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## ORIGINAL ARTICLE

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### Effects of Lianhua Qingwen on Pulmonary Oxidative Lesions Induced by Fine Particulates (PM2.5) in Rats

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**Key words:** fine particulates (PM2.5); pulmonary lesion; oxidative stress;  
Lianhua Qingwen; rats

**Objective** To investigate the antagonistic effects of different doses of Lianhua Qingwen on pulmonary injury induced by fine particulates PM2.5 in rats.

**Methods** Fine particulates suspended in the environment were collected. Forty-eight healthy adult wistar rats were randomly divided into 6 groups with 8 rats in each group. Four groups of rats were exposed to PM2.5 by intratracheally dripping suspensions of fine particulates PM2.5 (7.5 mg/kg) as dust-exposed model rats. Among them 24 rats in three groups received Lianhua Qingwen treatment (crude drug) at a dose of 2 g/kg, 4 g/kg, 8 g/kg per day for 3 days before dust exposure and were defined as low-dose, middle-dose and high-dose Lianhua Qingwen treatment groups respectively. The other dust-exposed model rats without treatment were assigned as PM2.5 control group. The un-exposed rats were set as saline control group (1.5 ml/kg saline) and blank control group. All rats were killed after 24 hours of the exposure. Lung tissue, serum and bronchoalveolar lavage fluid (BALF) were collected. The levels of malonaldehyde (MDA), lactate dehydrogenase (LDH), and glutathione peroxidase (GSH-PX) in blood serum and BALF, and superoxide dismutase (SOD) in blood serum were measured using fluorescent quantitation PCR; Expression of NF-E2-related factor 2(NRF-2), heme oxygenase 1 (HO-1) and quinone oxidoreductase 1 (NQO1) in lung tissues were measured using Western blot. Pathological changes of lung tissues in each group were also examined.

**Results** Pathology revealed thickened alveolar septum, congestion of capillary, interstitial edema and infiltration of lymphocyte and neutrophil surrounding bronchiole in the PM2.5 control group, which were

significantly relieved in the Lianhua Qingwen treatment groups. Compared to the blank and saline control groups, the PM2.5 control group had significantly higher levels of LDH and MDA ( $p < 0.01$ ) and lower level of GSH-PS ( $p < 0.01$ ) in BALF, significantly higher levels of LDH and MDA ( $p < 0.05$ ) and lower level of GSH-PS ( $p < 0.05$ ) in rat serum. The levels of MDA in blood serum and BALF were significantly lower in each treatment group than that in PM2.5 control group (all  $P < 0.05$ ). In both middle-dose and high-dose treatment group the measurements of LDH in serum and BALF as well as GSH-PX in serum were significant difference from those of PM2.5 control group (all  $P < 0.05$ ). Expressions of NRF-2, HO-1 and NQO1 in lung tissues were significantly different among middle-dose and high-dose treatment group compared with those in PM2.5 control group (all  $P < 0.05$ ).

**Conclusion** Fine particulates PM2.5 in environment may induce pulmonary oxidative lesions in rats. Middle-dose and high-dose Lianhua Qingwen has antagonist effece on the injuries induced by fine particulates.

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**A**S fine particulates knowledge grows, the impact of environmental fine particulates (PM2.5) on public health is attracting more attention. The overseas and domestic studies on pulmonary lesion induced by fine particulates show that fine particulates getting into pulmonary interstitium can induce chronic inflammation and oxidative stress injury in pulmonary tissues,<sup>1</sup> and dramatically increase morbidities and mortalities of chronic obstructive pulmonary diseases (COPD), asthma and other respiratory diseases.<sup>2</sup> Further studies were needed for better treatment of oxidative stress injury induced by PM2.5. Lianhua Qingwen Capsule, a formula of traditional Chinese medicine including Forsythia, Honeysuckle, Ephedra, Male Fern Rhizome, Herba Houttuyniae, Pogostemon Cablin, Rheum Officinale, Rhodiola Rosea, Menthol and Licorice Root, has been demonstrated to have antiviral activity and regulate pulmonary immunity, and has been used in inflammation diseases such as acute pulmonary infection. This *in vivo* animal study investigated oxidative lesion of pulmonary tissues induced by PM2.5 using rat contamination models, in order to explore the relationship between the dosage of Lianhua Qingwen and its effect on pulmonary injuries induced by PM2.5.

## MATERIALS AND METHODS

### Rats, exposure and intervention

This animal study was approved by the Animal Care and Utilization Committee of Hebei General Hospital. Forty-eight healthy adult Wistar rats from Experimental Center, Hebei Medical University (male, weight 200–250g, and age 8–10 weeks) were matched and maintained on a chow diet in a 12-h light/12-h dark environment at 25°C. The rats were randomly divided into 6 groups, with 8 rats in each group. Two groups were used for blank control and saline control; the other four groups were exposed with PM2.5,

and three of them were treated with Lianhua Qingwen.

PM2.5, provided by Shijiazhuang Environmental Monitoring Center, was made into suspension of 5 mg/ml for the experiment. When sampling, fine particles were released from filter which had been heated at 100°C for 24 hours, and put into deionized water and sonicated 3×15 minutes with a sonicator. The fine particles suspension was treated through multilayer gauze to filter glass fiber, and then dried by vacuum-freeze machine and stored at -20°C until experiment.

Four groups of rats were exposed to PM2.5 by intratracheally dripping suspensions of PM2.5 at 7.5 mg/kg as dust exposed model rats. Among them 24 rats in three groups were assigned into the low-dose group, middle-dose group and high-dose group, and were intragastrically treated with 2 g/kg·d, 4 g/kg·d and 8 g/kg·d crude Lianhua Qingwen (Shijiazhuang Yiling Pharmaceutical Co., Ltd, China) suspension respectively for 3 days before dust exposure. There was one group of 8 dust exposed model rats that did not receive the treatment and was defined as PM2.5 control group. In saline control group, the rats were only given 1.5 ml/kg normal saline *via* trachea once instead of PM2.5 suspension. In blank control group, the rats were given no treatment and no PM2.5 exposure.

### Sample collection

All rats were killed 24 hours after PM2.5 exposure by cutting off femoral artery. 10% chloral hydrate were injected intraperitoneally for anesthesia. Supernatant of blood was reserved after centrifugation. Left lung lobes underwent bronchoalveolar lavage, and bronchoalveolar lavage fluid (BALF) was taken for centrifugation.

### Histopathology

Left lungs of rats in each group were sliced and HE staining was performed after dehydration with dehydrated alcohol and paraffin embedding. Pathological changes of samples

were observed under light microscope (BX51; Olympus, Tokyo, Japan).

### PCR

Lactic dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) in serum and BALF were measured using a PCR kit (Jiancheng Biologics Co., Ltd. Nanjing) by Fluorescent Quantitation PCR System (ABI prime 7300; American ABI Company, USA).

### Western blot

Fresh lung tissues were sampled and packed in 1.5 ml EP tubes and immediately stored in liquid nitrogen. NF-E2-related factor 2 (NRF-2) is a central regulator of cellular antioxidant response. Heme oxygenase enzyme-1 (HO-1) can decompose heme to produce bilirubin, which has a high antioxidative effect. NQO1 has a protective mechanism against injury to oxidant response. Levels of NRF-2, HO-1 and NQO1 were detected using Western-blot Vertical Cells (DYCZ-24D type, Beijing Liuyi Instrument Plant, Beijing) and Gel Imaging System (BioDoc-It, American UVP Company, USA). Antibodies, including anti-NRF-2(1:1000), anti-HO-1 (1:250), and anti-NQO1 (1:1000), were purchased from Abcam (Cambridge, UK).

### Statistical analysis

Statistical analysis was performed using SPSS software version 19.0 (Chicago, IL). All the measurement data were expressed as mean and standard deviation; one-way analysis

of variance was used for measurements comparison among groups. When variance was found not homogeneous, the Dunnett T3 test was use for multiple comparisons. And *t* test was used for comparison between two groups.  $P < 0.05$  was considered statistical significance.

## RESULTS

### Pathological changes of pulmonary tissues

There was no abnormal histological change under light microscope in blank control group (Fig. 1A) and saline control group (Fig. 1B). For PM2.5 control group, alveolar septum became thickening; congestion of blood capillary and interstitial edema were found; a large number of lymphocytes infiltration and neutrophils infiltration were found surrounding bronchiole (Fig. 1C). The microscopic manifestations of pulmonary tissues in low-, middle- and high-dose Lianhua Qingwen treatment groups were shown in Fig. 1D, 1E and 1F, which relieved in a dose-dependent manner.

### LDH, MDA, GSH-PX and SOD in serum and BALF

The measurements of LDH, MDA, GSH-PX in rats BALF are shown in Table 1, where the LDH and MDA in PM2.5 group were significant higher than those in controls ( $P < 0.01$ ), but were significantly reduced in each of treatment group, with the lowest level detected in the high dose Lianhua Qingwen group ( $P < 0.05$ ). GSH-PX in BALF of PM2.5 group was significantly lower than that in the controls ( $P < 0.01$ ), but was elevated in each of the treatment group, with the highest

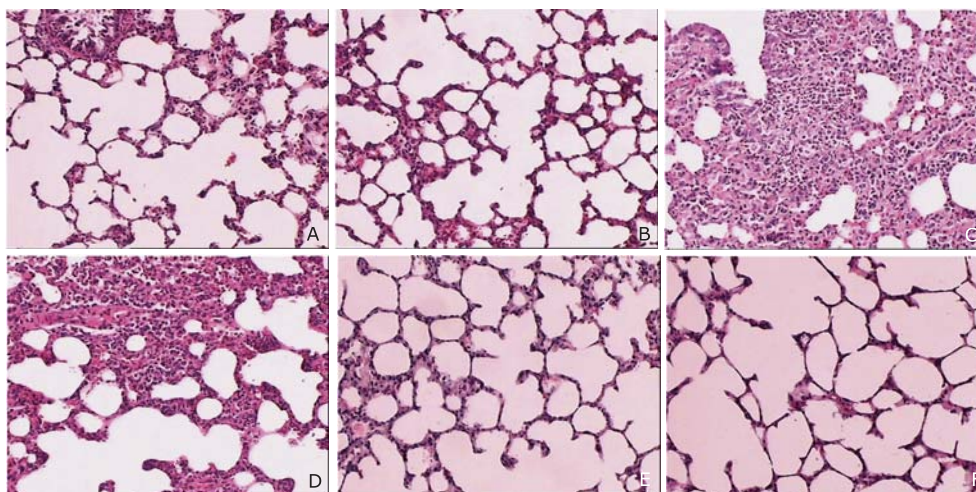


**Figure 1.** Pathological changes of rats pulmonary tissue in variable doses of Lianhua Qingwen under light microscope. HE  $\times 100$  Compared to the blank control group (A) and saline control group (B), PM2.5 control group (C) showed thickened alveolar septum, congestion of capillary, interstitial edema, lymphocytes infiltration and neutrophils infiltration surrounding bronchiole. The above changes were relieved in that of low-dose (D), middle-dose (E) and high-dose treatment group (F) compared with PM2.5 control group(C) in a dose-dependent manner.

level detected in high dose group ( $P<0.05$ ), despite no significant difference among three treatment groups ( $P>0.05$ ). The measurements of LDH, MDA, GSH-PX and SOD in rats serum were shown in Table 2, where the LDH, MDA in PM2.5 group were significant higher than that in controls ( $P<0.05$ ), but were significantly reduced in middle- and high-dose treatment group, with the lowest level detected in the high-dose group ( $P<0.05$ ). SOD and GSH-PX in serum of PM2.5 group were significantly lower than that in the controls ( $P<0.05$ ), but only GSH-PX was detected to be significantly elevated in Middle- and high-

dose group ( $P<0.05$ ).

### Protein expression in pulmonary tissues

Western blot showed that protein expressions of HO-1, NRF-2 and NQO-1 in pulmonary tissues increased remarkably in middle- and high-dose Lianhua Qingwen groups (Fig. 2, Table 3). HO-1, NRF-2 and NQO-1 protein expression increased after treatment of middle- and high-dose Lianhua Qingwen, and statistical difference was found ( $P<0.05$ ). These results suggested that Lianhuaqingwen could protect pulmonary tissues against oxidative injury induced by PM2.5.

**Table 1.** Comparisons of LDH, MDA and GSH-PX in rat bronchoalveolar lavage fluid among the study groups and the controls ( $n=8$ ,  $\bar{x} \pm s$ )

| Groups         | LDH (U/L)       | MDA (mmol/L) | GSH-PX (U/ml)  |
|----------------|-----------------|--------------|----------------|
| Blank control  | 204.34±78.15    | 0.38±0.23    | 200.73±22.58   |
| Saline control | 321.13±147.62   | 0.38±0.27    | 214.09±22.72   |
| PM2.5 control  | 643.92±127.91** | 0.98±0.25**  | 139.23±12.09** |
| Low-dose       | 421.48±205.00*  | 0.67±0.20*   | 167.73±12.09*  |
| Middle-dose    | 304.17±191.01*  | 0.53±0.22*   | 177.55±25.15*  |
| High-dose      | 367.64±181.42*  | 0.43±0.11*   | 184.91±26.61*  |

\* $P<0.05$  as comparing with PM2.5 control group; \*\* $P<0.05$  as comparing with blank control group or saline control group. LDH: lactate dehydrogenase; MDA: malonaldehyde; GSH-PX: glutathione peroxidase.

**Table 2.** Comparisons of LDH, MDA, GSH-PX and SOD in rat serum among the study groups and the controls ( $n=8$ ,  $\bar{x} \pm s$ )

| Groups         | MDA(mmol/L) | LDH (U/L)         | GSH-PX(U/ml)    | SOD (U/ml)     |
|----------------|-------------|-------------------|-----------------|----------------|
| Blank control  | 5.17±2.40   | 4062.38±1638.27   | 855.46±154.57   | 207.55±34.29   |
| Saline control | 5.71±3.42   | 4328.13±1323.75   | 698.39±208.90   | 195.38±22.35   |
| PM2.5 control  | 9.54±3.90** | 6119.75±1292.98** | 229.82±144.74** | 163.22±30.37** |
| Low-dose       | 5.36±4.16*  | 5506.13±1181.54   | 314.52±89.96    | 178.94±21.74   |
| Middle-dose    | 4.29±2.96*  | 4668.00±1478.22*  | 387.32±81.44*   | 175.34±26.20   |
| High-dose      | 4.11±1.78*  | 4067.38±1272.76*  | 373.88±88.12*   | 184.49±23.09   |

\* $P<0.05$  as comparing with PM2.5 control group. \*\* $P<0.05$  as comparing with blank control group or saline control group. SOD: superoxide dismutase.

**Table 3.** Protein expressions of HO-1, NQO1 and NRF-2 in rat pulmonary tissues of the study groups and the PM2.5 control group ( $n=8$ ,  $\bar{x} \pm s$ )

| Groups        | HO-1                     | NQO1         | NRF-2                    |
|---------------|--------------------------|--------------|--------------------------|
| PM2.5 control | 1.370±0.195              | 2.116±0.286  | 1.490±0.172              |
| Low-dose      | 1.654±0.197              | 3.271±0.404* | 1.255±0.142              |
| Middle-dose   | 2.695±0.322 <sup>Δ</sup> | 2.774±0.285* | 1.989±0.256 <sup>Δ</sup> |
| High-dose     | 4.403±0.628 <sup>Δ</sup> | 4.980±0.507* | 3.692±0.486 <sup>Δ</sup> |

\* $P<0.05$  as comparing with PM2.5 control group.  $\Delta P<0.05$  as comparing with PM2.5 control group by Dunnett T3 test.

HO-1: heme oxygenase enzyme; NQO1: quinone oxidoreductase; NRF-2: NF-E2-related factor 2.

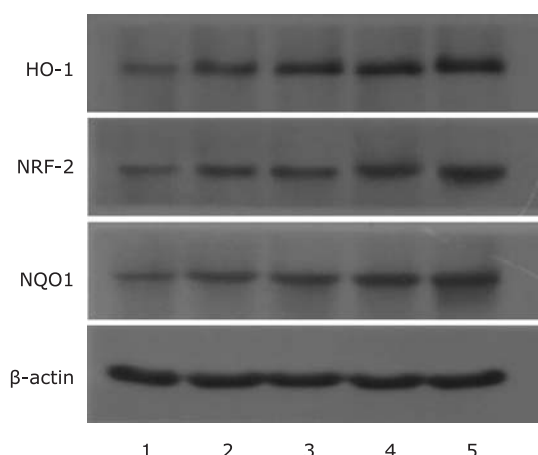


**Figure 2.** Protein expressions of HO-1, NRF-2 and NQO1 in pulmonary tissues by Western blot.

HO-1, NRF-2 and NQO1 protein expression were significantly higher in the groups of middle- and high-dose Lianhua Qingwen than controls ( $P < 0.05$ ). 1: Control group; 2: PM2.5 control group; 3: Low-dose group; 4: Middle-dose group; 5: High-dose group.

## DISCUSSION

In recent years, studies have shown that PM2.5 can induce chronic inflammation and oxidative stress injury for pulmonary tissues, reduce lung function, affect lung development at some extent, and was associated with remarkable increase of morbidities and mortalities from COPD, asthma and other respiratory diseases.<sup>1</sup> Elevated concentration of PM2.5 in environment could increase mortality of people who was suffering from heart and lung diseases, including lung cancer, and might also increase all-cause mortality.<sup>2</sup>

In this study we found that intratracheal instillation of PM2.5 suspension for 24 hours induced oxidative injury of pulmonary tissues in rats. LDH is a kind of metabolic enzyme with abundant content in cells, and can be released into extracellular space after damage of cell membrane. Changes of LDH can indirectly reflect extent of cytotoxic damage. MDA can be used to evaluate cellular oxidative injury and oxidative level.<sup>3</sup> GSH-P and SOD are important enzymes with anti-oxidation effects in human body. We found that exposure to PM2.5 suspension for 24 hours significantly increased metabolic enzyme LDH in BALF, indicating PM2.5 induced oxidative injuries of pulmonary parenchyma and biological membrane. This results was consistent with studies by Xiao *et al*<sup>4</sup> and Cao *et al*<sup>5</sup> Besides, we found that, in PM2.5 control group, oxidative stress product MDA increased in serum and BALF, and GSH-P in BALF and serum and SOD in serum decreased, which

indicated the elevated peroxidation level and that GSH-P and SOD were consumed as *in vivo* antioxidant system to protect against oxidative injury induced by PM2.5. These results were also consistent with the previous studies.<sup>6-8</sup>

With consideration of components and formula of Lianhua Qingwen Capsule from the point of view of Traditional Chinese Medicine (TCM), it has been used in the treatment of inflammation diseases such as acute pulmonary infection, for its antiviral activity and regulating pulmonary immunity. In this study, different doses of Lianhua Qingwen were used to treat PM2.5 exposed rats. Compared to the PM2.5 control group, oxidative stress marker, LDH and MDA in both BALF and serum were significantly reduced in middle- and high-dose pre-treated groups, GSH-P activity was significantly increased in middle- and high-dose pre-treated groups as well, which indicated that middle- and high-dose Lianhua Qingwen could protect against or treat pulmonary oxidative injury. No statistical difference of GSH-P in rat serum was found between low-dose Lianhua Qingwen pre-treated group and the control groups ( $P > 0.05$ ). These results suggested that middle- or high-dose Lianhua Qingwen could increase GSH-P activity in BALF, and it might help to treat pulmonary oxidative injury induced by PM2.5 in a dose-dependent manner.

NRF-2 is a central regulator of cellular antioxidant response, and regulates expression of antioxidant protein and phase II detoxification enzyme with interaction of antioxidant responsive element (ARE). After NRF-2 binding with ARE promoter site, downstream target genes were activated that there were expression of catalase (CAT), SOD, glutathione S-transferase (GST), quinone oxidoreductase (NQ1), heavy chain  $\gamma$ -glutamate synthetase ( $\gamma$ -GCSH), light chain  $\gamma$ -glutamate synthetase ( $\gamma$ -GCSH) and HO-1.<sup>9-10</sup> HO-1 can decompose heme to produce bilirubin, which has a high antioxidative effect.<sup>11</sup> NQO1 can catalyze quinones, an important part of PM2.5, to directly revert to hydroquinone, most of which can be stably combined and excreted in cells. Consequently, production of unstable semiquinone materials was avoided.<sup>12</sup> In this study we found that the NQO1 protein expression increased in all three Lianhua Qingwen pre-treated groups, and HO-1, NRF-2 protein expression significantly increased in the middle- and high-dose treated group. These results suggested that Lianhua qingwen could protect pulmonary tissues against oxidative injury induced by PM2.5.

Qu *et al*<sup>13</sup> found that PM2.5 had acute toxic effects on rat pulmonary tissues. On pulmonary pathological slide, inflammatory changes including hyperplasia of neutrophil, monocyte and lymphocyte were observed. In our study, no

abnormal histological change of rat lungs was observed in blank control group and saline control group. For PM<sub>2.5</sub> control group, histological changes such as thickened alveolar septum, congestion of blood capillary and interstitial edema, distinctive lymphocytes infiltration and neutrophils infiltration were found surrounding bronchiole. This suggested that fine particulates had a toxic damage to lung tissues. These pathological changes were relieved in the Lianhua Qingwen pretreated groups. This demonstrated the protective effect of Lianhua Qingwen on pulmonary injury induced by PM<sub>2.5</sub>.

In conclusion, our study demonstrated the stress injury of pulmonary tissues induced by PM<sub>2.5</sub> in rats, and provided evidence that Lianhua Qingwen in middle- and high-dose could alleviate this injury. Given the damage of PM<sub>2.5</sub>, our observations suggested it would be necessary to perform clinical trial in the future to study the effects of Lianhua Qingwen on patients with respiratory diseases that are related to PM<sub>2.5</sub>, as well as the underlying mechanism.

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