Protection against Acute Pingyangmycin-induced Lung Injury: A novel Role for Low-dose Radiation

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Abstract: Endotracheal challenge in mice with pingyangmycin (bleomycin A5, PYM) is a well-established model for acute lung injury resulting in pulmonary fibrosis. Immune hormesis induced by low-dose radiation has been proven effective in lymphocyte, macrophage, and natural killer cells. This study examines the effects of low-dose radiation on pingyangmycin-induced pulmonary fibrosis and the relationship between cytokine levels and pulmonary fibrosis induced by pingyangmycin. Kunming strain male mice were exposed to whole-body low-dose radiation (total dosage: 75 mGy). After 6 h, the mice were subjected to inhalation of PYM atomization at a concentration of 2 mg/mL. The effect of low-dose radiation on pulmonary damage was analyzed by observing HE slices under a light microscope and analyzing the cytokine levels (IL-6) in the bronchoalveolar lavage fluid using ELISA. TNF-α and TGF-β levels were detected by immunohistochemistry. During the early stage of pingyangmycin-induced lung injury, the experimental group had lower grade of alveolitis compared with the control group ($P < 0.05$). In addition, the IL-6 level in the experimental group was lower than that of the control group and was close to that of the blank group. The experimental group also had lower TGF-β and TNF-α expression compared with the control group ($P < 0.05$). Low-dose radiation (75 mGy) can reduce alveolitis grade, IL-6 secretion, and TGF-β and TNF-α expression.

[Hongsheng Yu, Zimin Liu, Xiaoyun Yu, Aiqin Song, Ning Liu, Hao Wang. **Protection against Acute Pingyangmycin-induced Lung Injury: A novel Role for Low-dose Radiation.** *Life Sci J* 2013; 10(3): 1204-1209]. (ISSN: 1097-8135). http://www.lifesciencesite.com 178

Keywords: Pingyang mycin-induced; low dose radiation; lung injury; cytokine

1. Introduction

The available information on hormesis has been significantly improved since it was first reported by Luckey in 1982 (Day et al., 2007; Ramola et al., 2010, 2012; Rautela et al., 2012; Walsh and Kaiser, 2011). Epidemiology shows a decrease, rather than an increase, in the incidence of tumor in people working in high natural background radiation areas, victims receiving low-dose radiation from atomic bomb explosions and atomic accidents, medical staff in radiology departments, and patients receiving radiation treatment (Ramola et al., 2010, 2012; Rautela et al., 2012). A number of studies showed that the immune function in such people was upregulated, consequently inhibiting tumor growth (Walsh and Kaiser, 2011; Little, 2009, 2010, 2012). Immune hormesis induced by low-dose radiation has been proven effective in lymphocyte, macrophage, and natural killer cells (Day et al., 2007; Day et al., 2006; Meng et al., 2012; Halliday and Rana, 2008). Endotracheal challenge in mice with bleomycin (BLM) is a well-established model of acute lung injury (ALI) resulting in pulmonary fibrosis, which resembles idiopathic pulmonary fibrosis (Cucoranu et al., 2005; Hagimoto et al., 2002). Pulmonary fibrosis occurs in three stages: alveolar epithelial cell death, inflammation, and enhanced collagen deposition with fibroblast and smooth muscle cell proliferation (Crystal et al., 2002; Leslie, 2005; Raghu et al., 2011). However, the effect of low-dose radiation on lung injury and the relationship between cytokine level and lung injury induced by bleomycin A5 (BLM-A5) has not been reported. This study aims to find theoretical evidence for the clinical use of low-dose radiation by exploring its effect on lung injury, IL-6 levels, and TNF-α and TGF-β expression.

2. Materials and Methods

2.1. Objects

Kunming strain male mice were randomly divided into three groups: normal control group (20 mice), low-dose radiation with BLM-A5 group (P + L) (20 mice), and BLM-A5 group (P) (20 mice). All mice were raised routinely and provided with unlimited water and food. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Qingdao University.

2.2. Radiation conditions

The mice were placed in a cardboard box. A cobalt-60 radiation machine was used to expose the

mice to 75 mGy of whole-body radiation at a source-to-surface distance of 212 cm and a dose ratio of 12.5 mGy/min. A water phantom of 30 cm was placed intermedially to filter the ray.

2.3. Atomization inhalation conditions

The mice in the P + L group were placed in a glass trough with a length of 40 cm and a width of 30 cm. The contact nebulizer was inserted through a small blowhole on the plastic plate placed on the glass trough. BLM-A5 was mixed with physiological saline to form a solution with a concentration of 2 mg/mL. The duration of the atomization inhalation was 3 min. Similarly, the mice in the P group were subjected to atomization inhalation of BLM-A5, but they were not exposed to low-dose radiation.

2.4. Specimen collection

The operation was performed on the following days (d): d1, d7, d14, d21, and d28.

After being injected with anesthesia, the mice were fixed on a cystosepiment using pins. The skin tissue was cut to expose the abdominal wall. Then, the sternum was cut to expose the double lungs. The left lung was ligated, and a needle of 17 order was ligated to the trachea. About 0.3 mL of physiological saline was injected slowly, and needle aspiration was performed three times to obtain 0.8 mL of alveolar lavage fluid. The fluid was then placed in a test tube and stored in a refrigerator at –20 ℃.

Right lungs were harvested and placed in 10% formalin solution to obtain the lung tissue specimens.

2.5. ELISA

Mice IL-6 kits were obtained from Beijing Jingmei Biological Engineering Co., Ltd. (China). Each kit contained standard substance, dilute specimens, concentrated biotinylated antibody, diluted biotinylated antibody, concentrated enzyme combination, diluted enzyme combination, concentrated cleaning solution, color development reagent, stop buffer, antibody-coated batten, and bakelized paper.

The necessary battens were removed from the sealed bag and equilibrated to room temperature. About 100 μL of standard substance was added to each hole except for the blank group. Then, the reaction holes were sealed using bakelized paper, and the battens were incubated at 37 ℃ for 90 min. The battens were washed four times. Then, 100 μL of biotinylated antibody operating fluid was added to each hole except for the blank group. The reaction holes were sealed using gummed paper, and the battens were incubated at 37 ℃ for 60 min. The battens were washed four times before 100 μL of enzyme combination operating fluid was added to each hole except for the blank group. The reaction holes were again sealed using gummed paper, and the battens were incubated at 37 ℃ for 30 min. The battens were washed four times before 100 μL of color development reagent was added to each hole. The battens were kept in a dark place and were incubated at 37 ℃ for 10 min to 20 min. Finally, 100 μL of stop solution was added to each hole), and OD450 was measured immediately after mixing.

2.6. Detection of TGF-β and TNF-α

The two-step patterning experimental process was performed as follows:

(a) The specimens fixed by formalin were douched with lotic water. After 4 h, the specimens were dehydrated in an automatic machine, imbedded in paraffin, and serially cut to obtain slices with thickness of 1 μm to 2 μm. The slices were placed in a 60° temperature box overnight.

(b) The slices were deparaffinized and hydrated by flushing with xylene every 5 min; with ethanol once, 95% ethanol twice, and 85% ethanol once for 2 min; and with water for 5 min. The slices were flushed with distilled water three times.

(c) The antigen was repaired by submerging the slices into 0.01 M of folic acid salt buffer and placing them inside the microwave at 100 firepower for 2.5 min and at 30 firepower for 7 min. This process was repeated three times, and then the slices were cooled naturally. Then, the slices were washed with phosphate buffered saline (PBS) four times for 1 min to 2 min each time. The first set of antibodies (Beijing Zhongshan Jinqiao Biological Technology Co., Ltd., China) was added, and the slices were incubated in a bath box at 37 ℃ for 1 h. The slices were again washed with PBS four times for 1 min to 2 min each time.

(d) The second set of antibodies (rabbit antibody, rat IgG produced by Beijing Zhongshan Jinqiao Biological Technology Co., Ltd., China) was added. Each slice was incubated in a bath box at 37 ℃ for 20 min and then washed with PBS four times for 1 min to 2 min each time. Color modification was then performed using diaminobenzidine. Each slice was washed in distilled water, dyed with hematoxylin-eosin (HE), rendered transparent with xylene, and closed with neuter gum.

2.7. Determination of results

The slices were classified according to cytoplasm and cell membrane color as follows: (–) without color, (±) light-yellow, (+) buffy, (++) deep buffy, (+++) brown, and (+ – +++) positive expression. The slices were observed using 20× or 40× microscopes (Japan OLYMPUS BH-2). After the positive regional location was measured, the extracted image by color kinescope was inputted into the Video Pro 32 color image analysis system. Then, the positive regions were subdivided accurately, and the gray values (Grey) were measured. The data were analyzed using statistical methods. The HE slices were observed under a light microscope. The classification and scoring standards of the HE slices based on alveolitis grade according to the Szapriel method are as follows: (–): no

alveolitis, grade 0; (+): mild alveolitis–monocytes infiltrating the alveolar septum 450 (www.springerlink.com/content/1613-9089), which becomes wider, localizing and approaching the pleura, and less than 20% of the whole lung and the structure of pulmonary alveoli are normal, grade 1; (++): moderate alveolitis–the proportion of the lung close to the pleura ranges from 20% to 50%, grade 2; (+++): severe alveolitis, the affected proportion of the lung exceeds 50%, grade 3.

2.8. Statistical analysis

SPSS 11.5 was used for statistical analysis. Quantitative data were expressed by mean ± standard deviation. Comparison between means was achieved by a q-test. Semi-quantitative data were analyzed by ridit scoring.

3. Results

3.1. IL-6

Results revealed that the IL-6 level in each group was statistically significant. The maximum IL-6 level was found in the P group, followed by the P + L group and the blank group, in which IL-6 level was the lowest. IL-6 levels on d1 and d7 in the P+L and the P groups were statistically significant. By contrast, IL-6 levels on d14, d21, and d28 in the P+L and the P groups were not statistically significant, indicating that IL-6 secretion can be decreased by low-dose radiation. However, IL-6 levels during the late stage of chemical lung injury in the two groups were not statistically significant (Figure 1A).

3.2. TGF-β and TNF-α

Semi-quantitative analysis revealed that TGF-β and TNF-α expression on d1, d14, and d28 were not statistically significant ($P > 0.05$). However, compared with the blank group, TGF-β and TNF-α expression in the P + L and the P groups were statistically significant ($P < 0.05$). Analysis of the detection results of TGF-β and TNF-α gray value indicate that the quantitative analysis results of TGF-β and TNF-α on d1, d14, and d28 were statistically significant ($P < 0.05$, Figures 1B and 1C).

3.3. Alveolitis grade

The P and the P + L groups had a higher alveolitis grade than the control group ($P < 0.01$). The P + L group had a lower alveolitis grade than the P group ($P < 0.05$ on d1 and d7; $P > 0.05$ on d14, d21, and d28; Figure 1D).

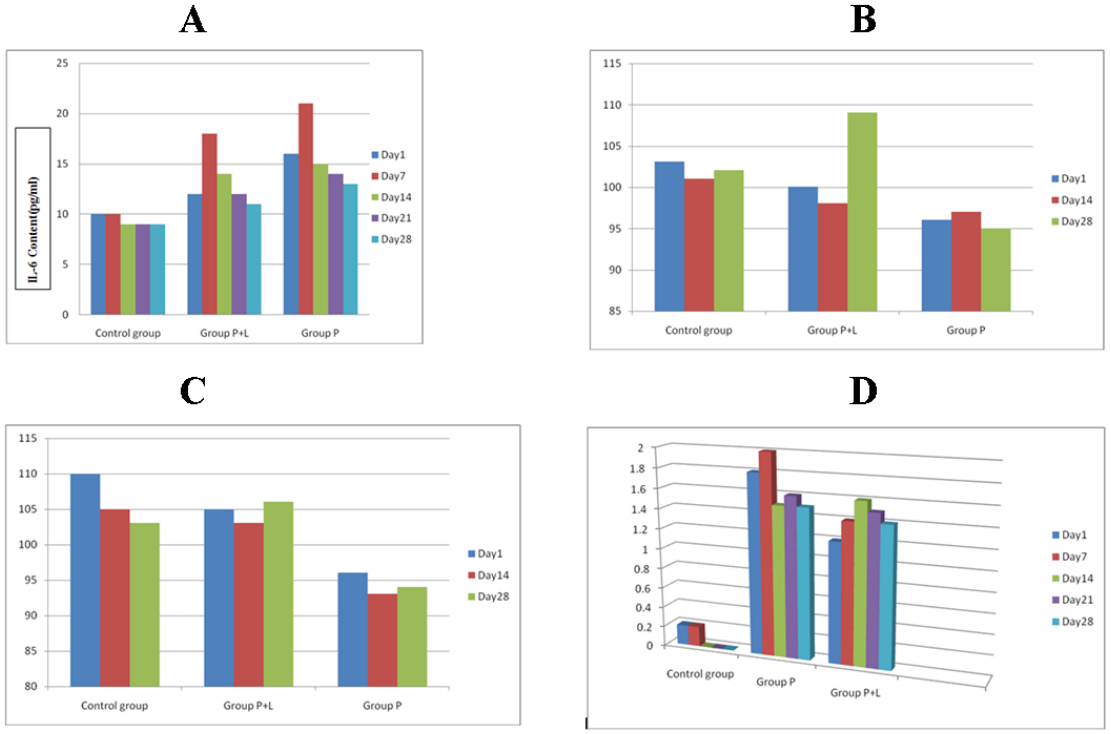

Figure 1 A: Testing results of IL-6: The IL-6 level was the highest in Group P, took the second place in Group P + L, the lowest was in the blank group. IL-6 levels on the first day and the seventh day between Group P+L and Group P were of statistical significance, meanwhile, IL-6 levels on the d14, the d21 and the d28 between Group P+L and Group P were of none statistical significance. **B:** Chart testing results of TGF-β Value: The semiquantitative analysis results of TGF-β on the first day, the fourteenth day and the twenty-eighth day were of none statistical significance ($P > 0.05$). But Group P + L and Group P compare with the blank group respectively were of statistical significance ($P < 0.05$). The analysis on detection results of TGF-β gray value is that the quantitative analysis results of TGF-β on the first day, the fourteenth day and the twenty-eighth day were of statistical significance ($P < 0.05$). **C:** Chart

testing results of TNF-α gray value: The semiquantitative analysis results of TNF-α on the first day, the fourteenth day and the twenty-eighth day were of none statistical significance ($P > 0.05$). But Group P + L and Group P compare with the blank group respectively were of statistical significance ($P < 0.05$). The analysis on detection results of TNF-α gray value is that the quantitative analysis results of TNF-α on the first day, the fourteenth day and the twenty-eighth day were of statistical significance ($P < 0.05$). **D:** The alveolitis grades score: The alveolitis grades score in group P was higher than that in control group ($P < 0.01$), the alveolitis grades score in group P+L was higher than that in control group ($P < 0.01$) too. The alveolitis grades score in group P+L was lower than that in group P ($P < 0.05$ at Day1 and Day 7, $P > 0.05$ at Day14, Day21 and Day 28).

4. Discussion

The disease course of ALI is characterized by three phases: exudative, proliferative, and fibrotic. However, the inflammatory and repair mechanisms occur in parallel, rather than in series (Frutos-Viva et al., 2004; MacCallum and Evans, 2005). The exudative phase encompasses the first seven days after injury, whereas the proliferative phase spans days 7 to 21. The fibrotic phase occurs two to four weeks after the initial pulmonary injury (Frutos-Viva et al., 2004; MacCallum and Evans, 2005).

Elevated TNF-α and IL-6 levels were found in the BLM-treated rats. TNF-α and IL-6 have multiple effects on acute inflammation and infiltration by neutrophils and lymphocytes (Frutos-Vivar et al., 2004; MacCallum and Evans, 2005). TNF-α also contributes to the pathophysiology of interstitial lung disease by inducing the apoptosis of epithelial cells and the sequential release of TGF-β, IL-1β, and IL-1 receptor antagonist (Janes et al., 2006). In addition, the production of reactive oxygen and nitrogen species is related to apoptosis in alveolar epithelial cells (Kuwano et al., 2003), TGF-β release from pulmonary epithelial cells (Gharaee-Kermani et al., 2009), and TGF-β1 activation through the disruption of its interaction with latency-associated peptide (Hagimoto et al., 2002).

TNF-α is an important factor that causes fibrosis and promotes inflammatory reaction. TNF can initiate the synthesis and release of cytokines such as IL-1, IL-6, and MCP creating a "water fall effect" of the cytokines. Thus, proinflammatory cytokines such as TNF and IL-1 play a key role in the pathogenesis of radiation pneumonitis. Atamas and other experts (Atamas et al., 2002; Atamas and White, 2003) observed that IL-6 and TNF-α are released by alveolar macrophages during pulmonary fibrosis in rats, and that the IL-6 level was significantly higher in the groups treated with BLM than in the control group. The IL-6 level peaked on d7 and decreased afterward. However, it still remained on a high level, indicating that IL-6 participates in the early stage of alveolitis and the later stage of pulmonary fibrosis. Homer and other experts (Homer et al., 2011; Pechkovsky et al., 2010) found that mine dust, quartz dust, and asbestos dust induce macrophages to secrete TNF-α and IL-6. Studies (Wynn, 2011; Todd et al., 2012) showed that the TNF-α acceptor and IL-6 levels in the blood plasma of macrophages are much higher than that of the control group. Results indicate that the TNF-α acceptor and IL-6 levels in blood plasma are relevant to the fibrosis of pneumonoconiosis. TGF-β promotes the division, growth, maturation, and differentiation of fibroblasts. Fibroblasts synthesize a great quantity of type I, III, and IV collagen proteins, particularly type IV, to increase the collagen of lung mesenchymal cells. Meanwhile, TGF-β can inhibit the synthesis of collagenase and plasminogen activator. Moreover, TGF- β can increase the formation of protease inhibitors to decrease mesochymal EMC degradation, resulting in regulation imbalance. TGF-β also plays an important role in pulmonary fibrosis by promoting the synthesis and release of PDGF, IGFs, TNF, IL-1, and IL-6 by phagocytes and mononuclear macrophages, thereby increasing their bioactivity. TGF-β1 plays an important role in lung radiation damage (Bonner, 2004). Research results indicate that the cell can secrete numerous kinds of cytokines. The biological effects of different cytokines on reciprocal chiasmata overlap and affect one another, constructing a complex lung cytokine network (Ihn, 2002; Biernacka et al., 2011).

The experiment results show that the IL-6 level was highest in the P group, followed by the P + L group and the blank group, in which IL-6 was the lowest. IL-6 levels in the P+ L and the P groups on d1 and d7 were statistically significant. By contrast, IL-6 levels in the P+ L and the P groups on d14, d21, and d28 were not statistically significant. This result indicates that the IL-6 levels in Group P + L and Group P in the early stage of chemical pulmonary injury are statistically significant. Low-dose radiation can decrease IL-6 secretion. However, IL-6 levels in both groups during the later stage of chemical pulmonary injury were not statistically significant. Immunohistochemistry results indicate that the semi-quantitative analysis results of TGF-β and TNF-α on d1, d14, and d28 were not statistically significant ($P > 0.05$). However, compared with the blank group, TGF-β and TNF-α expression in the P + L and the P groups were statistically significant ($P < 0.05$). Analysis of the detection results of TGF-β and TNF-α gray value indicate that the quantitative analysis results of TGF-β and TNF-α on d1, d14, and d28 were statistically significant ($P < 0.05$).

The experiment indicates that BLM-A5 can

cause pulmonary injury in mice. During the early stage, low-dose radiation (75 mGy) can decrease the secretion of IL-6 and the generation of TGF-β and TNF-α, indicating that low-dose radiation can mitigate pulmonary injury induced by BLM-A5 in mice.

Acknowledgements
This work was supported by National National Scentific Foundation of China (No: 30030781).

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8/5/2013