Ecotoxicology and Environmental Safety 168 (2019) 9–16

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Cold stress provokes lung injury in rats co-exposed to fine particulate matter and lipopolysaccharide

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ARTICLE INFO

Keywords:
Cold stress
Fine particulate matter
Lipopolysaccharide
Nrf2/HO-1

ABSTRACT

Cold exposure aggravates respiratory diseases, which are also influenced by the exposures to particulate matter and endotoxin in the air. The aim of this study was to investigate the potential interactions among cold stress, fine particulate matter ($PM_{2.5}$, particles with aerodynamic diameter of 2.5 μm or less) and lipopolysaccharide (LPS, pure chemical form of endotoxin) on rat lung and to explore the related possible mechanisms of the interactions. Wistar rats were randomly grouped to be exposed to, 1) normal saline (0.9% NaCl), 2) $PM_{2.5}$, 3) LPS, and 4) $PM_{2.5}$ and LPS ($PM_{2.5}$ + LPS) through intratracheal instillation under cold stress (0 °C) and normal temperature (20 °C). Lung function, lung tissue histology, inflammatory response and oxidative stress levels were measured to examine the lung injury and to investigate the potential mechanisms. Exposure to $PM_{2.5}$ or LPS substantially changed pulmonary function [indicated by peak inspiratory flow (PIF) and peak expiratory flow (PEF)], inflammatory cytokine levels [indicated by interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)] and lung histology, compared to the non-exposed groups. Exposure to $PM_{2.5}$ + LPS under cold stress induced the most significant changes, including the increases of IL-6, TNF-α and thiobarbituric acid–reactive substances (TBARS), the decreases of PIF and PEF and more severe lung injury, among all exposure scenarios. Glutathione peroxidase activity and, nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) were found to be suppressed under cold stress, whereas Nrf2 and HO-1 levels were observed to be upregulated by exposure to $PM_{2.5}$ or LPS under normal temperature. In conclusion, cold stress may aggravate the lung injury in rats induced by simultaneous exposure to $PM_{2.5}$ and LPS. The progress may involve the suppressing of Nrf2/HO-1 signal pathway.

1. Introduction

Cold stress is a known environmental factor that induces and aggravates respiratory diseases, such as acute lung injury, chronic obstructive pulmonary disease (COPD), asthma and pneumonia (Hansel et al., 2016; Koskela, 2007). It was reported that cold stress caused bronchoconstriction and suppressed immune responses, which resulted in local inflammation and increased risk of respiratory infections (Koskela, 2007). Our previous study found that cold stress suppressed the phagocytosis function of alveolar macrophage (Luo et al., 2017), which is a critical component of the lung defending system. Airborne particulate matter is also a well-known risk factor of respiratory diseases (Jo et al., 2017; Zhao et al., 2017). Evidence from human studies indicated the interactive effect of cold stress and exposure to particulate matter on morbidity and mortality due to respiratory diseases (Wu et al., 2014; Zhang et al., 2015). However, the underling mechanism has not yet been fully understood.

Meanwhile, besides metal and polycyclic aromatic hydrocarbons (PAHs), endotoxin on particulate matter was also found to promote inflammatory reactions, which were associated with decreased forced expiratory volume in one second (FEV1) and increased asthma symptoms in children (Rabinovitch et al., 2005). A recent systematic review summarized the potential association between low levels of airborne endotoxin (< 100 EU/m^3) and respiratory effects (Farokhi et al., 2018).

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https://doi.org/10.1016/j.ecoenv.2018.10.064
Received 29 May 2018; Received in revised form 11 October 2018; Accepted 16 October 2018
Available online 25 October 2018
0147-6513/

Lipopolysaccharide (LPS), a pure chemical form of bacterial endotoxin, was reported to induce acute lung injury in mouse and the reaction was observed to be exacerbated by cold stress (Joo et al., 2016). Nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signal pathway is a critical antioxidant pathway in the lung. This pathway was found to be upregulated during the exposure to $PM_{2.5}$ or LPS (Thimmulappa et al., 2006), probably because exposure to $PM_{2.5}$ or LPS induced the production of reactive oxygen species (ROS) (Feng et al., 2016; Yan et al., 2008). Since cold stress was found to suppress the antioxidant enzymes like glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in rat lungs (Luo et al., 2014), we posited that the Nrf2/HO-1 signal pathway might be influenced by the cold stress during exposure to $PM_{2.5}$ or LPS, promoting exacerbated inflammatory response in the lung. To test this hypothesis, we exposed rats to $PM_{2.5}$, LPS and simulated cold stress; evaluated the inflammatory levels and oxidative stress; and explored the changes of the Nrf2/HO-1 signal pathway in the lung. To the best of our knowledge, this was the first study investigating the potential interactive effects of cold stress and simultaneous exposures to $PM_{2.5}$ and LPS in rats and the possible mechanisms.

2. Materials and methods

2.1. Materials

Antibodies against Nrf2 and HO-1 were purchased from Abcam (Cambridge, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and horseradish peroxidase-conjugated secondary anti-rabbit was purchased from Elabscience Biotechnology (Wuhan, China), while anti-mouse antibody was obtained from Signalway Antibody (College Park, Maryland, USA). LPS was supplied by Sigma (St. Louis, MO, USA) and the endotoxin kit (BTN120415) was purchased from Balb Technology (Beijing, China). Kits used for thiobarbituric acid–reactive substances (TBARS) and GSH-Px measurement were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. $PM_{2.5}$ preparation

$PM_{2.5}$ were collected on glass fiber filters at two different locations in Lanzhou City, on the top of two buildings 5 km to each other and 100 m away from major arterial streets. Two mini-volume $PM_{2.5}$ samplers (Airmetrics, Springfield, OR, USA) were used to collect $PM_{2.5}$ from September 2015 to August 2016 at a flow rate of 8 L/min. Particles collected on the filter were released by a sonication bath in a laboratory. Briefly, filters were cut into 2 cm^2 and placed in a glass beaker with ultra-pure water. Particles were released from filters by agitating for 20 min with an ultrasonic shaker at 140 w and repeated for 3 times. After filtered through six layers of gauze, the solution was dried by a vacuum freeze drier (Christ/ALPHA2–4 LD, Germany) at −80 °C. Particles were kept at −20 °C till use.

2.3. Analysis of PAHs in $PM_{2.5}$

Two milligram of $PM_{2.5}$ sample was re-suspended in 200 mL dichloromethane by ultrasonication (8210R-MTH, Branson ultrasonics Cor., USA) for 30 min. After filtrated with 47-millimeter Teflon filter (Merck Millpore Ltd., Iceland), the filtrated fluid was evaporated to 0.5 mL by a TurboVap II (Caliper Life Sciences, MA, USA) with nitrogen gas. The extract was used for the analysis of 17 PPAHs (parent PAHs) and 19 oxygenated-PAHs (OPAHs) with gas chromatographic mass spectrometry (Agilent 7820 A GC coupled with an Agilent 5977E MSD, Agilent Technologies, USA) in selected ion monitoring mode and electron impact ionization, which were repeated for three times. The details of the 36 PAHs could be found in the Supplementary materials. The analytical method was reported previously (Gou et al., 2017; Noth et al., 2016).

2.4. Endotoxin analysis

$PM_{2.5}$ sample was re-suspended in pyrogen-free phosphate-buffered saline to a concentration of 1 mg/mL. Then, ultrasound water bath was employed for 1 h at 22 °C (Falcon-Rodriguez et al., 2017). The endotoxin in $PM_{2.5}$ dilution was analyzed with the Chromogenic Endpoint Tachypleus Amebocyte Lysate Kit (BTN120415, Balb Technology Co., LTD, Beijing, China), which was repeated for three times.

2.5. Animal grouping and exposure protocols

Fifty-six male Wistar rats [10 weeks old, weighing 160–200 g, batch number: SCXK (Gan) 2015-001] were purchased from Veterinary Institute, Chinese Academy of Agricultural Sciences (Lanzhou, China). Animals were provided with drinking water and a standard diet in normal temperature (20 ± 2 °C) and relative humidity 50% ± 5% with a 12-h light-dark cycle (9:00–21:00 light, 21:00–9:00 dark). After two weeks of blank control period, these rats were randomly divided into eight groups (7 rats per group), including cold stress groups (control group, LPS exposure group, $PM_{2.5}$ exposure group and LPS + $PM_{2.5}$ exposure group) and normal temperature groups (control group, LPS exposure group, $PM_{2.5}$ exposure group and LPS + $PM_{2.5}$ exposure group).

Groups exposed to normal temperature were kept under identical conditions as in blank control period. Groups exposed to cold stress were placed in a climate simulator (GDJS-500L, Pulingte Co., Tianjin, China) controlling temperature at 0 °C ± 2 °C and relative humidity of 50 ± 5% for 8 h a day. Each rat of every cold stress group was kept in a single wire cage when exposing to cold stress. The exposure to cold stress was repeated every other day for three times. Rats were anesthetized before exposed to $PM_{2.5}$ or LPS. Animals in the LPS-exposed groups were administered a single dose of 0.2 mg LPS in 200 μL of sterile normal saline (0.9% NaCl) via intratracheal instillation. For $PM_{2.5}$-exposed groups, rats were instilled 0.8 mg $PM_{2.5}$ suspended in 200 μL sterile normal saline. The combined exposure of $PM_{2.5}$ and LPS ($PM_{2.5}$ + LPS) was carried out by instillation of 0.8 mg $PM_{2.5}$ and 0.2 mg LPS suspended in 200 μL NS to the trachea of the rat. The control groups received the same volume of sterile normal saline. In cold stress exposed groups, LPS and/or $PM_{2.5}$ exposures were carried out in the middle of each cold exposure period (at the 4th h of 8 h cold exposure). LPS and/or $PM_{2.5}$ exposures were also repeated every other day for three times. Rats were sacrificed at the 24th h after the last treatment. The experiment protocols were approved by Lanzhou University according to the rules of the Ethics Committee of Animal Care and Experimentation, National Institute for Environmental Studies, China.

2.6. Pulmonary function test

After treatments, each rat was placed in a main chamber of the body plethysmograph immediately. Peak inspiratory flow (PIF) and peak expiratory flow (PEF) were measured as indicators of pulmonary function, using a barometric whole-body plethysmography (Buxco, EMKA Technologies, Paris, France) according to the published method (Witzenrath et al., 2006). All the pulmonary function tests were carried out by well-trained technicians in a double-blind manner.

2.7. Histological assessment

After sacrifice, the right lung was harvested, of which the upper lobes were fixed in 4% paraformaldehyde and embedded in paraffin overnight. Three rat lungs of each group were randomly selected for histological study. Two histological slides were used for each rat. Four micrometer paraffin sections were stained with hematoxylin and eosin (H&E) according to the standard procedure. A pathological image

acquisition and analysis system (DP-72, Olympus, Japan) was used to observe and record the pathologic changes of the lung tissue. Lung injury was scored according to a published method (Matute-Bello et al., 2001). Each slide was evaluated by investigators in a double-blind manner. To generate the lung injury score, a total of 300 alveoli were counted on each slide at 400 × magnification. Injury scoring criteria includes alveolar septae (0 = all septae are thin and delicate; 1, 2, 3 = congested alveolar septae in less than 1/3, 1/3–2/3, greater than 2/3 of the field), intra-alveolar fibrin (0 = no intra-alveolar fibrin; 1, 2, 3 = fibrin strands in less than 1/3, 1/3–2/3, greater than 2/3 of the field); alveolar hemorrhage (0 = no hemorrhage; 1, 2, 3 = at least 5 erythrocytes per alveolus in 1–5, 5–10, more than 10 alveoli) and intra-alveolar infiltrates (0, 1, 2, 3 = less than 5, 5–10, 10–20, more than 20 intra-alveolar cells per field). The total injury score for each rat lung was calculated according to the following formula: injury score = [(alveolar hemorrhage points/number of fields) + 2 × (alveolar infiltrate points/number of fields) + 3 × (fibrin points/number of fields) + (alveolar septal congestion/ number of fields)]/total number of alveoli counted.

2.8. Inflammatory cytokine measurements and oxidative stress evaluation

The left lung of each rat was collected and made into 10% lung tissue homogenate with sterile normal saline at 0 °C. The supernatant was collected immediately after centrifuged at 4 °C and kept at −80 °C before analysis. The total protein concentration was measured by Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, USA). Following manufacturer's instructions, the levels of TNF-α and IL-6 were measured as inflammatory markers, and GSH-Px was measured to evaluate the level of antioxidant enzyme in rat lung. TBARS was measured as an indicator of oxidative stress. According to the kit instruction, 0.1 mL of the lung homogenate supernatant and 0.1 mL 8.1% sodium dodecyl sulfate were mixed up and added to 3 mL 0.67% solution containing thibabituric acid and 20% glacial acetic acid. Following by adding 1 mL N-butyl pyrimidine, the mixed solution was subjected to heat at 95 °C for 40 min and then centrifuged at 2800 × *g* for 10 min. The absorbance of the supernatant was taken at 532 nm using a spectrophotometer.

2.9. Western-blot analysis Nrf2 and HO-1

Western blot analysis of Nrf2 and HO-1 in lung tissue was performed according to standard protocols (Lv et al., 2016). In brief, lung tissue was harvested and lysed with RIPA buffer. The level of total proteins was calculated by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Thirty microgram of protein sample diluted with sodium dodecyl sulfate polyacrylamide gel electrophoresis was loaded onto 10% gels and transferred on to polyvinylidene fluoride membrane and blocked with 2% bovine serum albumin in tris-buffered saline and Tween 20 at room temperature for 1 h. Then, the membranes were incubated with anti-Nrf2 and anti-HO-1 at 4 °C overnight. Immunodetection was performed with a horseradish secondary antibody using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology, Haimen, China). The membranes were scanned and the band densities were calculated using Image J software (https://imagej.nih.gov/ij/).

2.10. Statistical analysis

Data were reported as mean ± standard deviation. Differences between groups were determined by one-way analysis of variance (ANOVA) test and Fisher post hoc test with SPSS 22.0 software for Windows (SPSS, Chicago, IL). The minimum significance level was set at a p value of 0.05 for all analysis. A significance level of $p < 0.01$ and $p < 0.001$ were reported as well.

Table 1
Components in fine particulate matter.

	Concentration		Ratio
PPAHs (ng/mg)	210.98 ± 19.43	PPAHs/ΣPAHs	0.16 ± 0.03
OPAHs (ng/mg)	1085.78 ± 323.34	OPAHs/ΣPAHs	0.84 ± 0.03
Endotoxin (EU/mg)	0.90 ± 0.02		

Parent PAHs (PPAHs); Oxygenated PAHs (OPAHs). Results expressed the three repeated measurements of $PM_{2.5}$ sample, reported as mean ± standard deviation.

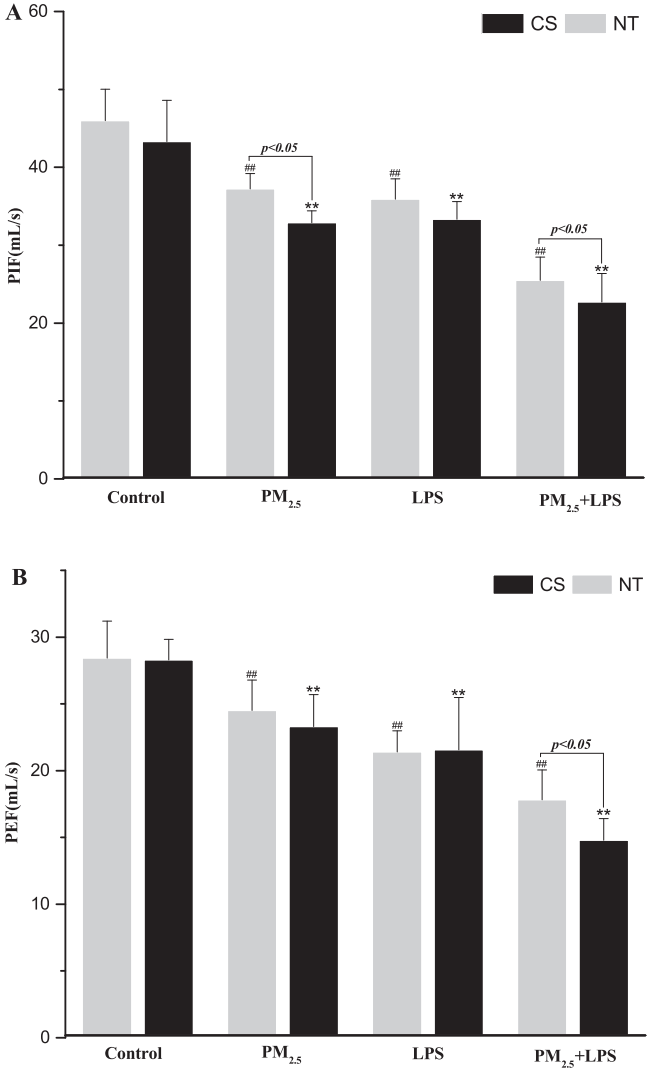

Fig. 1. Effect of cold stress, $PM_{2.5}$ and LPS on pulmonary function in rats. A) Peak Inspiratory Flow (PIF); B) Peak Expiratory Flow (PEF). Normal Temperature (NT); Cold Stress (CS); Lipopolysaccharide (LPS); Fine Particulate Matter ($PM_{2.5}$). Results were reported as mean ± standard deviation. n = 7 per group. $^{\#\#}$ indicates $p < 0.01$ when compared with the control group under normal temperature; ** indicates significant difference at $p < 0.01$ when compared with the control group under cold stress. Solid lines with P value show the groups that are compared.

3. Results

3.1. $PM_{2.5}$ Composition

Results of $PM_{2.5}$ composition analysis (including PPAHs, OPAHs and endotoxin) can be found in Table 1. The sum of 17 PPAHs in archived $PM_{2.5}$ was 211.00 ng/mg, with anthracene contributing the most

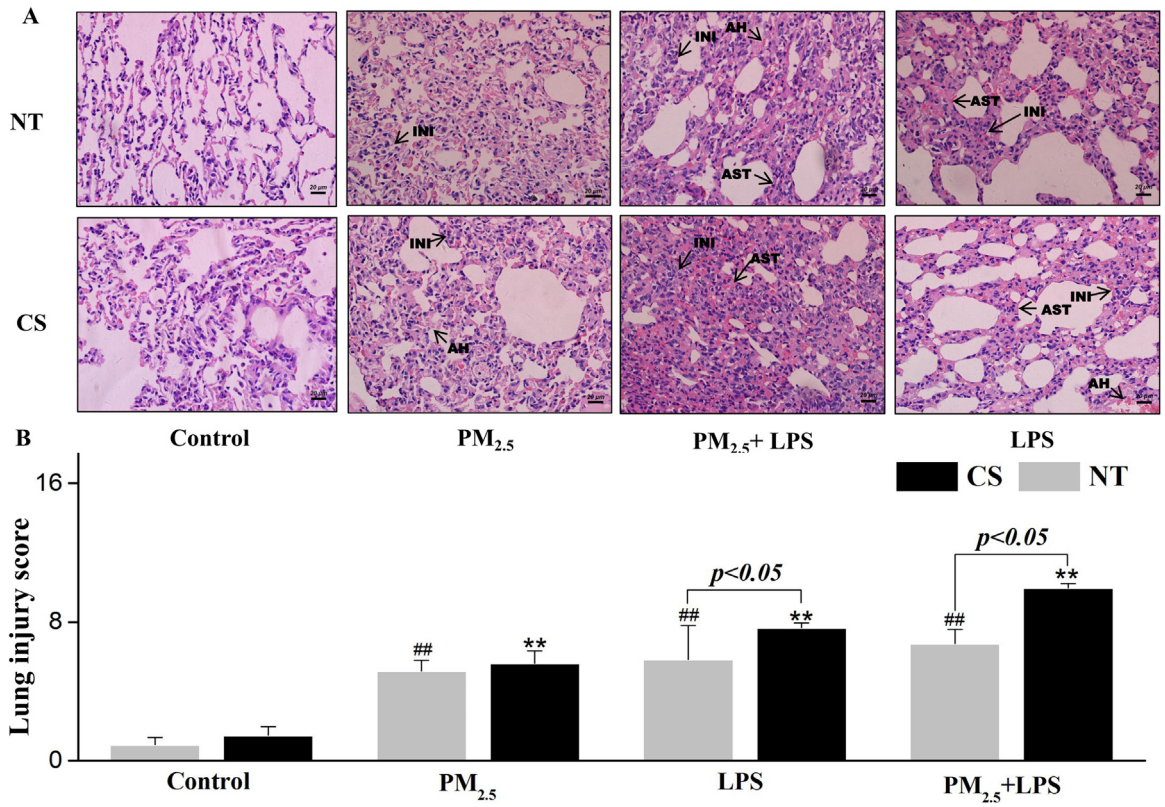

Fig. 2. Effect of cold stress, $PM_{2.5}$ and LPS on histological changes in rat lung. A) Lung histology images; B) Lung injury score. Normal Temperature (NT); Cold Stress (CS); Lipopolysaccharide (LPS); Fine Particulate Matter ($PM_{2.5}$). Lung specimens were stained with hematoxylin and eosin. (INI) indicates intra-alveolar neutrophil infiltration; (AST) indicates alveolar septae thickening; (AH) indicates alveolar hemorrhage. Original magnification is 400 ×. The results of lung injury score were expressed as mean ± standard deviation. $n = 3$ per group. $^{\#\#}$ indicates $p < 0.01$ when compared with the control group under normal temperature; ** indicates significant difference at $p < 0.01$ when compared with the control group under cold stress. Solid lines with p value show the groups that were compared. Scale bar is 20 μm.

(48.22 ng/mg). The total concentration of OPAHs was 1085.80 ng/mg. Among 19 OPAHs measured, 2-ethylanthraquinone was most abundant (253.92 ng/mg). The details for specific PAHs concentration in $PM_{2.5}$ of this study and standard reference materials 1649a could be found in Supplementary material. The endotoxin concentration on $PM_{2.5}$ was 0.90 EU/mg.

3.2. Pulmonary function after exposure

Cold stress by itself did not result in any significant effects on PIF or PEF without exposure to $PM_{2.5}$ or LPS (Fig. 1A & B). Exposures to $PM_{2.5}$, LPS or $PM_{2.5}$ + LPS all reduced lung function (PIF and PEF) regardless of the temperature, with simultaneous exposures to $PM_{2.5}$ + LPS resulting in the most significant reduction, compared to the control groups. PIF seemed to be more sensitive to cold stress than did PEF when exposed to $PM_{2.5}$ or LPS alone. The cold stress did not induce significant differences in PEF when rats were exposed to $PM_{2.5}$ or LPS (Fig. 1B), but the difference was significant when they were exposed to $PM_{2.5}$ + LPS. Moreover, simultaneous exposures to $PM_{2.5}$ + LPS under the cold stress resulted in the lowest PIF and PEF.

3.3. Lung histology changes after exposure

No obvious pathological change was observed in lungs of control group under normal temperature and cold stress (Fig. 2A). The lung injury in the groups treated with $PM_{2.5}$ and/or LPS under both temperature conditions was characterized by patchy areas of neutrophilic infiltrates with thickening of the alveolar septae and areas of hemorrhage. The majority of alveolar septae were filled with infiltrated cells and thickened with edema. Both leukocyte infiltration and alveolar septae thickening were especially obvious in $PM_{2.5}$ + LPS group under cold stress. The histopathology score plot (Fig. 2B) also showed significant lung injury after the treatment of cold stress, $PM_{2.5}$ and LPS. Particularly combined with cold stress, LPS and $PM_{2.5}$ induced the most severe injury in rat lung.

3.4. Inflammatory cytokine changes after exposure

In this study, TNF-α and IL-6 were measured to examine the proinflammatory effect of exposure to cold stress, $PM_{2.5}$ and LPS. Among normal temperature groups, exposure of $PM_{2.5}$ and/or LPS increased both TNF-α and IL-6 with the largest increase occurred in $PM_{2.5}$ + LPS group (Fig. 3). Compared with the control group, treating with LPS or $PM_{2.5}$ exposure increased TNF-α and IL-6 level by more than 100 ng/mL and 50 ng/mL, respectively. Cold stress increased lung TNF-α and IL-6 expression in every treatment group, although the significant increase only happened in $PM_{2.5}$ + LPS group. Compared with other groups, the group exposed to $PM_{2.5}$ + LPS under cold stress had the highest level of TNF-α and IL-6.

3.5. Oxidative stress and anti-oxidant function evaluation

Lung tissue GSH-Px level in cold stress exposed rats exhibited great reduction compared with those under normal temperature, particularly when exposed to $PM_{2.5}$ or LPS (Fig. 4A). Both LPS and $PM_{2.5}$ induced the reduction of GSH-Px level in rat lung, but the significant reduction was only found in rats with combined exposure of $PM_{2.5}$ + LPS (normal temperature and cold stress). The lowest GSH-Px level was found in the group with $PM_{2.5}$ + LPS exposure under cold stress. Rat lung level of TBARS was measured to study the lipid peroxidation after co-exposure

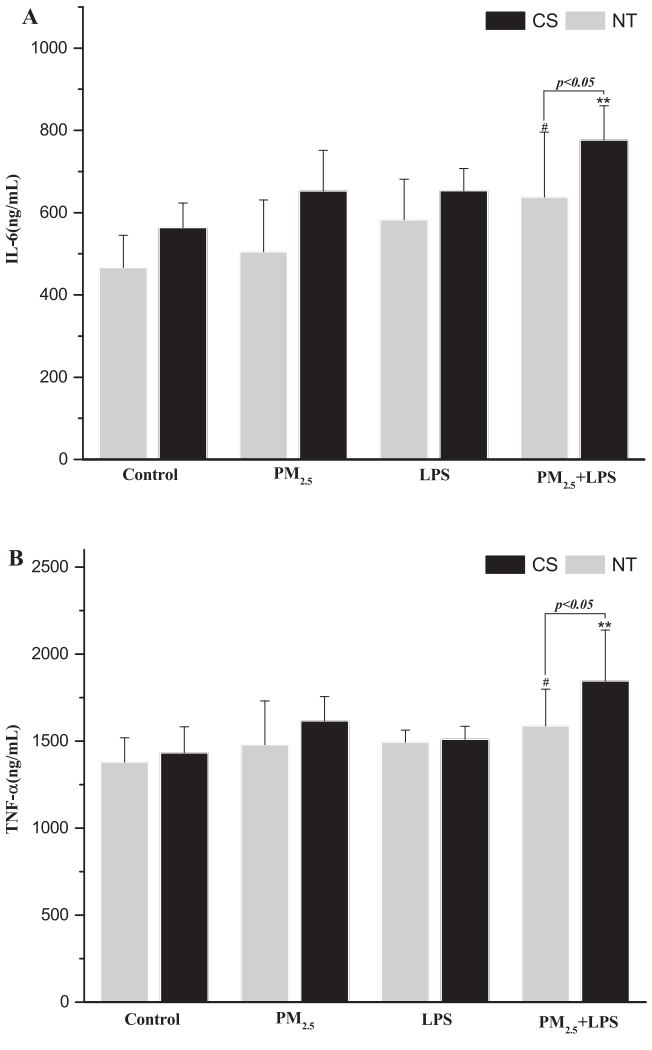

Fig. 3. Effect of cold stress, $PM_{2.5}$ and LPS on proinflammatory cytokines in rat lung. A) Interleukin-6 (IL-6); B) Tumor Necrosis Factor-α (TNF-α). Normal Temperature (NT); Cold Stress (CS); Lipopolysaccharide (LPS); Fine Particulate Matter ($PM_{2.5}$). Results were reported as mean ± standard deviation. n = 7 per group. # indicates $p < 0.05$ when compared with the control group under normal temperature; ** indicates significant difference at $p < 0.01$ when compared with the control group under cold stress. Solid lines with p value show the groups that were compared.

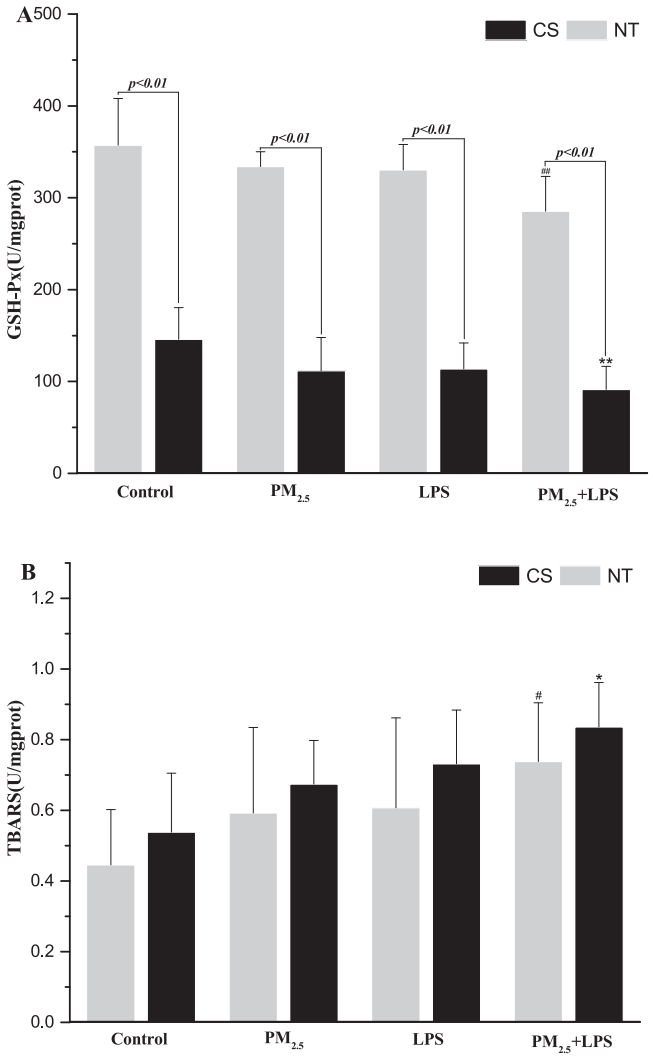

Fig. 4. Oxidative stress levels in rat lungs after exposure to cold stress, $PM_{2.5}$ and LPS. A) Glutathione Peroxidase (GSH-Px); B) Thiobarbituric Acid–reactive Substances (TBARS). Normal Temperature (NT); Cold Stress (CS); Lipopolysaccharide (LPS); Fine Particulate Matter ($PM_{2.5}$). Results are reported as mean ± standard deviation. n = 7 per group. # and ## indicate significant difference at $p < 0.05$ and $p < 0.01$ when compared with the control group under normal temperature; * and ** indicate significant difference at $p < 0.05$ and $p < 0.01$ when compared with the control group under cold stress. Solid lines with p value show the groups that were compared.

to cold stress, $PM_{2.5}$ and LPS (Fig. 4B). Compared with normal temperature groups, TBARS level increased in cold stress exposed groups. It was significantly higher in groups with co-exposure of $PM_{2.5}$ + LPS, compared to control group regardless of temperature condition. Exposure to $PM_{2.5}$ or LPS alone increased TBARS level, but the effects were not significant.

3.6. Nrf2/HO-1 level after exposure

As indicated in Fig. 5, cold stress significantly suppressed the expression of Nrf2 and HO-1 in all groups. Under cold stress, higher expression of Nrf2 and HO-1 was found in LPS treating group, but there was no substantial difference compared with $PM_{2.5}$ + LPS group. The lowest expression was found in cold stress exposed group (without $PM_{2.5}$ or LPS) for HO-1. Regardless temperature conditions, both $PM_{2.5}$ and LPS upregulated Nrf2 and HO-1, while they were the highest in group with exposure of $PM_{2.5}$ + LPS under normal temperature.

4. Discussion

Aerosolized endotoxin is often found on the surface of particulate matter, which is related to its respiratory health effect, even if at a low level (Farokhi et al., 2018). In some special places, like livestock farms and composting sites, aerosolized endotoxin has been found at high level (Jonges et al., 2015). Since the lung effect of either LPS or $PM_{2.5}$ could be exacerbated by cold stress, we hypothesized that it would be much worse when these three factors combined. Interestingly, the most severe lung injury was indeed observed in the group that was exposed to $PM_{2.5}$ and LPS simultaneously under cold stress, which may involve suppressing of Nrf2/HO-1 signal pathway.

Previously, we reported higher production of IL-6, TNF-α in rat lung after exposure to $PM_{2.5}$ at a total dose of 8 mg/rat (Luo et al., 2014). In this study, the total dose of $PM_{2.5}$ exposure was only 2.4 mg/rat, which may explain the unobvious increasing level of IL-6 and TNF-α in rat lung, even if combined with cold stress. In a similar study, two weeks' cold stress aggravated the inflammatory responses in an LPS-induced

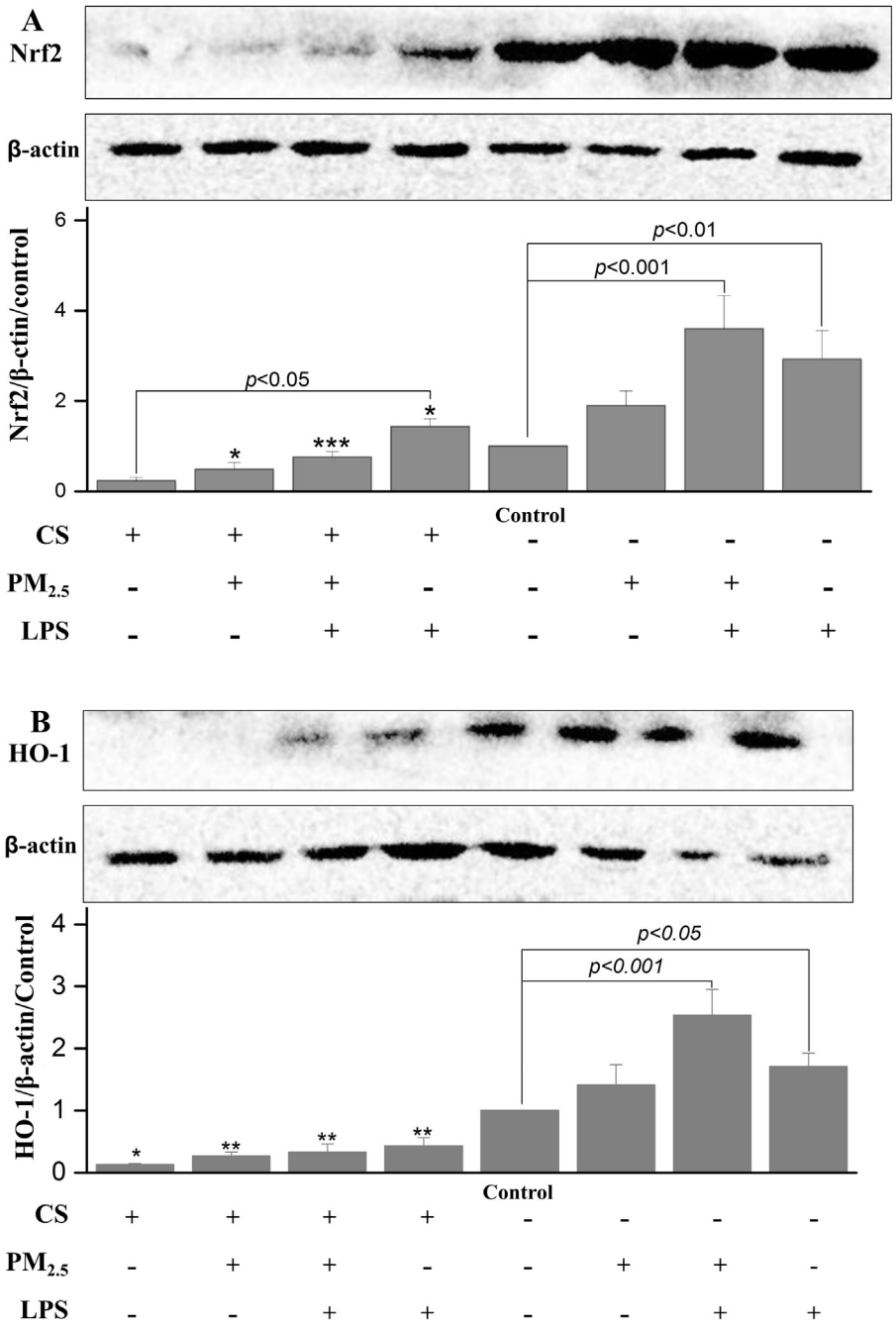

Fig. 5. Nrf2 and HO-1 expression in rat lung. A) Nuclear factor E2-related factor 2 (Nrf2); B) Heme Oxygenase-1 (HO-1). Cold Stress (CS); Lipopolysaccharide (LPS); Fine Particulate Matter ($PM_{2.5}$). The intensity of the bands was quantified by densitometric analysis. The bar represents the value of Nrf2/β-actin and HO-1/β-actin compared to blank. The results of quantitative analysis, with the images depicting the results of three independent western blot experiments (mean ± standard deviation, $n = 3$). The symbol (−) indicates the absence of treatment, the symbol (+) indicates the presence of treatment. * and ** indicate significant difference at $p < 0.05$ and $p < 0.01$ when compared with the same treatment group without cold stress. Solid lines with p value show the groups that were compared.

mouse model of acute lung injury (Joo et al., 2016). But we only exposed rats to cold stress for only three times, which may not be long enough to aggravate the LPS induced inflammatory responses in the rat lung. Besides, IL-6 and TNF-α may not be sensitive to indicate the lung inflammatory response of LPS, since IL-12 and IL-17 were found increased in the study of Joo. et al. (2016) and lower lung function was observed after LPS exposure in this study. However, as hypothesized, we did find higher expression of TNF-α and IL-6 and much lower instant lung function in the rats treated with cold stress, $PM_{2.5}$ and LPS. In that, cold stress suppressed the respiratory immune system by destroying the cilium and inhibiting leukocyte migration (Polderman, 2009; Shephard and Shek, 1998), alveolar macrophage activity and phagocytic function (Luo et al., 2017). Although it has been reported that endotoxin could be 10 fold-higher in $PM_{2.5–10}$ than in $PM_{2.5}$ (Heinrich et al., 2003), the deeper penetration capacity of $PM_{2.5}$ combined with LPS into the lung may provoke serious adverse effect. Hence, we believe that cold stress may aggravate the effect of $PM_{2.5}$ and LPS on rat lung.

In this study, we analyzed 36 PAHs (including 17 parent PAHs and 19 oxygenated PAHs) in the $PM_{2.5}$ samples. The total concentration of detected PAHs in $PM_{2.5}$ (in 1.30 μg/mg $PM_{2.5}$) was higher than that was in standard reference materials 1649a (Bergvall and Westerholm, 2008), indicating a great ROS production potentiality in the body (Shang et al., 2014). TBARS was often used as equivalent of MDA, which was often used as an indirect indicator of oxidative stress (Bayatli et al., 2013). We found TBARS was higher in both $PM_{2.5}$ and LPS exposed groups, indicating ROS probably involved in $PM_{2.5}$/LPS induced lung inflammation, which was consistent with findings of other studies (Chuang et al., 2011; Yan et al., 2008). Suppressed antioxidant enzyme activities, such as GSH-Px activity in this study and SOD activity in other studies (Li et al., 2017; Lin et al., 2016; Luo et al., 2014) confirmed ROS involvement in the lung pathological changes induced by $PM_{2.5}$/LPS exposure (Yanagisawa et al., 2003). Meanwhile, cold

exposure leads to hypothermia and cell damages, which may also increase ROS production (Pizanis et al., 2011; Rauen and de Groot, 2004). By activating the iron channel of cold-sensing transient receptor potential subfamily member 1 (TRPA1), cold stress induced higher production of nitric oxide and ROS in A549 cell (Sun et al., 2014, 2016). Normally, ROS are at low level and often contained by a wide array of antioxidant enzymes like SOD and GSH-Px (Zalba et al., 2007). Suppression of these enzymes would impair the regulation of ROS. Therefore, ROS may be involved in the interactive effect of cold stress, $PM_{2.5}$ and LPS on rat lung.

Studies reported that $PM_{2.5}$ induced activation of Nrf2-mediated defenses, such as increasing Nrf2 and HO-1 expression to mitigate the oxidative stress both in vitro and vivo (Deng et al., 2013; Yan et al., 2008; Zhou et al., 2017). The LPS-induced expression of proinflammatory cytokines in the Nrf2 + /+ mice were suppressed by Nrf2 activator through increasing antioxidative genes expression such as HO-1 (Thimmulappa et al., 2006). Therefore, Nrf2 is very important in increasing the expression of detoxifying enzymes and facilitate the removal of ROS. In this study, the higher expression of Nrf2 and HO-1 in rat lung after $PM_{2.5}$ and LPS exposure may indicate the activation of Nrf2/HO-1 signal pathway. Interestingly, we observed lower Nrf2 and HO-1 expression in all groups under cold stress, compared to groups under normal temperature. As Nrf2 contains a transcription-activation domain and positively regulates HO-1 transcription, inhibiting Nrf2 gene could result in the impairment of transcriptional responses to inducers of HO-1 and thus inhibit the production of HO-1 (Alam et al., 1999; Ishii et al., 2000; Morse et al., 2009). In a vitro study, HO-1 expression declined dramatically with a temperature reduction of 7 °C (from 37 °C to 30 °C) and became undetectable with further temperature decrease (Balogun et al., 2003). In addition, Nrf2-Keap1 inhibition was reported to be related to cardiac injury after two weeks' cold stress in a recent study (Cong et al., 2018). The possible explanation could be that cold stress may inhibit the production of Nrf2 and HO-1 or induce faster depletion of them. Therefore, the inhibited expression of Nrf2 and HO-1 in cold stress exposure may promote higher ROS activity and consequently lead to aggravated inflammatory response in the lungs of $PM_{2.5}$ or/and LPS exposed groups. However, this mechanism is very tentative. To prove that, we look forward to use Nrf2 + /+ rat and HO-1 stimulator, and measure target genes of Nrf2, like NQO1 and GCLC.

5. Conclusion

In summary, this pilot study reported that cold stress was observed to aggravate the lung injury induced by simutaneous exposures to $PM_{2.5}$ and LPS, which may involve suppressing the Nrf2/HO-1 signal pathway.

Acknowledgement

This study was supported by grants from the Natural Science Foundation of China (No. 41405108) and the Fundamental Research Fund for the Central Universities, Lanzhou University, China (lzujbky-2017-86). We greatly appreciate Mr. Charles Perrino for his help in PAHs and OPAHs analysis, which was carried out in Prof. S. Katharine Hammond's Laboratory at University of California, Berkeley.

Disclosure Statement

The authors state no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.10.064.

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