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Protection of Pirfenidone against an early phase of Oleic acid induced acute lung injury in rats

Shuang Mei, **Wei Yao**, **Yuanjue Zhu and Jinyuan Zhao**

Research Center of Occupational Medicine, Third Hospital of Peking University and Department of Respiratory medicine, Peking Union Medical College Hospital

Running title: Protection of PFD from an early phase of ALI by OA in rats

Corresponding Author

Name: Jinyuan Zhao

Address: 49North Garden Road, Haidian District, Research Center of Occupational Medicine, the Third Hospital of Peking University, Beijing 100083 China

Tel: 8610-62017691-2651

Fax: 8610-82074373

E-mail:zhaojinyuan@sina.com

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Abbreviations: PFD, pirfenidone; ALI, acute lung injury; OA, oleic acid; ESR, electron spin resonance; PO_2, Partial pressure of oxygen; ARDS, acute Respiratory Distress Syndrome; ROS, reactive oxygen species; PBN, a-phenyl-N-*tert*-butyl nitrone; DETAPAC, diehtylonethiaminepentaacetic acid; DPPH, Diphenyl-picri-hydrazyl; TGF-β1, transforming growth factor beta1;

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Abstract

The potential role of pirfenidone (PFD), an antifibrotic compound with anti-inflammatory effects, in several models of acute lung injury (ALI) has gained increasing attention. Yet the protective effect of PFD in oleic acid (OA) induced ALI remains unknown. We hypothesized that PFD protects from OA induced ALI in rats, and we hoped to obtain the optimum preclinical conditions with PFD in ALI. Sprague-Dawley rats were randomized into 5 groups (5 rats per group): normal control group; OA treated group (0.15ml/kg); 3 PFD treated groups (20mg/kg, 40mg/kg and 80mg/kg, p.o., respectively). Arterial blood gases, lung wet/dry weight ratio and postmortem histological changes were determined at 0.5h, 1h, 2h, 6h and 24h after OA challenge. Electron spin resonance (ESR) spectroscopy was used for free radicals detection and measurement. Experiments were examined based on the orthogonal test L4 (4^2) setting two factors (A: PFD dose; B: PFD valid time) with four different levels. The results of orthogonal test showed the sequence of effect of PFD was at 0.5h (oxygen radicals), 1h (histological changes), 2h (lung edema) and 6h (Partial pressure of oxygen, PO_2) after OA challenge and the 40mg/kg of PFD was the most effective dose in this study. We conclude that PFD protects against OA-induced ALI in rats. The mechanism of these protective effects partly involves decrease of oxygen radicals. The data of this study proves that orthogonal test will be a powerful method to help obtain the optimum experimental conditions with PFD in ALI in the future.

Introduction

Acute Respiratory Distress Syndrome (ARDS) is a complex syndrome of non-cardiagenic edematous acute lung injury characterized by diffuse pulmonary infiltration, increased pulmonary capillary permeability and severe hypoxaemia (Repine, 1992). ARDS is the most severe and final stage of ALI. ALI and ARDS are among the main causes of death in intensive care units, with a mortality rate of 30%-40% (Frutos-Vivar et al, 2004). They are caused by an inciting insult, such as systemic release of endotoxin, trauma or aspiration which then triggers a series of pathologic events involving neutrophils aggregation and activation, release of proteases and lipid mediators and production of free radicals and cytokines etc (Repine, 1992). OA-induced lung injury is well known as a model of ARDS (Leeman, 1991). Though extensive studies on OA-induced lung injury have been made for decades, its intrinsic mechanism still has remained obscure. Oxidative stress is thought to play an important role in OA-induced lung injury (Yang et al, 2003; Tampo et al, 1999; Zhang et al, 2000). The NADPH oxidase-dependent pathway from activated polymorphonuclear leukocytes and endothelial cells (Sanders et al, 1999) contributes quite much to the generation of reactive oxygen species (ROS) in OA-induced lung injury. A mechanism of OA-induced lung injury is that the intravenous administration of OA can induce neutrophils activation through aggregation and attachment to endothelial cells (Moriuchi et al, 1998), activated neutrophils then can directly cause tissue injury by releasing ROS such as superoxide anions, hydroxy anions etc. which may lead to lipid peroxidation and integrity damage of the biomembranes. In turn, loss of the functional integrity of the biomembranes results in a rapid increase of alveolar-capillary permeability, lung edema and severe hypoxia (Kumar et al, 2000). Although ROS has been studied in the research of ARDS for many years, there is still absence of

detailed and direct data of ROS because of its high activity and short life. In former studies, ROS is generally analyzed by measuring their secondary or end products, for example, hydrogen peroxide, oxidized proteins, or peroxidized lipids, i.e**.** Malondialdehyde etc. (Kumar et al, 2000; Zhang et al, 2000). This study used electron spin resonance (ESR) spectroscopy directly to measure ROS in the lung tissues of ARDS rats induced by OA, which first shows a true situation of ROS generation during ALI and ARDS by chemicals. Thereby opens a new "window" in the pathogenesis exploration of ALI and ARDS.

To date, there is no specific pharmacologic approach for prevention and treatment of ALI and ARDS. Lung protective low tidal volume strategy and activated protein C have been proven to be effective in reducing mortality (Michael et al, 2003). High-dose glucocorticoids as anti-inflammatory drugs, alone or in combination with other immunosuppressive agents, also continue to be prescribed while there is a high incidence of drug-related adverse effects (Iuchi et al, 2003). Unfortunately, trials of high-dose steroid therapy failed to show an improvement in mortality of patients at risk of ARDS or with early ARDS (Rocco et al, 2003).Thus, future studies should focus on newer forms of therapy and, if possible, early course of the disease. PFD (5-methyl-L-phenyl-2-(1H)-pyridone, trade name: Deskar), a new experimental drug, was chosen as a therapeutic agent for ALI and ARDS in this study which has been showed that could attenuate the severity of experimental ALI by Lipopolysaccharide challenge (Cain et al,1998; Spond et al,2003; Oku et al.,2002; Corbel et al,2001) and Bleomycin induction (Iyer et al, 1995, 1998, 2000; Schelegle et al, 1997; Mansoor et al, 1999).

Yet, because different stimulus may respond differently, the effect of PFD on lung injury may also be different from stimulus to stimulus. To better understand the potential role of PFD in OA-

induced ALI, we assayed blood gas, lung wet/dry weight ratio, pulmonary histology and oxygen radicals in lung tissue at different time points. To obtain the optimum experimental conditions with PFD in ALI in the future, we chose two factors in this study including PFD dose and PFD valid time with four different levels of each.

Material and methods

Animals

This study was approved by the Institutional Animal Care and Use Committee of Health Sciences Center, Peking University. Male Sprague-Dawley rats (250-280g) were purchased from the Experimental Animal Center of Health Sciences Center, Peking University (Beijing, China) and allowed to acclimate upon arrival for 3 days before experimentation. Animals were fed rodent chow and water *ad. libitum*.

Reagents

PFD (Tocris Cookson, Ellisville, MO) was dissolved in a 0.4% methylcellulose vehicle solution (0.4% hydroxypropyl methylcellulose in H_2O; Dow Chemical Co.). A-phenyl-N-*tert*-butyl nitrone (PBN) was purchased from Sigma Chem. Co., USA. OA was purchased from golden dragon Chem. Co., Beijing, China. Diehtylonethiaminepentaacetic acid (DETAPAC) and ethyl acetate were the products of Beijing Chemical Agent Manufactory, China, which all were analytical reagent level.

Study design

The protocol was divided into 5 time points (0.5h, 1h, 2h, 6h and 24h) after the injection of OA. At each time point, there were 5 groups (5 rats per group): (1). control group only received0.4% methylcellulose vehicle solution; (2).ARDS group administered OA (0.15ml/kg, iv) and 0.4% methylcellulose vehicle solution; (3). 3 PFD treated groups (20mg/kg, 40mg/kg and 80mg/kg, p.o., respectively). The animal model of ARDS was verified by blood gas analysis and pathological examination of lung.

After anesthetized by inhalation of isoflurane, rats from ARDS group and PFD treated groups

were injected OA via tail vein, while control rats were only administered same volume of saline intravenously. The rats of PFD treated groups were immediately once given PFD (20 mg/kg, 40 mg/kg or 80mg/kg, p.o.) after OA injection, while control rats and ARDS rats were only received 0.4% methylcellulose vehicle solution. Animals were allowed to recover on a heated pad, returned to houses and permitted access to food and water. Then, at the set time point, rats were anesthetized by intraperitoneal injection of urethane (1.0g/kg). After reaching anesthesia, Surgery was carried out in a sterile setting with rats in the supine position. A carotid catheter with heparinized saline was inserted for measurement of blood gas which was analyzed at 37°C with blood gas analyzer (CiBA Corning 170, USA).

Lung Wet/dry ratio

After the experiment protocol was completed, the samples of all animals were rapidly obtained from the right upper lobe and desiccated in an oven at 60 °C for 72h to examine the lung wet/dry weight ratio.

Lung histopathology

Lung sections from the right lower lobe were fixed into 10% buffered formalin for 24h. Lung tissues were embedded in paraffin and 4-um sections were stained with Hematoxylin and eosin. The stained sections were scored by pathologists who were blinded to the experimental protocol. The specimens were evaluated and the severity of injury was judged by the previous criteria (Su et al, 2003).

ESR spectra of oxygen radicals from lung tissues

The samples of lung tissues from the left upper lobe were removed and homogenised with100mM PBN as a spin trap and 2mM DETAPAC. An X-band ESR spectrometer (Varian

E-109E, USA) was used to detect the levels of free radicals in lung tissues directly, with measurement conditions as: X-band, central magnetic field 3445G, scan width 200G, microwave power 20 mW, frequency 100 kHz, modulation amplitude 2.5G, time constant 0.128s at 37 °C (Capani et al, 2001). Before measurement, ESR spectrometer was standardized with Diphenyl-picri-hydrazyl (DPPH) to make sure that the equipment was in the same conditions in every time experiments.

Statistics analyses

Statistics analyses were performed by SPSS11.5 software and the results were presented as mean±SED. Continuous parametric data was subjected to analysis of variance (ANOVA) followed by the Student-Newman-Keuls post-hoc test for between-groups difference. Kruskal-Wallis test was used to detect the lung injury score differences among groups. A "p" value $\leqslant 0.05$ was regarded as the statistical significance.

Orthogonal test

To obtain the optimum pharmacological condition of PFD, experiments were examined based on the orthogonal test L4 (4^2) (Wang et al, 2004) setting two factors (A: PFD dose; B: PFD valid time) with four different levels of each (Table1).

Results

General situation of the rats

One hundred and twenty-nine rats were entered into the study. The rat body weights were similar among groups. Two rats of the OA group and one rat of the 80mg/kg PFD group died, respectively at 24h after injection. At 6h, one rat of the OA group died. Other rats of all experimental groups survived. All animals tolerated either the vehicle or the drug without obvious adverse events. Plasma PFD concentrations did not seem to relate to differences in therapeutic effects.

Blood gas analysis

Figure1 showed that hypoxemia was much more evident in ARDS group after injection of OA, especially 2h later after OA injection than that in the control group. The changes of PO_2 corresponded with diagnostic criterion of ARDS by the meeting of the American-European consensus conference on ARDS (Bernard et al, 1994) suggested the animal model of ARDS was successful. PO_2 in the PFD groups, especially the 40mg/kg group, was significantly more elevated than that in the OA group (Figure2). There was no difference in effect on ALI among 3 PFD treated groups, except for the 80mg/kg group at 1h after OA challenge. That $PaCO_2$ and PH did not change among different groups at each time point ($PaCO_2$ and PH data were not shown).

Evaluation of lung water

After administration with OA, lung wet/dry weight ratio was much more pronounced in the OA group than that in the control group. The summit of lung wet/dry weight radio in the OA group was found at 1h after OA injection (Figure3). Lung wet/dry weight ratio in the PFD groups, especially the 40mg/kg group, was significantly lower than that in the OA group (Figure4) at all

time points. There was no difference in effect on ALI among three PFD treated groups.

Lung histopathology

The differences were illustrated in histological sections (which were in the Additional file). There was patchy hemorrhage observed grossly and light microscopic findings in the OA group including hemorrhage and edema, thickened alveolar interstitial and the existence of inflammatory cells in alveolar spaces. It is obvious that lung histologic changes were already present at 0.5 h after injection of OA and were most serious until 24 h after OA challenge. In PFD groups (especially the 40mg/kg group), these changes and pathologic score (Figure5) were far less marked than those in the OA group. There was no difference in effect on ALI among 3 dose PFD treated groups.

ESR spectra of oxygen radicals from lung tissues

ESR spectra of spin trapping adduct of oxygen radicals with PBN extracted from the lung tissue were shown in Figure7 and Figure8. The peak height of spectrum represented relative concentration of oxygen radicals. The result showed that no signal of oxygen radicals could nearly be seen in normal lung tissues. The significant release of oxygen radicals was measured at 1 h and 6h after intravasculous injection of OA. Free radical levels in the OA group enhanced considerably over the control group at each time point. Free radicals in PFD treated groups (especially the 40mg/kg group) were sharply lower than those in the OA group at all time points (Figure9). There was no difference in effect on ALI among PFD treated groups, except for the 80mg/kg group 24h after OA challenge.

Orthogonal test

Table2 showed the best experimentally pharmacological dose of PFD was 40mg/kg body

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weight. The time sequence of the most outstanding pharmacological effects of PFD was reducing free radical contents in lung tissues at 0.5h, improving pathological changes at 1h, alleviating lung edema at 2h and raising PaO_2 levels at 6h after OA challenge.

Discussion

It is well known that ARDS is a serious disease with a high mortality though extensive supportive treatments were given. Because ROS are believed to play a pivotal role in the pathogenesis of ARDS, preventing the damage by ROS would be a key measure to the treatment of ARDS (MacNee, 2001; Morcillo et al, 1999). Therefore, we examined the utility of PFD to inhibit oxygen radicals in OA induced ARDS model. Meanwhile we first used ESR spectroscopy in ARDS to measure ROS directly in OA-induced rat model of ARDS in this study. ESR spin trapping technique provides a sensitive, direct and accurate means for studying short life-span free radicals such as superoxide anions and hydroxyl radicals etc. which have unpaired electrons. Spin trap (such as PBN) could trap the active short-lived free radicals to form a relative long-lived free radical product, spin adducts (such as PBN-OOH), which can be easily detected by ESR. Therefore, ESR has been considered one of the most effective techniques to detect short life-span free radicals (Finkelstein et al, 1980). In our study, the increase of oxygen radicals which was examined by an X-band ESR spectrometer at 1h and 6h after OA challenge provides strong evidence that oxidative stress could be involved in the lung injury during OA-induced ARDS. The comparison between ESR spectra and blood gas analysis results (PO_2) indicates that the change of ROS was earlier than that of pathophysiology, because the concentration of ROS increased significantly at 1h after OA exposure and began to decrease at 2h, whereas the reduction of PO_2 started from 2h. Nevertheless, our results by ESR are a confirmation of previous studies (Yang et al, 2003; Tampo et al, 1999; Zhang et al, 2000) which favor the role of free radicals in OA-induced lung injury is critical.

The data from this study demonstrates that PFD reduces the severity of experimental lung injury

by several physiological gravimetric and histological criteria. We propose it was due to the association between PFD and decrease of oxygen radicals in this study. Firstly, we directly found PFD decreased oxygen radicals in ALI by an X-band ESR spectrometer. The free radicals in PFD treated groups, especially the 40mg/kg group, were sharply lower than that in the OA group at all time points. Secondly, previous reports (Iyer et al, 1998; Misra and Rabideau, 2000) show PFD suppresses free radicals through directly scavenging and indirectly modulating activities of prooxidant and antioxidant enzymes. Thirdly, the sequence of effect of PFD is at 0.5h (oxygen radicals), 1h (histology), 2h (lung edema) and 6h (PO2) after OA challenge by the orthogonal test, which indicates decrease of free radical precedes improvement of other lung injurious parameters. There are two possible mechanisms for the beneficial effects of PFD on OA-induced ALI: (1).PFD inhibits direct organ injuries by releasing oxygen radicals. ROS, such as superoxide anion, hydroxyl radical and hydrogen peroxide, causes cellular injury through lipid peroxidation which can alter both structure and function of alveolar-capillary permeability leading to the damage of integrity of the cell membranes, an acute increase in lung edema and decrease in gas exchange (Yang et al, 2003; Tampo et al, 1999; Zhang et al, 2000). As reported previously (Misra and Rabideau, 2000), PFD as a potent scavenger of hydroxyl radicals and inhibitor of membrane lipid peroxidation directly improves lung injury by ROS. In addition to the direct scavenger of hydroxyl radicals, PFD modulates oxidative stress indirectly by regulating enzymatic activities of prooxidant and antioxidant enzymes. PFD can alleviate the lung injury by superoxide anion, hydroxyl radical and hydrogen peroxide through regulating activities of myeloperoxidase and superoxide dismutase (Iyer et al, 1998); PFD may also enhance the production of intracellular glutathione by reducing transforming growth factor beta1 (TGF-β1) levels (Corbel et al, 2001),

because it is reported (Arsalane et al, 1997) that TGF-β1 can damage the alveolar epithelial integrity and cause lung edema by depletion of intracellular glutathione which is a soluble antioxidant and a scavenger of superoxide anion, hydroxyl radical and hydrogen peroxide (Halliwell and Gutteridge, 1991). So PFD can ameliorate lung injury by direct and indirect decrease of free radicals in the lung tissue. (2). PFD reduces the inflammatory cascade caused by free radicals as a signal molecule. As an intracellular signal molecule, free radicals activated redox-sensitive nuclear factor-kappa B complexes (Kikumori et al, 1998) that coordinate the induction of multiple genes encoding inflammatory mediators (Balibrea and Arias-Diaz**,** 2003) including cell-adhesion molecules, enzymes, cytokines, and chemokines, which seems to be, at the molecular level, a signature event of ARDS leading to the rapid activation of intracellular signaling pathways. The interplay among above inflammatory mediators leads to inflammatory cells recruitment and activation in the lung, lung parenchymal cells injuries or activation and release of additional inflammatory mediators. Thus, an initial small quantity of free radicals can stimulate a cascade of events resulting in a significant increase in neutrophil recruitment and inflammatory mediators and the exacerbation of lung injuries via positive feedback loops. We speculate that PFD suppresses production of oxygen radicals and then inhibits nuclear factor-kappa B (Tsuchiya et al, 2004) and nuclear factor-kappa B induced several cytokines such as TNF-α relevant to ARDS pathology (Spond et al, 2003; Iyer et al, 2000; Corbel et al, 2001; Oku et al, 2002), which ameliorates lung edema and gas exchange. Therefore, we conclude the mechanism of these protective effects of PFD partly involves suppression of oxygen radicals in lung tissues.

PFD (10mg/kg and 30mg/kg) was documented to exert a dose-dependent anti-inflammatory in

the models of endotoxin-induced lung injury (Spond et al, 2003). A top dose of PFD (30mg/kg) did not completely inhibit the recruitment of inflammatory cells. Higher doses of PFD may bring more beneficial effects of inhibition. Therefore, we chose a higher dose of PFD (40 and 80mg/kg) in OA induced ALI. In our study, the protective effect of PFD on lung injury in the 40 mg/kg group was much better than that in the 20 mg/kg group, in which groups dose of PFD is in the effective dose range，while PFD in the 80mg/kg group had slightly protective effects on ALI except PO2 at 1h and oxygen radicals at 24h after OA challenge are closed to those in the OA group. The reasons may be that PFD has a very narrow effective dose range. If the dose of PFD is higher or lower than the effective dose range, it will result in activity of other signal pathways of effect and impair the protective effect of PFD. Regardless, to our knowledge these are the first reported observations that PFD, at 20 and 40 mg/kg, affords an effective protection in OA induced ALI in rats. Through the orthogonal test, we also found the same point as above conclusion that the best experimental dose of PFD is 40mg/kg body weight. That $PaCO_2$ and PH did not change among PFD treated groups indicates there are no changes in blood acid base status. Similar changes in $PaCO_2$ and PH have been observed in patients and rats after PFD treatment (Nagai et al, 2002; Mansoor et al, 1999).Our results in rats suggest PFD also has no other adverse effects within the dose range observed consistent with the previous reports in animals and humans (Gahl et al, 2002; Mansoor et al, 1999; Nagai et al, 2002; Giri et al, 2002). Plasma PFD concentrations did not seem to relate to differences in therapeutic effects similar to the previous reports from patients and mice with PFD (Nagai et al, 2002; Giri et al 2002). So we think the findings that a rat in the 80mg/kg PFD group died 24h after OA injection was not drug-related. No death in other PFD treated groups indicates that PFD improves animal survival without causing toxic and

adverse effect.

In summary, our results are the first to report the protective effect of PFD in OA induced ALI. We also found the effective protection of PFD is a narrow dose range out of which PFD was slightly able to block lung injury and the protective inhibition of free radicals with PFD serves as an important target to reduce inflammation and organ injury in ARDS. As this is an animal model of ALI induced by OA, we should be extremely cautious in extrapolating these data to the complex clinical situation. However, there is an important clinical relevance in this study which the treatment with PFD at the early phase of ALI may help prevent from lung tissue impedance and ameliorate symptoms of lung edema and hypoxaemia. The treatment also can avoid the side effects related to prolonged and high doses of steroid. Though these initial results were obtained from a limited data, the results of this study show that orthogonal test is more optimized and effective than other ways by pharmacological and validated experiments. It will be a powerful method to help obtain the optimum preclinical conditions with PFD in ALI in the future.

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Send reprint requests to:

Name:Jinyuan Zhao

Address: 49North Garden Road, Haidian District, Research Center of Occupational Medicine, the Third Hospital of Peking University, Beijing 100083 China

E-mail: zhaojinyuan@sina.com

Legends for figures

Figure1 the effect of PFD on PO2with oleic acid induced acute lung injury in rats. Sprague-Dawley rats were randomized into 5 groups (n=5 per group): normal control group; oleic acid group (0.15ml/kg); 3 PFD treated groups (20mg/kg, 40mg/kg or 80mg/kg, p.o. respectively). 0.5h, 1h, 2h, 6h and 24h represents oleic acid induced time point, respectively. *p<0.05 versus the control group. ** p<0.01 versus the control group. # p<0.05 versus the oleic acid group. ## p<0.01 versus the oleic acid group.+p<0.05 versus the 40mg/kg PFD group.

Figure2 Comparison of PaO2 among the oleic acid group, the 40mg/kg PFD group and the control group. 40mg/kg body weight PFD was the best experimental dose. *p<0.05 versus the control group. ** p<0.01 versus the control group. # p<0.05 versus the oleic acid group. ## p<0.01 versus the oleic acid group.

Figure3 the the effect of PFD on lung wet/dry weight ratio with oleic acid induced acute lung injury in rats. Sprague-Dawley rats were randomized into 5 groups (5 rats per group): normal control group; oleic acid group (0.15ml/kg); 3 PFD treated groups (20mg/kg, 40mg/kg or 80mg/kg, p.o., respectively). 0.5h, 1h, 2h, 6h and 24h represents oleic acid induced time point, respectively. *p<0.05 versus the control group. ** p<0.01 versus the control group. # p<0.05 versus the oleic acid group. ## p<0.01 versus the oleic acid group.

Figure4 Comparison of lung wet/ dry weight ratio among the oleic acid group, the 40mg/kg PFD group and the control group. The best experimental dose of PFD was 40mg/kg body weight.*p<0.05 versus the control group. ** p<0.01 versus the control group. # p<0.05 versus the oleic acid group. ## p<0.01 versus the oleic acid group.

Figure5 the effect of PFD on pathologic score with oleic acid induced acute lung injury in rats. Sprague-Dawley rats were randomized into 5 groups (5 rats per group): normal control group; oleic acid group (0.15ml/kg); 3 PFD treated groups

(20mg/kg, 40mg/kg or 80mg/kg, p.o., respectively). 0.5h, 1h, 2h, 6h and 24h represents oleic acid induced time point, respectively. *p<0.05 versus the control group. ** p<0.01 versus the control group. # p<0.05 versus the oleic acid group. ## p<0.01 versus the oleic acid group.

Figure6 Comparison of pathological score among the oleic acid group, the 40mg/kg PFD group and the control group. The best experimental dose of PFD was 40mg/kg body weight. ** p<0.01 versus the control group. ## p<0.01 versus the oleic acid group.

Figure7 effect of pirfenidone on oxygen radicals detected by an X-band ESR spectrometer. 1=control group; 2=oleic acid treated group; 3=20mg/kg pirfenidone group; 4=40mg/kg pirfenidone group; 5=80mg/kg pirfenidone group. 0.5h, 1h, 2h, 6h and 24h represents oleic acid induced time point, respectively.The peak height of spectrum represented relative concentration of oxygen radicals (gauss). The result showed that no signal of oxygen raidcals could nearly be seen in normal lung tissues; A significantly release of oxygen radicals in the oleic acid group was detected at 1 h and 6h after intravasculous injection of oleic acid. Free radical levels in the oleic acid group rose considerably over the control group at each time point. Free radicals in PFD treatment groups (especially, 40mg/kg) were sharply lower than that in the oleic acid group at all time points. There is no difference in effect on ALI among 3 dose PFD groups, except for the 80mg/kg PFD group at 24h after oleic acid challenge.

Figure8 the effect of PFD on oleic acid induced acute lung injury in rats. Sprague-Dawley rats were randomized into 5 groups (5 rats per group): normal control group; oleic acid group (0.15ml/kg); 3 PFD treated groups according to the doses divided (20mg/kg, 40mg/kg or 80mg/kg, p.o., respectively). 0.5h, 1h, 2h, 6h and 24h represents oleic acid induced time point, respectively. *p<0.05 versus the control group. ** p<0.01 versus the control group. # p<0.05 versus the oleic acid group. ## p<0.01 versus the oleic acid group. ++p<0.01 versus the 40mg/kg PFD group.

Figure9 Comparison of oxygen radicals among the oleic acid group, the 40mg/kg PFD group and the control group. The best experimental dose of PFD was 40mg/kg body weight.** $p<0.01$ versus the control group. ## $p<0.01$ versus the oleic acid group.

Table1 Factors and levels of orthogonal design L4 (4^2) with pirfenidone

Level	A PFD Dose (mg/kg)	level	B PFD valid time (h)
0	0	1	0.5
1	20	2	1
2	40	3	2
3	80	4	6

Experiments were examined based on the orthogonal test L4 (4^2) setting two factors (A: PFD dose; B: PFD valid time) by four different levels for obtaining the optimum conditions.

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Table 2 Orthogonal test for the optimum experimental conditions with pirfenidone

level	A	B	Free radicals (gauss)	Wet/dry lung weight radio	pathologic score	PO2 (mmHg)
1	0	1	74.4	10.07	12.75	63.67
2	0	2	149	10.58	9.25	56
3	0	3	94.2	9.21	10.5	47.33
4	0	4	122.6	9.35	15	67.67
5	1	1	44.8	6.49	7	78.33
6	1	2	91.4	6.89	5.25	80.33
7	1	3	55.8	4.67	4.5	81
8	1	4	64.6	6.60	9	88
9	2	1	36	5.41	5.25	83.33
10	2	2	88.6	6.21	4.75	87.33
11	2	3	44.2	4.31	4	83.67
12	2	4	54	5.97	7.75	89.33
13	3	1	49.4	6.78	8.25	77
14	3	2	92.6	7.27	6.5	73
15	3	3	61.2	5.51	6.25	78
16	3	4	67	6.97	9.5	81

Variance analysis of orthogonal test

Free radicals	Ⅰj	440.2	204.6	A2：B1
	Ⅱj	256.6	421.6	
	Ⅲj	222.8	255.4	
	Ⅳj	270.2	308.2	
	Rj	217.4	217	
wet to dry lung weight radio	Ⅰj	39.21	28.75	A2：B3
	Ⅱj	24.66	30.95	
	Ⅲj	21.9	23.70	
	Ⅳj	26.53	28.89	
	Rj	17.31	7.25	
pathological score	Ⅰj	47.5	33.25	A2：B2
	Ⅱj	25.75	25	
	Ⅲj	21.75	25.25	
	Ⅳj	30.5	41.25	
	Rj	25.75	16.25	
PO2	Ⅰj	234.67	302.33	A2：B4
	Ⅱj	327.66	296.66	
	Ⅲj	343.66	290	
	Ⅳj	299	326	
	Rj	108.99	36	

Notes: $^{Ⅰ}j, ^{Ⅱ}j, ^{Ⅲ}j, ^{Ⅳ}j$ represent respectively the sum of the mean value of PFD dose and PFD valid time of all16 experiments; R_j denotes the variance; The optimal experimental conditions were presented by A: B

Figure1

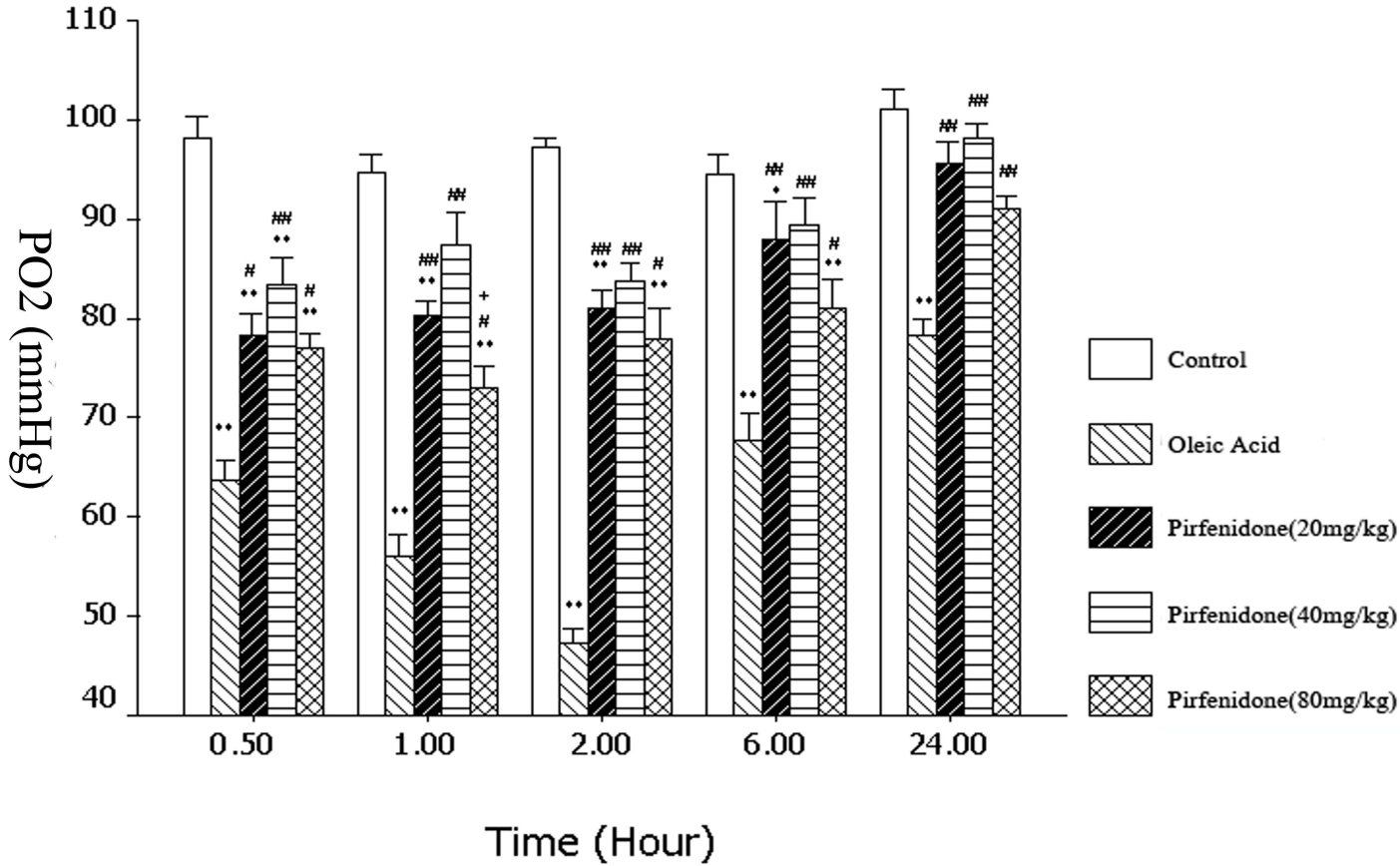

PO2 (mmHg)
110
100
90
80
70
60
50
40
0.50
1.00
2.00
6.00
24.00
Time (Hour)
Control
Oleic Acid
Pirfenidone(20mg/kg)
Pirfenidone(40mg/kg)
Pirfenidone(80mg/kg)

Figure2

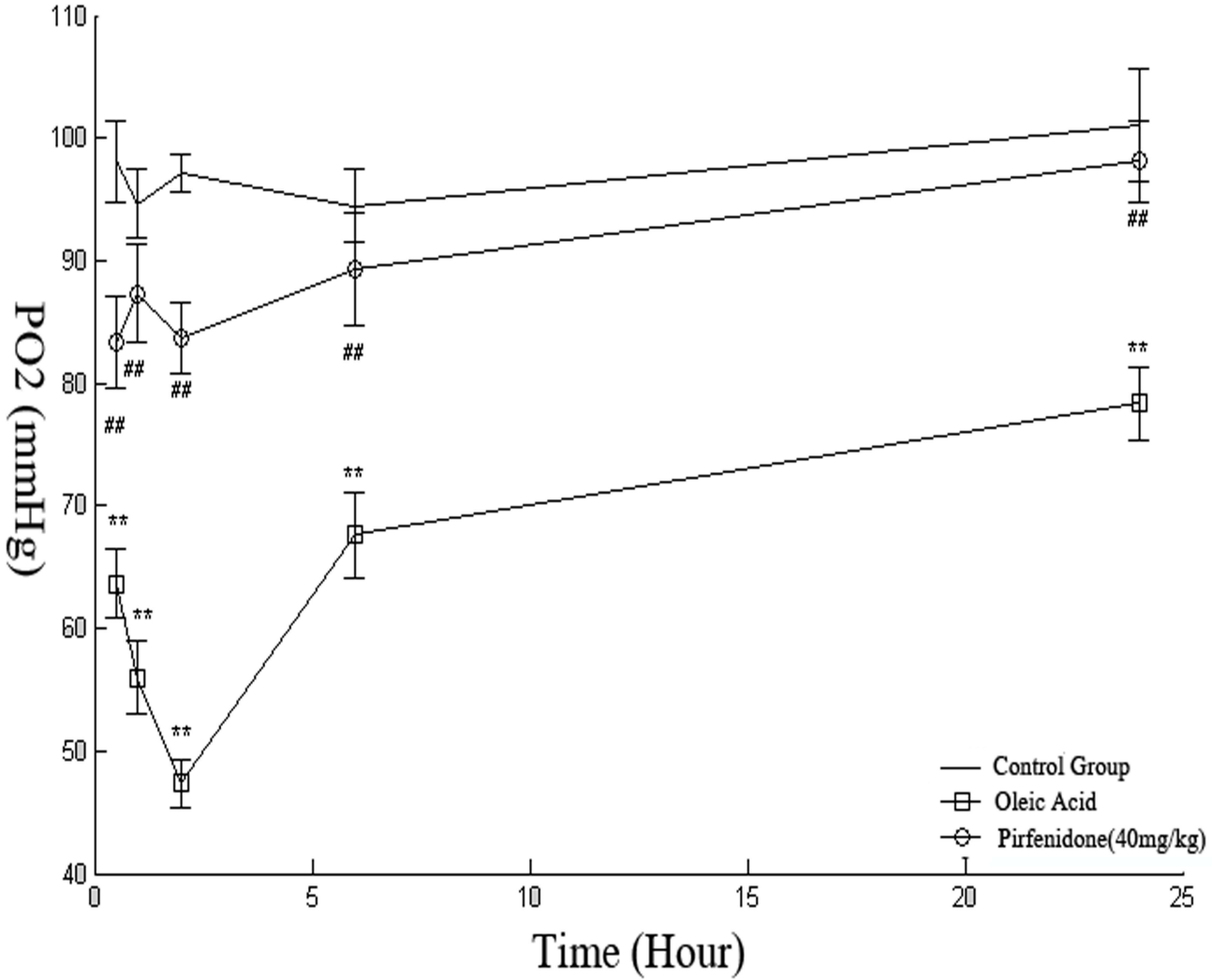

PO2 (mmHg)
Time (Hour)
Control Group
Oleic Acid
Pirfenidone(40mg/kg)
110
100
90
80
70
60
50
40
0
5
10
15
20
25
##
**

Figure3

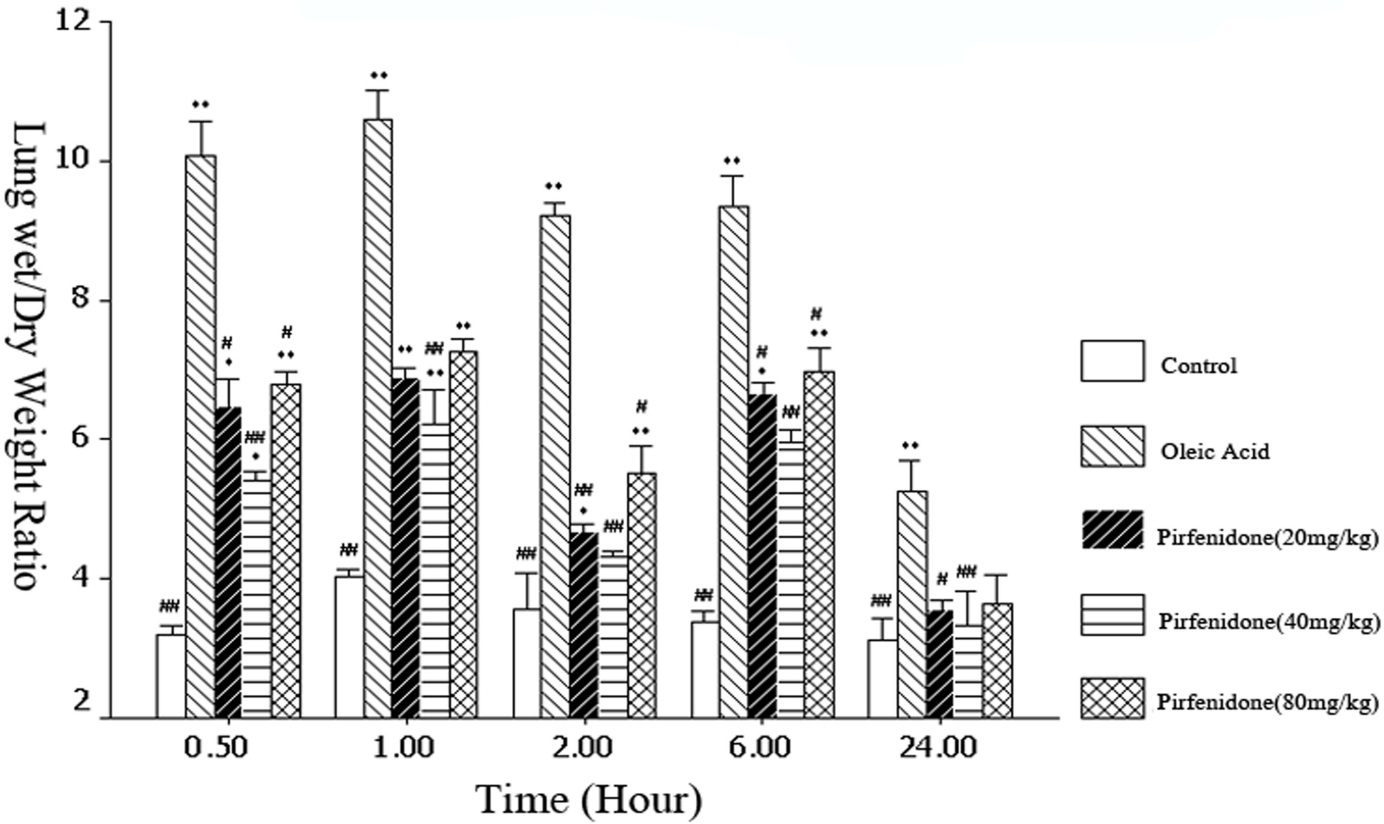

Lung wet/Dry Weight Ratio
12
10
8
6
4
2
0.50
1.00
2.00
6.00
24.00
Time (Hour)
Control
Oleic Acid
Pirfenidone(20mg/kg)
Pirfenidone(40mg/kg)
Pirfenidone(80mg/kg)

Figure4

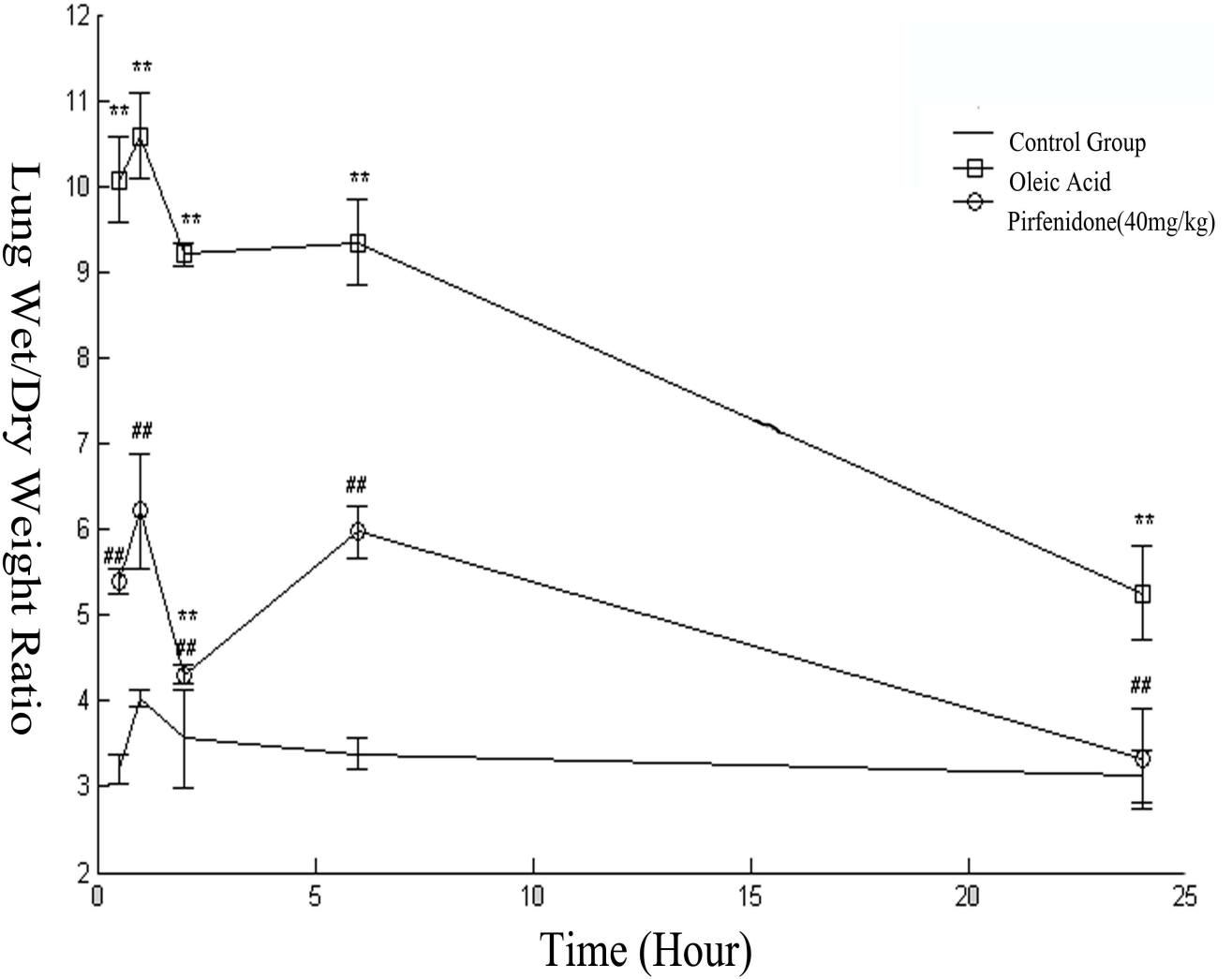

Lung Wet/Dry Weight Ratio
Time (Hour)
Control Group
Oleic Acid
Pirfenidone(40mg/kg)
12
11
10
9
8
7
6
5
4
3
2
0
5
10
15
20
25
**
##

Figure5

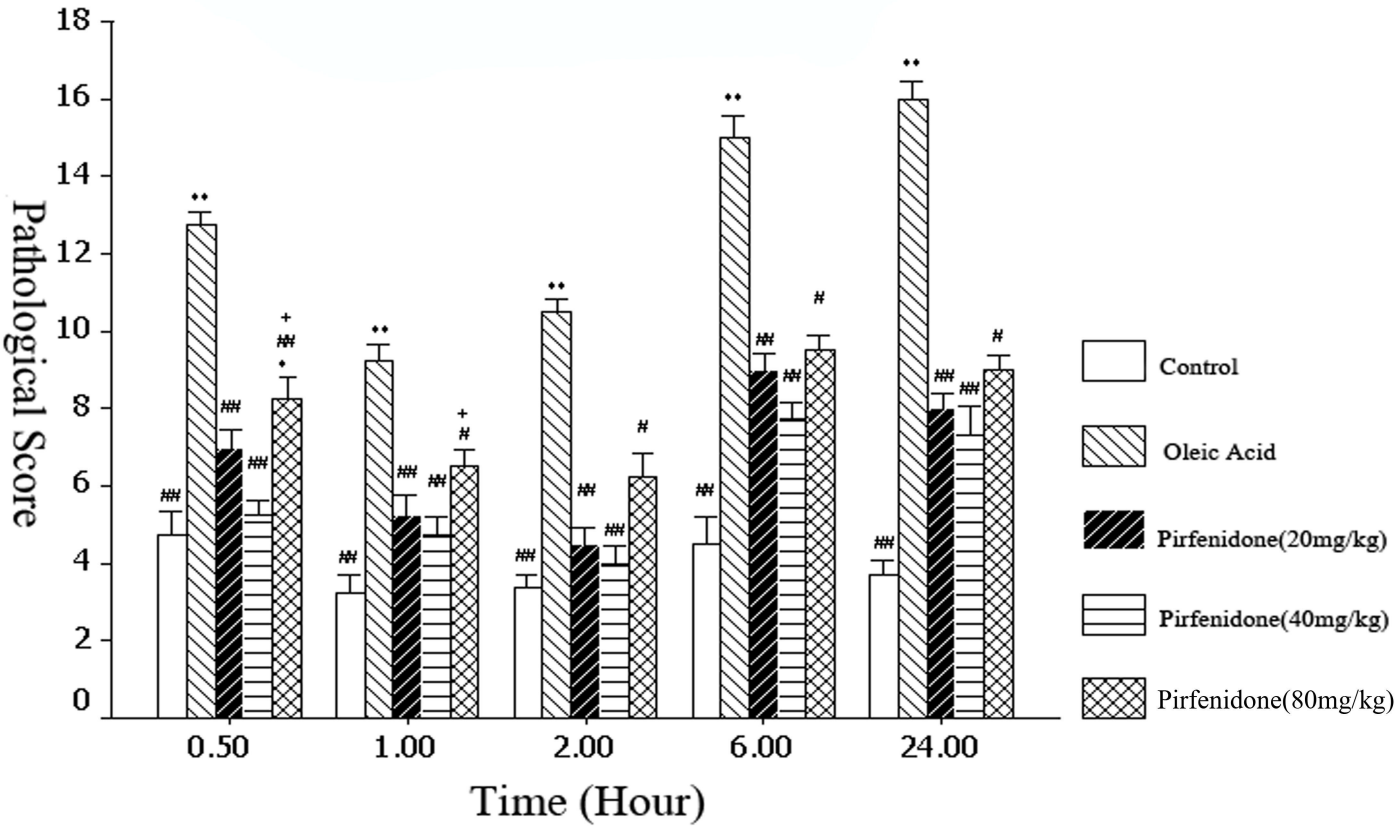

Pathological Score
18
16
14
12
10
8
6
4
2
0
0.50
1.00
2.00
6.00
24.00
Time (Hour)
Control
Oleic Acid
Pirfenidone(20mg/kg)
Pirfenidone(40mg/kg)
Pirfenidone(80mg/kg)

Figure6

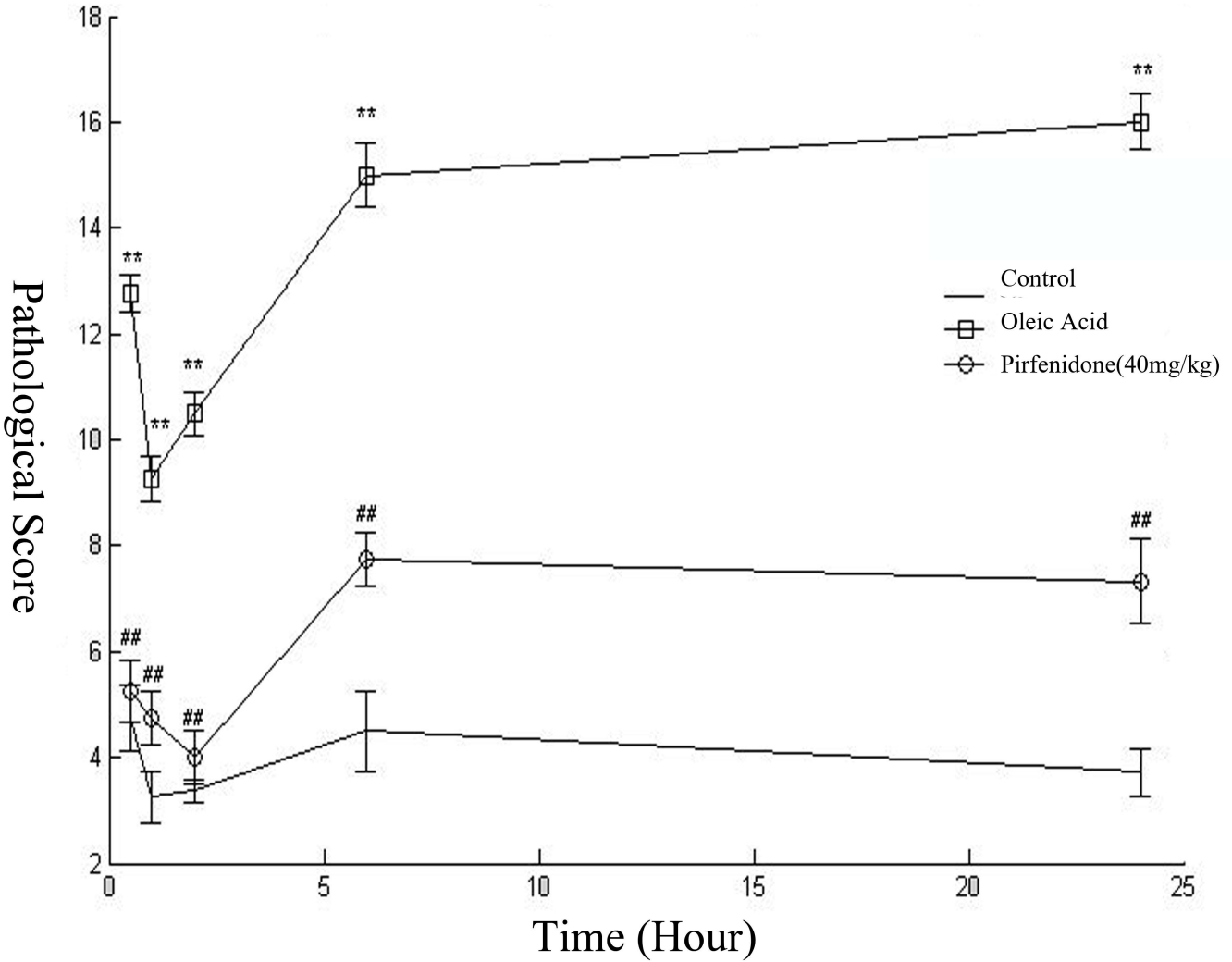

Pathological Score
Time (Hour)
Control
Oleic Acid
Pirfenidone(40mg/kg)
18
16
14
12
10
8
6
4
2
0
5
10
15
20
25
**
##

Figure7

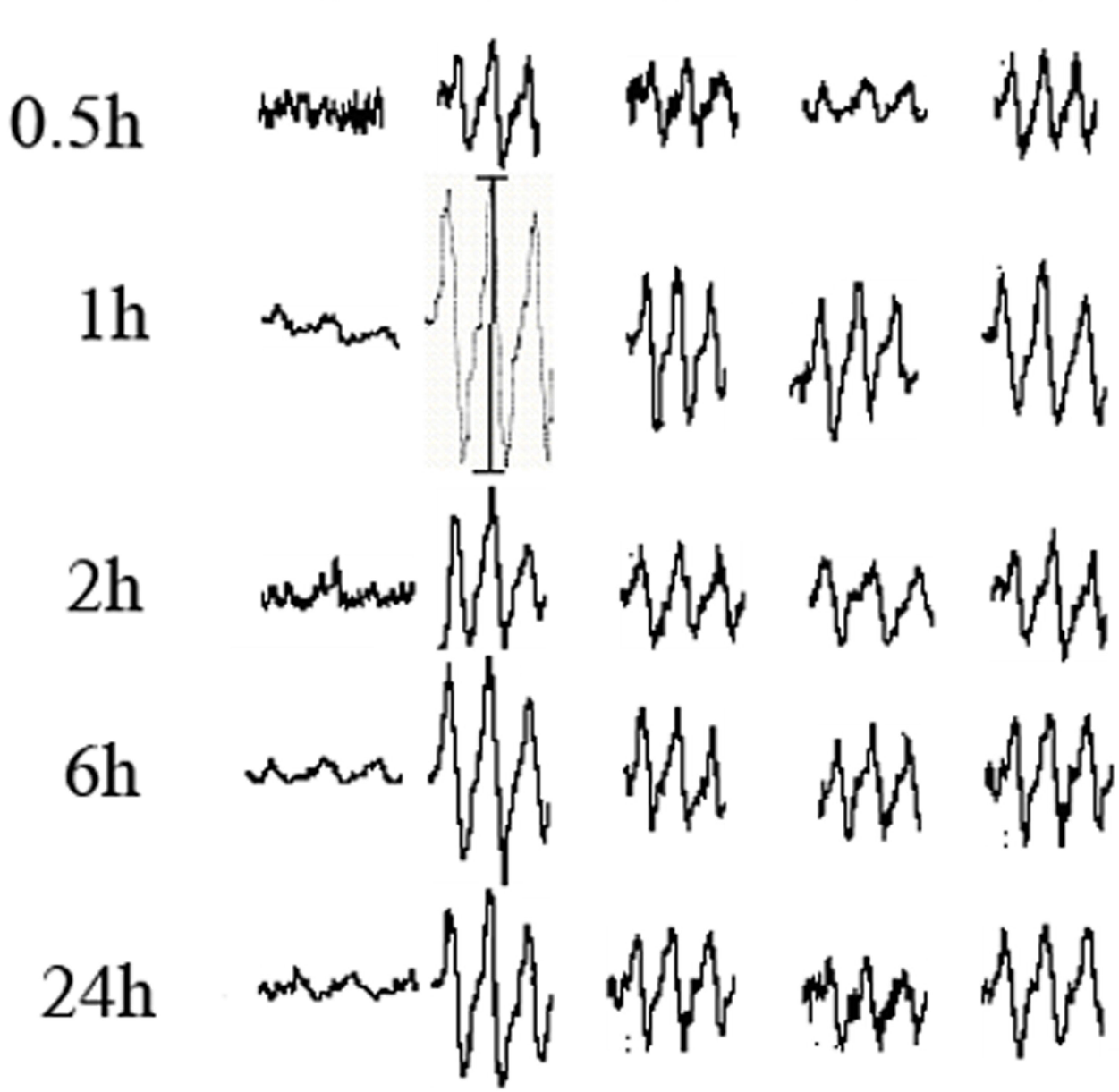


Figure8

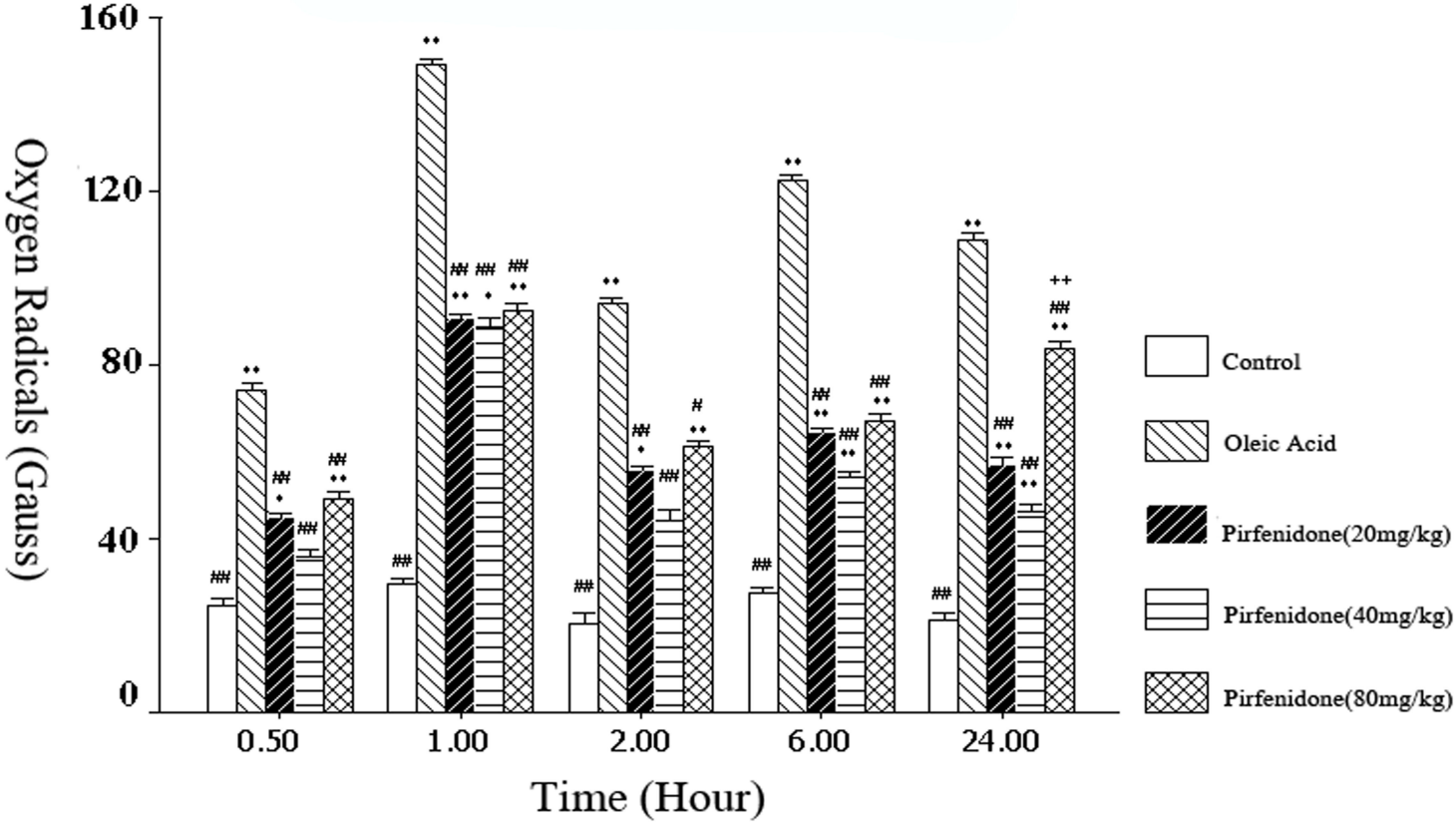
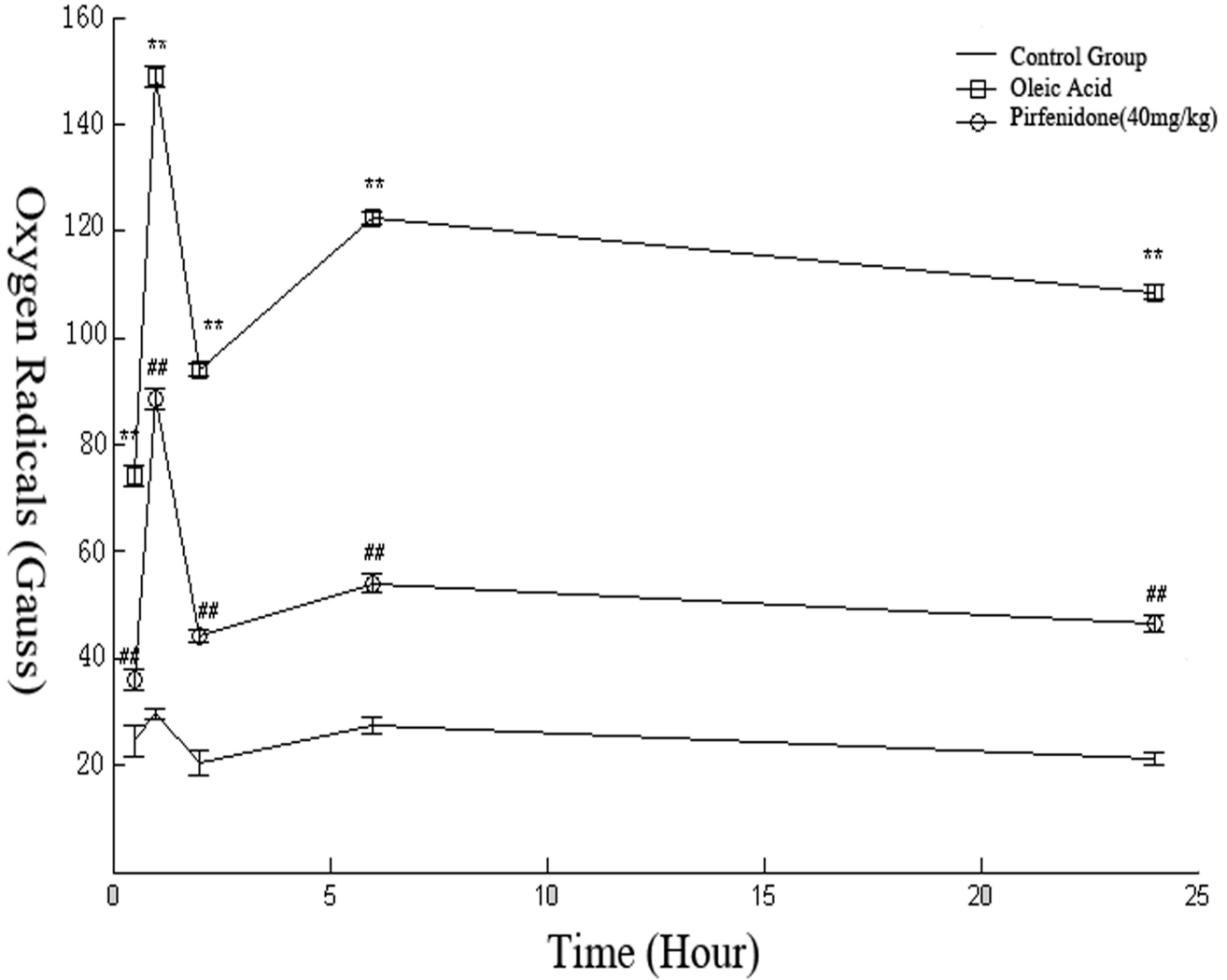

160
120
80
40
0
Oxygen Radicals (Gauss)
0.50
1.00
2.00
6.00
24.00
Time (Hour)
Control
Oleic Acid
Pirfenidone(20mg/kg)
Pirfenidone(40mg/kg)
Pirfenidone(80mg/kg)

Figure9


Control Group
Oleic Acid
Pirfenidone(40mg/kg)
Oxygen Radicals (Gauss)
Time (Hour)
160
140
120
100
80
60
40
20
0
5
10
15
20
25
**
##