necroptosis participate in the inflammatory damage to chicken AECII induced by $PM_{2.5}$ from broiler houses.

Oxidative stress is one of the main causes of the onset of several diseases (Brownlee, 2001; Ayer and Zhang, 2008). Increasing evidence has demonstrated that $PM_{2.5}$ can cause oxidative stress in cells and reduce cell viability (Deng et al., 2013). In this study, we found that $PM_{2.5}$ caused a significant increase in intracellular ROS levels, which led to the disruption of oxidative homeostasis and subsequent oxidative stress. A recent report suggested that cigarette smoke extracts could mediate endothelial cell pyroptosis via the ROS/NLRP3 axis, which in turn, led to atherosclerosis (A et al. 2019). Another article showed that excess ROS altered the integrity of the mitochondrial membrane and induced necroptosis (Jia et al., 2018). Therefore, we speculated that ROS induced by $PM_{2.5}$ from broiler houses caused pyroptosis and necroptosis in chicken AECII. TBHQ is a strong antioxidant that activates the Nrf2-ARE signaling pathway and reduces oxidative damage (Nouhi et al., 2011). It is a key signaling pathway of antioxidative stress in the body that can induce the production and release of antioxidant enzymes in cells. These enzymes can react with oxidative stress factors to remove excess ROS, thus exhibiting neutralization and detoxification effects (Taguchi et al., 2011). In the present study, TBHQ significantly reduced the level of intracellular ROS and protected the integrity of cell membranes, suggesting that it can prevent the death of chicken AECII induced by $PM_{2.5}$ from broiler houses. Additionally, TBHQ observably decreased the expression levels of pyroptosis-related genes (*NLRP3, Caspase-1, IL-18, IL-1β*) and necroptosis-related genes (***RIPK3***) in chicken AECII after exposure to $PM_{2.5}$. Consequently, these results indicated that TBHQ played an important role in attenuating $PM_{2.5}$-induced pyroptosis and necroptosis in chicken AECII by inhibiting the generation of ROS.

CONCLUSIONS

In conclusion, this study demonstrated that $PM_{2.5}$ from broiler houses can induce both pyroptosis and necroptosis for the first time. Pyroptosis was caused by the activation of the NLRP3 inflammasome. We also demonstrated that necroptosis induces lytic cell death in $PM_{2.5}$-treated AECII through the RIPK3-MLKL pathways. Moreover, we show that ROS play a key role in positively regulating lytic cell death of $PM_{2.5}$-treated AECII. These data illustrate a new molecular mechanism of TBHQ ameliorating broiler lung injury caused by $PM_{2.5}$ and provide new scientific evidences for the prevention and treatment of $PM_{2.5}$-mediated inflammation-associated diseases of the chicken lung.

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DISCLOSURES

The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2021.101593.

REFERENCES

Abdel-Wahab, M. H. 2003. Testicular toxicity of dibromoacetonitrile and possible protection by tertiary butylhydroquinone. Pharmacol. Res. 47:509–515.

Ayer, R. E., and J. H. Zhang. 2008. Oxidative stress in subarachnoid haemorrhage: significance in acute brain injury and vasospasm. Cerebral Vasospasm 104:33–41.

Beker, A. S., L. Vanhooser, J. H. Swartzlander, and R. G. Teeter. 2004. Atmospheric ammonia concentration effects on broiler growth and performance. J. Appl. Poult. Res. 13:5–9.

Bove, P. F., B. R. Grubb, S. F. Okada, C. M. P. Ribeiro, T. D. Rogers, S. H. Randell, W. K. O'Neal, and R. C. Boucher. 2010. Human alveolar type II cells secrete and absorb liquid in response to local nucleotide signaling. J. Biol. Chem. 285:34939–34949.

Brownlee, M. 2001. Biochemistry and molecular biology of diabetic complications. Nature 414:375–392.

Carlo, M. D., and R. F. Loeser. 2010. Increased oxidative stress with aging reduces chondrocyte survival: correlation with intracellular glutathione levels. Arthritis Rheum 48:3419–3430.

Case, C. L., and R. Craig. 2013. Analyzing Caspase-1 activation during legionella pneumophila infection in macrophages. Methods Mol. Biol. 954:479–491.

Cambra-López, M., A. J. A. Aarnink, Y. Zhao, S. Calvet, and A. G. Torres. 2010. Airborne particulate matter from livestock production systems: a review of an air pollution problem. Environ. Pollut. 158:1–17.

Dai, P. Y., D. Shen, Q. Tang, K. Huang, and C. M. Li. 2020. $PM_{2.5}$ from a broiler breeding production system: the characteristics and microbial community analysis. Environ. Pollut 256 113368.1-113368.11.

Deng, X. B., F. Zhang, W. Rui, F. Long, L. J. Wang, Z. H. Feng, D. L. Chen, and W. J. Ding. 2013. $PM_{2.5}$-induced oxidative stress triggers autophagy in human lung epithelial A549 cells. Toxicol. Vitro 27:1762–1770.

Dickens, L. S., I. R. Powley, M. A. Hughes, and M. Marion. 2012. The 'complexities' of life and death: death receptor signalling platforms. Exp. Cell Res. 318:1269–1277.

Duewell, P., H. Kono, K. J. Rayner, C. M. Sirois, and E. Latz. 2010. Erratum: NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature 466:652.

Ewald, W. 2015. On the tricks alveolar epithelial cells play to make a good lung. Am. J. Respir. Crit. Care Med. 191:504–513.

Edelson, J. D., J. M. Shannon, and R. J. Marson. 1988. Alkaline phosphatase: a marker of alveolar type II cell differentiation. Am. Rev. Respir. Dis. 138:1268–1275.

Forman, H. J., H. Q. Zhang, and K. J. A. Davies. 2015. Oxidative stress response and Nrf2 signaling in aging. Free Radic. Biol. Med. 88:314–336.

Galluzzi, L., O. Kepp, and G. Kroemer. 2009. RIP kinases initiate programmed necrosis. J. Mol. Cell Biol. 1:8–10.

Hamscher, G., H. T. Pawelzick, S. Sczesny, H. Nau, and J. Hartung. 2003. Antibiotics in dust originating from a pig-fattening farm: a new source of health hazard for farmers? Environ. Health Perspect. 111:1590–1594.

Hirose, M., S. Takahashi, K. Ogawa, M. Futakuchi, T. Shirai, M. Shibutani, C. Uneyama, K. Toyoda, and H. Iwata. 1999. Chemoprevention of heterocyclic amine-induced carcinogenesis by phenolic compounds in rats. Cancer Lett. Iwata 143:173–178.

Holler, N., R. Zaru, O. Micheau, M. Thome, A. Attinger, S. Valitutti, J. L. Bodmer, P. Schneider, B. Seed, and J. Tschopp. 2000. Fas triggers an alternative, Caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat. Immunol. 1:489–495.

Jia, Y., F. Wang, Q. Guo, M. M. Li, L. Wang, Z. L. Zhang, S. Y. Jiang, H. H. Jin, A. P. Chen, S. Z. Tan, F. Zhang, J. J. Shao, and S. Z. Zheng. 2018. Curcumol induces RIPK1/RIPK3 complex-dependent necroptosis via JNK1/2-ROS signaling in hepatic stellate cells. Redox Biol 19:375–387.

Kepp, O., L. Galluzzi, L. Zitvogel, and G. Kroemer. 2010. Pyroptosis - a cell death modality of its kind? Eur. J.Immunol. 40:627–630.

Lagrange, B., S. Benaoudia, P. Wallet, F. Magnotti, A. Provost, F. Michal, A. Martin, F. D. Lrenzo, B. F. Py, A. Molinaro, and T. Henry. 2018. Human Caspase-4 detects tetra-acylated LPS and cytosolic francisella and functions differently from murine Caspase-11. Nat. Commun. 9:1–14.

Liang, X. L., W. S. Quan, L. S. Jin, J. K. Fung, M. Zhang, A. Tanaka, A. M. K. Choi, and J. Yang. 2013. P62 sequestosome 1/light chain 3b complex confers cytoprotection on lung epithelial cells after hyperoxia. Am. J. Respir. Cell Mol. Biol. 48:489–496.

Liu, W., Y. H. Chen, M. Jiao, M. F. Wu, F. F. Bi, C. C. Chang, H. Li, and L. J. Zhang. 2018. Ablation of Caspase-1 protects against TBI-induced pyroptosis in vitro and in vivo. J. Neuroinflamm 15:48.

Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of pro-IL-β. Mol. Cell 10:417–426.

Miao, E. A., J. V. Rajan, and A. Aderem. 2011. Caspase-1-induced pyroptotic cell death. Immunol. Rev. 243:206–214.

Ministry of Agriculture of the People's Republic of China. 1999. NY/T 388−1999 Environmental quality standard for the livestock and poultry farm. No.11, Nongzhan Nanli, Chaoyang District, Beijing. (in Chinese).

Mizumura, K., S. Maruoka, Y. Gon, A. M. K. Choi, and S. Hashimoto. 2016. The role of necroptosis in pulmonary diseases. Respir. Investig. 54:407–412.

Mostafa, E. 2012. Air-polluted with particulate matters from livestock buildings. Air Quality − New Perspect 14:287–312.

Niu, L. L., L. P. Li, C. Xing, B. Luo, C. C. Hu, M. M. Song, J. P. Niu, Y. Ruan, X. H. Sun, and Y. Lei. 2021. Airborne particulate matter ($PM_{2.5}$) triggers cornea inflammation and pyroptosis via NLRP3 activation. Ecotoxicol. Environ. Saf 207:1–9.

Nouhi, F., S. K. Tusi, A. Abdi, and F. Khodagholi. 2011. Dietary supplementation with tBHQ, an Nrf2 stabilizer molecule, confers neuroprotection against apoptosis in amyloid β-injected rat. Neurochem. Res. 36:870–878.

Radon, K., C. Weber, M. Iversen, B. Danuser, S. Pedersen, and D. Nowak. 2001. Exposure assessment and lung function in pig and poultry farmers. Occup. Environ. Med 58:405–410.

Seon-Mi, Y., and S. J. Kim. 2014. Withaferin a-caused production of intracellular reactive oxygen species modulates apoptosis via PI3K/Akt and JNKinase in rabbit articular chondrocytes. J. Korean Med. Sci 29:1042.

Shen, D., S. Wu, P. Y. Dai, Y. S. Li, and C. M. Li. 2018. Distribution of particulate matter and ammonia and physicochemical properties of fine particulate matter in a layer house. Poult. Sci 97:4137–4149.

Siempos, I. I., K. C. Ma, M. Imamura, R. M. Baron, L. E. Fredenburgh, J. W. Huh, J. S. Moon, E. J. Finkelsztein, D. S. Jones, M. T. Lizardi, E. J. Schenck, S. W. Ryter, K. Nakahira, and A. M. K. Choi. 2018. RIPK3 mediates pathogenesis of experimental ventilator-induced lung injury. J. Clin. Invest. 3:e97102.

Taguchi, K., H. Motohashi, and M. Yamamoto. 2011. Molecular mechanisms of the Keap1−Nrf2 pathway in stress response and cancer evolution. Genes Cells 16:123–140.

Tang, Q., K. Huang, J. Liu, S. Wu, and C. M. Li. 2019. Fine particulate matter from pig house induced immune response by activating TLR4/MAPK/NF-κB pathway and NLRP3 inflammasome in alveolar macrophages. Chemosphere 236:124373.

Upton, J. W., W. J. Kaiser, and E. S. Mocarski. 2010. Virus inhibition of RIP3-dependent necrosis. Cell 7:302–313.

Wallach, D., T. Kang, C. P. Dillon, and D. R. Green. 2016. Programmed necrosis in inflammation: toward identification of the effector molecules. Science 352 aaf2154.1-aaf2154.8.

Wang, W., Q. X. Shi, S. C. Wang, H. F. Zhang, and S. W. Xu. 2020. Ammonia regulates chicken tracheal cell necroptosis via the LncRNA-107053293/MiR-148a-3p/FAF1 axis. J. Hazard. Mater 386:121626.

Wang, X. B., Y. Bian, R. Zhang, X. D. Liu, L. Ni, B. T. Ma, R. Zeng, Z. W. Zhao, X. T. Song, and C. W. Liu. 2019. Melatonin alleviates cigarette smoke-induced endothelial cell pyroptosis through inhibiting ROS/NLRP3 axis. Biochem. Biophys. Res. Commun. 519:402–408.

Wu, W., P. Liu, and J. Y. Li. 2012. Necroptosis: an emerging form of programmed cell death. Crit Rev. Oncol. Hematol 82:249–258.

Wu, X. X., H. Y. Zhang, W. Qi, Y. Zhang, J. Li, Z. G. Li, and Y. Lin. 2018. Nicotine promotes atherosclerosis via ROS-NLRP3-mediated endothelial cell pyroptosis. Cell Death Dis 9:171.

Zhao, X. M., Z. Chen, J. B. Zhao, P. P. Zhang, Y. F. Pu, S. H. Jiang, J. J. Hou, Y. M. Cui, X. L. Jia, and S. Q. Zhang. 2016. Hsp90 modulates the stability of MLKL and is required for TNF-induced necroptosis. Cell Death Dis 7:e2089.

Zhao, Y. X., H. R. Zhang, X. N. Yang, Y. H. Zhang, S. Feng, and X. X. Yan. 2019. Fine particulate matter ($PM_{2.5}$) enhances airway hyperresponsiveness (AHR) by inducing necroptosis in BALB/c mice. Environ. Toxicol. Pharmacol 68:155–163.