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Ultrastructural observation on pulmonary fibrosis in E9 rats treated with compound *Carapax trionycis* formula

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ABSTRACT

Objective: To investigate ultrastructural changes in pulmonary tissue of a rat model of pulmonary fibrosis following treatment with compound *Carapax trionycis* (*C. trionycis*; Biejia in Chinese) formula. **Methods:** Sixty male Sprague-Dawley rats were randomly divided into four groups (n=15): compound *C. trionycis* formula high-, middle-, and low-dose groups as well as model group. Pulmonary fibrosis was induced by bleomycin. Five rats from each group were sacrificed on day 7, 14 and 28 of the drug treatment, respectively. The pulmonary tissue was harvested followed by hematoxylin-eosin staining and subsequent transmission electron microscopy. The Szapiel's method was used to assess the degree of alveolitis and pulmonary fibrosis. **Results:** Compared with the model group, the compound *C. trionycis* formula groups had slighter pulmonary alveolitis after the 7-day treatment and also had alleviated alveolar inflammation and pulmonary fibrosis after the 14-day treatment. After the 28-day treatment, the compound *C. trionycis* formula groups showed deposition of a small amount of fibrous tissue and lesions occupying less than 21% of the whole lung area, while the model group showed focal or diffuse fibrous deposition, narrow alveolar cavity, disordered lung structure, and lesions in larger than 51% of the whole lung area. **Conclusions:** The compound *C. trionycis* formula can inhibit the proliferation of collagen fibers and resist pulmonary fibrosis.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is one of chronic, progressive interstitial lung diseases. The 5-year mortality rate for IPF patients is 50%-70%. IPF has a high mortality rate and lacks effective treatment methods in clinic[1]. It has been reported that compound *Carapax trionycis* (*C. trionycis*; Biejia in Chinese) formula can lower free radical-induced oxidative damage to pulmonary tissue structure and decrease the activity of connective tissue growth factor within the pulmonary tissue by modulating free radical levels *in vivo*, thereby blocking the progression of pulmonary fibrosis[2]. In this study, we prepared a rat model of bleomycin-induced pulmonary fibrosis to investigate the effects of the compound *C. trionycis* formula against pulmonary fibrosis and the ultrastructural changes in pulmonary tissue after drug treatment.

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2. Materials and methods

2.1. Rats

Sixty male Sprague-Dawley rats of SPF/VAF grade, weighing 200-220 g, were raised in a laboratory of biosafety level. The rats were on embryonic day 9 (E9 rats) and allowed free access to water.

2.2. Drug

Bleomycin (specification 15 mg/ampoule, lot No.: X22270), used to induce pulmonary fibrosis, was purchased from BLM Group (Osaka, Japan). Compound *C. trionycis* formula (lot No.: 2002050), used to treat pulmonary fibrosis, was produced by Inner Mongolia Furui Medical Science Co., Ltd (Huhhot, China).

2.3. Experimental design

The rats were randomly divided into four groups, with 15 rats in each group. Following anesthesia by intraperitoneal injection of 1.5% (v/v) pentobarbital, the rats from each group were

administered bleomycin at a dose of 5 mg/kg body weight via the tracheal cannula. Massage was necessary to ensure the uniform distribution of bleomycin in the pulmonary tissue. The rats developed the signs of pulmonary fibrosis after 4 weeks post the administration. In the model group, the rats were intragastrically administered 3 mL physiological saline once daily. In the compound *C. trionycis* formula high-, middle- and low-dose groups, the rats were intragastrically administered the compound *C. trionycis* formula respectively at a dose of 1.40, 0.70 and 0.35 g/kg body weight once daily. Five rats from each group were sacrificed on day 7, 14 and 28 of the drug treatment, respectively. The pulmonary tissue was harvested for hematoxylin-eosin staining and transmission electron microscopy. Alveolitis and pulmonary fibrosis degree were assessed using the Szapiel's method.

3. Results

3.1. Alleviating action of the compound C. trionycis formula against pulmonary fibrosis

Pulmonary fibrosis degree was relieved by the compound *C. trionycis* formula as displayed by hematoxylin-eosin staining. The details are shown in Table 1. On day 7 of the drug treatment, obvious acute pulmonary inflammation was observed in the rats of the model group, as manifested by swollen and widened alveolar septum with infiltration of a large number of mononuclear cells and a small number of neutrophil granulocytes, and by appearance of a few pulmonary interstitial fibroblasts. At this time, the degree of alveolar inflammation was milder in the compound *C. trionycis* formula middle- and low-dose groups than that in the model group and compound *C. trionycis* formula high-dose group.

On day 14 of the drug treatment, the alveolar inflammation was alleviated in the model group compared with that on day 7, and fibroblasts appeared in alveolar septum. However, the degree of both alveolar inflammation and pulmonary fibrosis was alleviated in the compound *C. trionycis* formula groups compared with that in the model group.

On day 28 of the drug treatment, fibrous tissue deposited in a large focus-like or diffuse manner in the model group, leading to alveolar fusion, narrow alveolar cavity, disordered lung structure, and lesions in larger than 51% of the whole lung area. In comparison, only a small amount of fibrous tissue deposited and the area of lesion accounted for less than 21% of the whole lung.

3.2. Ultrastructural changes in pulmonary tissue after the 7-day treatment with the compound C. trionycis formula

On day 7 of the drug treatment, swollen type Ⅰ alveolar epithelial cells, slightly aggregated type Ⅱ alveolar epithelial cells, mitochondrial swelling, crista fragmentation, and an increased number of enlarged lamellar bodies were observed in the rats of the model group. Under a magnification of 20 000-30 000, uniform point-like particles in bundles were observed on the cross-section of pulmonary septum, and a lot of lysosomes, phagocytosed particles, lymphocytes and red blood cells were found in the macrophages. At this time, in the lungs of the treatment groups, type Ⅰ alveolar epithelial cells were slightly swollen, the aggregation of type Ⅱ alveolar epithelial cells was not obvious, and intracellular lamellar bodies had a regular morphology and decreased quantity and volume (Figure 1a).

3.3. Ultrastructural changes in pulmonary tissue after the 14-day treatment of the compound C. trionycis formula

Table 1

Pulmonary fibrosis degree after different times of treatment with *C. trionycis* as displayed by hematoxylin-eosin staining.

Group	Day 7		Day 14		Day 28	
	Alveolar inflammation	Pulmonary fibrosis	Alveolar inflammation	Pulmonary fibrosis	Alveolar inflammation	Pulmonary fibrosis
Model group	+ +	−	±	+	−	+ + +
High-dose group	+	−	±	±	−	+ +
Middle-dose group	±	−	−	±	−	+
Low-dose group	±	−/±	±	±	−	+

The symbols −, ±, +, + +, and + + + represent a decreasing degree of pathological changes in order.

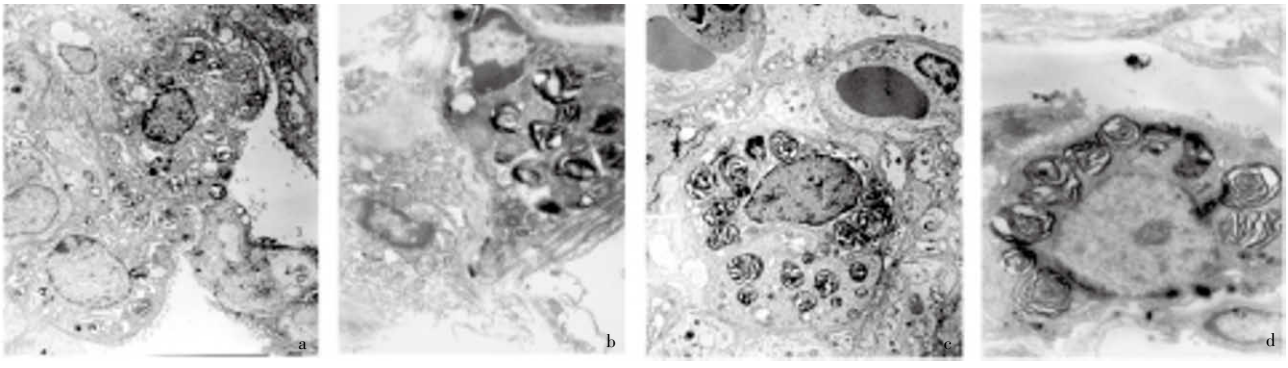

Figure 1. Ultrastructural changes in pulmonary tissue after different times of treatment.
On day 7 of the drug treatment, an increased number of lamellar bodies in type Ⅱ alveolar epithelial cells as well as intact capillary and alveolar basement membrane are observed in the compound *C. trionycis* formula group (×4 000) (Figure 1a). On day 14, alveolar cavities with little exudate and type Ⅱ alveolar epithelial cells which secrete well are observed in the compound *C. trionycis* formula group (×3 000) (Figure 1b). On day 28, collagen fibers are present around the pyknotic nuclei within the type Ⅱ alveolar epithelial cells in the model group (×4 000) (Figure 1c), while lamellar bodies in type Ⅱ alveolar epithelial cells are slightly larger, and no collagen fibers are present around the cells (×4 000) (Figure 1d).

On day 14 of the drug treatment, the lungs of the rats in the model group showed exfoliation of some type Ⅰ alveolar epithelial cells, alleviation of cell swelling and loose intercellular connections. Additionally, the aggregation of type Ⅱ alveolar epithelial cells became obvious and cellular nuclei exhibited an irregular morphology. Under a magnification of 20 000, collagen fibers in bundles appeared around the type Ⅱ alveolar epithelial cells and in the pulmonary interstitium. Comparatively, in the treatment groups, the number of pinocytotic vesicles in the type Ⅰ alveolar epithelial cells increased, and no changes were found in the nuclei and nucleoli of type Ⅱ alveolar epithelial cells. Collagen fibers were not observed in the basement membrane and interstitium (Figure 1b).

3.4. Ultrastructural changes in pulmonary tissue after the 28-day treatment of the compound C. trionycis formula

On day 28 of the drug treatment, degenerative type Ⅰ alveolar epithelial cells, proliferative type Ⅱ alveolar epithelial cells, swelling, disintegration and exfoliation of endothelial cells, and incomplete basement membrane were observed in the lungs of the rats in the model group (Figure 1c). However, in the treatment groups, the increase of type Ⅱ alveolar epithelial cells were not obvious, a few tenuous collagen fibers were found in the interstitium, and a small number of lamellar bodies were tightly arranged in the cells (Figure 1d).

4. Discussion

IPF is a progressive disease that finally leads to pulmonary dysfunction and is pathologically characterized by collagen deposition and destructed pulmonary tissue structure[3]. It has been reported that IPF patients should undergo lung transplantation as soon as possible after diagnosis because of a fact that medication therapy cannot prolong the life span of such patients at present[4-7]. However, the treatment is restricted by deficient donors and lifelong use of immunosuppressant after lung transplantation. Compound *C. trionycis* formula can strength body resistance to eliminate pathogenic factors and activate blood circulation to dissipate blood stasis; therefore, it is mainly used to treat pulmonary fibrosis. Recently, it has been reported that compound *C. trionycis* formula can effectively relieve pulmonary fibrosis[8].

Compound *C. trionycis* formula, composed of *C. trionycis* (Turtle shell), *Cordyceps* (Chinese caterpillar fungus), *Radix paeoniae* Rubra (Red peony root), *Rhizoma curcumae* (Zedoray rhizome), *Radix notoginseng* (Sanchi), *Placenta hominis* (Human placenta), *Radix codonopsis* (Tangshen), *Fructus forsythiae* (Weeping forsythia capsule), *Radix angelicae* Sinensis (Chinese angelica), *Radix isatidis* (Isatis root) and *Radix astragali* (Milkvetch root), can enhance immunity, alleviate swelling, act against inflammation, inhibit fibroblast proliferation, and prevent and treat pulmonary fibrosis[9]. In the formula, *C. trionycis* can soften hard lumps and dispel nodes. According to the modern pharmacology, *C. trionycis* can depress secretion of Kupffer's cells, activate hepatic stellate cells (HSC) by HSC autocrine pathway, and thereby inhibit activation of Kupffer's cells and HSC vascular endothelial growth factors to resist pulmonary fibrosis[10]. *Cordyceps* is capable of eliminating phlegm, regulate immunity, promoting collagen degradation, expanding bronchi and preventing emphysema. *Radix paeoniae* Rubra and *Radix notoginseng* can activate blood and remove blood stasis, enhance immunity and resist inflammation. *Placenta hominis* can invigorate vital energy and benefit blood. *Radix codonopsis* is capable of nourishing qi to invigorate the spleen. *Radix isatidis* has antiviral, antibacterial, antipyretic and detoxicant activities. A combined use of these components has an effect against pulmonary fibrosis[11].

In this study, the compound *C. trionycis* formula was used to treat IPF in rats. The observation on the ultrastructural changes showed that pulmonary fibrosis degree and collagen deposition were remarkably reduced. The results suggested that the formula can attenuate fiber accumulation and protect against pulmonary fibrosis, thereby playing an important role in prevention and treatment of IPF.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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