Monitoring Paraquat-induced Pulmonary Fibrosis in Rats with Micro-CT Finding and Histological Examination

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Received 13 May 2009; Accepted 31 Aug 2009

Abstract

The aim of this study is to monitor paraquat-induced pulmonary fibrosis in rats with micro-computed tomography (micro-CT) findings. Adult rats were divided into four groups and given a single oral dosage of aqueous solution of paraquat at 0 mg/kg, 40 mg/kg, 60 mg/kg, and 80 mg/kg, respectively. In the control group, the color and luster of the lung samples appeared normal and no petechia was observed. In the experimental groups, some petechia was observed in lung samples, and fibroblast and fibrinogen were found in histological sections, and the ground-glass predominance and reticulation in micro-CT findings increased during the period from the 1st week to the 3rd week, and decreased in the 4th week after the administration. In the experimental groups, the lung weight of adult rats increased when the dosage of paraquat was increased, but the body weight increments of adult rats decreased, therefore the ratios of lung weight over body weight increased with increased dosage. From the analysis of 3D micro-CT images, the results show that the effective capacity volume of the lungs gradually decreased with increased dosage, therefore micro-CT is seen as a powerful tool for fast diagnosis and longitudinal monitoring of pulmonary fibrosis.

Keywords: Micro-CT, Paraquat, Pulmonary fibrosis, Histological sections, 3D reconstruction

1. Introduction

Micro-CT is a powerful instrument used in animal molecular image systems for construction of animal models for human disease and drug research. It uses a non-invasive method and provides 3D structure and in vivo information, including perspectives on minute structures inside the object, and also facilitates comprehension of the structures of organs and tissue in experimental animals [1-4].

A normal lung is divided into the respiratory passage for air transportation, alveoli for gas-exchange, and pulmonary interstitial tissue composed of elastin, glycoprotein, and collagen for distribution. Collagen is excreted by fibroblasts, which are located in the interstitial tissue. When fibroblast cells are injured by various chemical and physical stimuli, they will be activated to excrete collagen to repair the pulmonary interstitial tissue, resulting in pulmonary fibrosis. Briefly, pulmonary fibrosis, manifesting as scars, is the result of tissue repair after the lung has been injured [5]. The lung is the organ most sensitive to paraquat in terms of reflecting symptoms and syndromes. Human and mouse histological sections both show the same type of pulmonary injury [6]. Because previous animal experiments all used invasive methods, animals needed to be sacrificed to obtain experimental data, and individual differences could not be determined. In addition, the interpretation of results of diagnosis on humans is very subjective and lacks evaluation references, so the outcomes are easily affected for the different readers [7-12].

There are many causes of pulmonary fibrosis, including asbestos, silicon, coal ash, harmful chemical gases, SARS, careless ingestion of some pesticides such as paraquat, administration of certain drugs, radiotherapy, immunotherapy, hyperbaric oxygen-therapy, sarcoidosis, autoimmune disease, many pulmonary infections (especially pulmonary tuberculosis), and other unclear reasons. Most reasons have been unknown until recently [13]. Progressive interstitial fibrosis disease, which widely infringes on the pulmonary alveoli wall, septa, blood vessels, lymph, and connective tissue around the trachea, leads to pulmonary fibrosis, which is a chronic inflammation; gases can’t exchange, and this leads to respiratory failure and functional disability. The symptoms include dry cough, heavy breathing and body weight loss. Prognosis is poor, patients may lose their ability to work, and they usually die in five to six years after
diagnosis, resulting in a great burden on the family and society. Therefore, attention has to be given to this problem.

Pulmonary fibrosis diagnostic aids include X-ray, high-resolution computed tomography, pulmonary function test, and histological sections. CT can show punctiform and reticulation signs in both sides of the lung field and honeycomb signs in the last phase. The pulmonary function test provides a means to identify diseases, predict their possible aggravation, and reflect the treatment effects. Detailed disease history, occupational history, and CT finding (high-resolution CT, HRCT) can enable preliminary diagnosis, but accurate diagnosis is based on the histological sections. At present, drugs to treat pulmonary fibrosis are limited. So, more effective and earlier diagnosis of this disease is the joint goal of medical professionals worldwide.

Paraquat was first synthesized in 1882, but its pesticidal properties were not discovered until 1959. A para-substituted quaternary bipyridyl cat-ion which was introduced commercially in 1962, it is very effective as a herbicide [14]. Paraquat-induced inflammation of alveoli and epidermal cells causes damage quickly [15]. Some studies indicated that paraquat-induced interstitial pneumonia in acute phase was of a higher grade, while type I pneumocytes were decreased and type II pneumocytes were increased, which could aggravate the degree of lung damage [16-18]. Therefore, some reports indicated that although paraquat is very effective as a herbicide, it was a serious hazard to humans and animals. Paraquat induces pulmonary fibrosis, one of the most severe results of exposure to paraquat, in humans, monkeys, dogs, and rats [19-21]. The mechanisms of paraquat-induced pulmonary fibrosis have not been fully elucidated. It has been well documented that paraquat-induced pulmonary fibrosis develops after the acute toxic phase [22]. It has also been reported that paraquat accumulates in the lungs and forms pulmonary edema that may progress to interstitial fibrosis [23]. Many countries have banned or severely restricted the use of paraquat because of the debilitating or life-threatening hazards from occupational exposure and the large number of reported accidental and suicidal fatalities [24,25].

The purpose of this study was to monitor paraquat-induced pulmonary fibrosis in rats with micro-CT, which uses a non-invasive and non-sacrificial method and reduces individual differences by monitoring the same animals. Based on micro-CT images, we construct an animal model for monitoring pulmonary fibrosis in rats and build an objective interpretation method by software circling of the finding data. This may lead to new applications in the assessment of treatment outcomes of pulmonary fibrosis and to the development of new drugs as a next step. Therefore, this study aimed to help with the diagnosis of pulmonary fibrosis and screening of drugs.

2. Materials and methods

2.1 Reagents

Reagents were obtained from Paraquat Sinon Corporation. The molecular weight of paraquat (C_{12}H_{14}C_{12}N_{2}) and paraquat dichloride (C_{12}H_{14}C_{12}N_{2}) are 186.3 and 257.2 daltons. They are colorless crystal and with fusion point around 300°C. Two chemical compounds are partially soluble in water (700g/L), poorly soluble in most organic solvents [26].

2.2 Experimental animals

Twenty-four rats (male, 180-200g) were divided into control, low-dosage, medium-dosage and high-dosage groups (6 rats/each group). These rats were acclimated (24 ± 2°C, 55 ± 15% RH) in racks under positive pressure, in a room with air purified through HEPA filters. The environmental conditions during our experiment were controlled with an illumination cycle 12 L: 12 D. Rats were fed with CRF-1 pellets (Oriental Yeast Co., Ltd., Japan), and with water filtered by Milli-Q filtrator (Millipore Corporation, Burlington, USA) ad libitum. The animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the Institute of Nuclear Energy Research (INER). The rats were weighed and imaged by micro-CT before administration, which was advantageous for comparison of body weights and micro-CT findings after administration.

2.3 INER's micro-CBCT system and micro-CT imaging

This study used the micro-cone-beam computed tomography developed by the INER [27]. The cone-beam micro-CT system consists of an X ray tube with 50 kV voltage (max.), 1 mA anode current (max.), and a 70 mm × 70 mm Gd$_2$O$_5$S(Tb)-CCD detector. A personal computer based on MS Windows 2000 was used for data acquisition, control, and reconstruction.

In this study, a CT volume was reconstructed by the Feldkamp algorithm [28]. Typical voxel volume and dimension of the reconstructed images were 100 × 100 × 100 µm$^3$ and 512 × 512 × 512 µm$^3$, respectively. Animal imaging was performed with x-ray operating at 40 kV and 0.6 mA. A total of 200 projections were acquired at every 1°. The exposure time to acquire a view of projection data was kept constant at 0.5 sec throughout all the CT scans.

2.4 Administration

The low-, medium-, and high-dosage groups were given a single oral dosage of aqueous solution of paraquat at 40 mg/kg, 60 mg/kg, and 80 mg/kg, respectively.

2.5 Experimental rat anatomy

Adult rats were sacrificed by forced inhalation of CO$_2$, then the right atriums of all the hearts were dissected and the left ventricles were perfused with normal saline solution until exsanguination was completed. The lung samples were quickly removed and observed, and pictures were taken to record the appearance. Finally, all specimens were soaked with 4% buffered formaldehyde and maintained in 4% formalin until the analyzed histological sections were performed [29].
2.6 Histological section and stain

Histopathology sections of 5 µm were taken from the tissues maintained in 4% formaldehyde and embedded with paraffin wax for histopathology stain. In this study, Hematoxylin & Eosin stain and Masson’s Trichrome stain [30] were applied in order to observe cell morphology and to evaluate criterion of pulmonary fibrosis.

2.7 Evaluation and quantization

The lung samples of sacrificed rats were observed by microscope using Hematoxylin & Eosin stain and Masson’s Trichrome stain according to criteria of pulmonary fibrosis on a scale of 0 to 8 suggested by [7] to determine whether petechia and edema were present or not. With diagnosis of histopathology in the normal condition, type I pneumocytes accounted for 90% or above, the grade of interstitial pneumonia was (–), and the alveoli were complete and clear, with walls being thin and brittle. When type I pneumocytes accounted for 80% and the grade of interstitial pneumonia was (+), the alveoli and walls were thickened slightly. Type I pneumocytes accounted for 70%, type II pneumocytes increased moderately, the grade of interstitial pneumonia was (++), the walls of the alveoli were thickened moderately, but there was no damage in the pulmonary structure. When type I pneumocytes accounted for 60%, type II pneumocytes increased obviously, the grade of interstitial pneumonia was (+++), there was slight damage to the pulmonary structure, and the formation of a small fibrotic area was observed. Type I pneumocytes and type II pneumocytes accounted for 50% each, the grade of interstitial pneumonia was (+++), there was substantial damage to the pulmonary structure, and a large fibrotic area was observed. When type II pneumocytes were over 50%, the grade of interstitial pneumonia was (++++) and the full visible field was fibrocytic.

Using histological sections to evaluate criteria of pulmonary fibrosis, and comparing the evaluation with the micro-CT finding to find their relationship, the results of this study showed that micro-CT finding could diagnose paraquat-induced pulmonary fibrosis in rats. The lung weights of adult rats also increased when the dosage of paraquat was increased, but the body weight increments of adult rats decreased; therefore, the ratio of lung weight to body weight increased as dosage increased. This ratio is another criterion to evaluate the toxicity of paraquat, and also to correct the difference among the experimental rats. Results were the same as when imaged by micro-CT, using the same rat to reduce individual differences.

Recent study indicated the effective capacity volume of lungs decreases because of pulmonary fibrosis [31]. In this study, quantitative analysis was used to calculate the effective capacity volume of lungs. The environmental quantitative analysis software used was MATLAB 7.1 and Amira 4.1 (American National Instruments Co., Taiwan). MATLAB 7.1 was used to program the analysis [32]. Amira 4.1 was used to produce the 3D structure model and to produce images [33].

In this study, a method that provides 3D quantitative structural data of the lungs and automatic operations, without manually defining regions of interest, is introduced. The basic concept of the method is to define the thoracic cavity through ribs automatically by in-house software and analyze the 3D volume histogram of the ribcage. Adequate parameters were chosen to segment the ribs from each image, and then to calculate a histogram of the selected region. The 3D quantitative technique is to measure the volume variance of air and soft tissue ratios in the lung.

3. Results

3.1 Lung samples observed

In the control group, the lung samples were in normal condition; the color and luster of the lung samples appeared normal and no petechia was observed (Figure 1). In the low-dosage group, the appearances of lung samples were almost in a normal condition (1st week) and the petechia was observed from the 2nd week. In the 4th week, some petechia was observed in the whole area of the lungs (Figure 2). In the medium-dosage group, some petechia was observed in part of the lungs in the 1st week. The 2nd week, some petechia was observed in the whole area of the left lung and upper part of the right lung. The 3rd week, severe bleeding was observed. In the 4th week, a lot of petechia was observed all over the lungs with severe bleeding of the right lung (Figure 3). In the high-dosage group, a lot of petechia was observed all over the lungs with severe hemorrhage of the right lung in the 1st week. 2nd and 3rd week, a lot of petechia was observed in the right lung. In the 4th week, a little petechia was observed in the right lung (Figure 4).

![Figure 1](image1.png)

Figure 1. The appearance of lung samples and histological sections in the control group. (a) Lung samples in the normal condition. (b) Histological sections in the normal condition.

3.2 The body weight increments of adult rats

As the dosage of paraquat was increased, body weights of adult rats increased, but the body weight increments decreased. The average increments of body weight in adult rats were 66.83 ± 3.06 grams, 62.00 ± 2.28 grams, 59.83 ± 2.32 grams, and 38.17 ± 2.48 grams in the control group, low-dosage group, medium-dosage group, and high-dosage group, respectively (Figure 5).

3.3 The lung weight of adult rats

As the dosage of paraquat was increased, the lung weight of adult rats decreased. After the dissection, the average lung
weights of adult rats were 1.44 ± 0.24 grams, 1.64 ± 0.40 grams, 1.94 ± 0.24 grams, and 2.72 ± 0.54 grams in the control group, low-dosage group, medium-dosage group, and high-dosage group, respectively (Figure 6).

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3.4 The ratio of lung weight over body weight in adult rats

As the dosage of paraquat was increased, the lung weights of adult rats also increased, but their body weight increments decreased, therefore the ratio of lung weight over body weight increased with increased dosage. After the dissection, the ratios of lung weight over body weight were 0.4644 ± 0.0012, 0.5205 ± 0.0011, 0.5915 ± 0.0008, and 0.8359 ± 0.0021 in the control group, low-dosage group, medium-dosage group, and high-dosage group, respectively. The ratio of each dosage is shown in Figure 7.

3.5 Histological sections

In the histological sections of the control group, type I pneumocytes accounted for 90% or above, the grade of interstitial pneumonia was (−), and the alveoli were complete and clear while their walls were thin and brittle (Fig. 1). In the low-dosage group, the appearance of histological sections was as follows: The 1st week, type I pneumocytes accounted for 90%, the grade of interstitial pneumonia was (±), and alveoli and walls were slightly thickened. The 2nd week, type I pneumocytes accounted for 80%, the grade of interstitial pneumonia was (±) with slight bleeding, and the alveoli were complete and walls were slightly thickened. The 3rd week, type I pneumocytes accounted for 70%, type II pneumocytes increased moderately, the grade of interstitial pneumonia was (+), and the alveoli were deformed, walls were thickened with hemorrhage, and inflammation as well as fibroblasts were visible. The 4th week, type I pneumocytes accounted for 80%, the grade of interstitial pneumonia was (±), the alveoli tended to be complete with thickened walls, and degradation as well as collagen appeared (Figure 2).

In the medium-dosage group, the appearance of histological sections was as follows: (1) The 1st week after the administration, type I pneumocytes accounted for 90%, the grade of interstitial pneumonia was (±), and the alveoli and walls were slightly thickened; (2) the 2nd week after the administration, type I pneumocytes accounted for 70%, type II pneumocytes had increased moderately, the grade of interstitial pneumonia was (+) with slight bleeding, the alveoli were deformed and walls were thickened with accumulation of lymphocytes, and inflammation as well as fibroblasts appeared; (3) the 3rd week after administration, type I pneumocytes accounted for 60%, type II pneumocytes had increased obviously, the grade of interstitial pneumonia was (++), the alveoli were deformed and walls were thickened with hemorrhage, inflammation as well as fibroblasts appeared; (4) the 4th week after administration, type I pneumocytes accounted for 60% and type II pneumocytes had increased moderately, the grade of interstitial pneumonia was (+) and bleeding and fibrosis ensued, the alveoli were deformed and walls were thickened, and degradation as well as collagen and macrophages appeared (Figure 3).

In the high-dosage group, the appearance of histological sections were as follows: The 1st week, type I pneumocytes accounted for 70%, type II pneumocytes had increased moderately, the grade of interstitial pneumonia was (±) with accumulation of lymphocytes, monocytes, and RBC. The 2nd week, type I pneumocytes accounted for 60%, type II pneumocytes had increased obviously, the grade of interstitial pneumonia was (+) with accumulation of inflammatory cells and RBC, the alveoli were deformed and walls were thickened with hemorrhage, inflammation as well as fibroblasts appeared. The 3rd week, type I pneumocytes accounted for 60%, type II pneumocytes had increased obviously, the grade of interstitial pneumonia was (++), the alveoli were deformed and walls were thickened with hemorrhage, inflammation as well as fibroblasts and collagen appeared. The 4th week, type I pneumocytes accounted for 70%, type II pneumocytes had increased moderately, the
grade of interstitial pneumonia was (+), the alveoli were
deformed and walls were thickened, and degradation as well
as fibroblasts and collagen appeared (Figure 4).

3.6 Micro-CT findings

In the control group, there were no differences among
images throughout the different weeks; however, in the
experimental group, the ground-glass predominance and
reticulation structure in micro-CT findings increased from
the 1st week to the 3rd week, and degraded in the 4th week
after administration (Figure 8). By taking advantage of
three-dimensional quantitative analysis, it was observed that
the effective capacity volume reduced by 14% in the
low-dosage group, 23% in the medium-dosage group, and
30% in the high-dosage group. As the dosage of paraquat
was increased, hemorrhages in adult rats also increased, but
the effective capacity of lungs decreased (Figure 9).

4. Discussion

4.1 Lung sample observation and histological sections of adult
rats

Many studies have indicated that paraquat-induced
pulmonary hemorrhage and pulmonary edema appear in lung
samples. As the dosage of paraquat in adult rats was
increased, pulmonary hemorrhages and pulmonary edema
also increased in direct proportion between dosages and
these occurrences [34-36]. In this study, the same condition
occurred. Histological sections were used as standard in

many investigations to verify paraquat-induced pulmonary
injury, and from 1985 to 2006, many studies indicated that
when the dosage of paraquat was increased, the grade of
interstitial pneumonia increased, type II pneumocytes
increased, type I pneumocytes decreased, pulmonary injuries
increased, alveoli walls thickened, and the fibrotic area
increased owing to increased fibroblast proliferation and
collagen production [37,38]. In this study, the same
conditions were observed.

4.2 The relation between body weight and lung weight, and the
ratio of lung to body weight in adult rats

Body weight loss was used as important index in the
study of Satomi et al. [26] and Smith et al. [35]. The
intoxicative capacity of paraquat was based on weight loss in
the study of Satomi et al. [36]. The variations of body
weights were used to evaluate the toxicity of paraquat
directly. In analyzing the results of this study, rats didn't
show significant loss of body weight owing to the food and
water given ad libitum. So, the body weight increments of
rats after different dosages of paraquat administration were
applied to evaluate the toxicity of paraquat. As the dosage of
paraquat increased, the body weight increments of adult rats
decreased owing to tachypnea, hypo-activity, and
hypo-appetite caused by the paraquat.

In addition, the studies of Hemmati et al. [37] and Ruiz
et al. [38] indicated paraquat-induced pulmonary hemorrhage
and pulmonary edema in adult rats. As the dosage of
paraquat increased, lung weight also increased, causing
increased pulmonary hemorrhage and pulmonary edema;
however, body weight increments decreased at the same time,
resulting in an increased ratio of lung weight over body
weight. This ratio is another criterion to evaluate the toxicity
of paraquat and to correct the differences among the
experimental rats. This has the same effect as imaging the
same rats by micro-CT to reduce individual differences.
4.3 Micro-CT findings

Katzenstein and Myers brought up standards of pulmonary fibrosis in CT findings, including the following: In mild grade, ground-glass predominance was observed; in moderate grade, reticulation and architectural distortion were observed; in severe grade, traction bronchiectasis and honeycombing of lungs were observed [10].

Recently, Zimmermann [31] reported that effective capacity decreased owing to pulmonary fibrosis, and Johnson provided a method to quantitate the reduced effective pulmonary capacity owing to pulmonary diseases by CT findings [39]. In the current study, a method was introduced providing 3D quantitative structural data of lungs automatically without manually defining regions of interest. The results showed that the effective capacity decreased by 14% in the low-dosage group, 23% in the medium-dosage group, and 30% in the high-dosage group. As the dosage of paraquat was increased, the hemorrhages in adult rats increased, but the effective capacity of lungs decreased, which matched the results of other studies. The results agree with body weight increments, lung weights, ratios of lung weight over body weight, and histopathology observed.

5. Conclusion

Generally, there was no significant loss of body weight; the weight of experimental animals did not decrease significantly. Therefore, the body weight increments of rats after different dosages of paraquat were applied to evaluate the toxicity of paraquat. As the dosage of paraquat was increased, lung weight of adult rats also increased, causing pulmonary hemorrhage and pulmonary edema to increase; however, body weight increments decreased, therefore the ratio of lung weight over body weight increased. This ratio is another criterion to evaluate the toxicity of paraquat and to correct the differences among the experimental rats. This has the same effect as imaging the same rats by micro-CT to reduce individual differences. In previous studies, when the dosages of paraquat were increased, the grade of interstitial pneumonia increased, type II pneumocytes increased, type I pneumocytes decreased, pulmonary injuries increased, alveoli walls thickened, and the fibrotic areas increased due to increased fibroblasts and collagen. In this study, the same conditions were observed. This study constructed an animal model of pulmonary fibrosis. The micro-CT images from 3D reconstruction show that the effective capacity of the lungs gradually decreased with increased dosages, therefore micro-CT can be regarded as a powerful tool for fast diagnosis and longitudinal monitoring of pulmonary fibrosis.

References


