Protection of Pirfenidone against an Early Phase of Oleic Acid-Induced Acute Lung Injury in Rats

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ABSTRACT

The potential role of PFD [5-methyl-L-phenyl-2-(1H)-pyridone], an antifibrotic compound with anti-inflammatory effects, in several models of acute lung injury (ALI) has gained increasing attention; however, 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 preconditioning conditions with PFD in ALI. Sprague-Dawley rats were randomized into five groups (five rats per group): normal control group, OA-treated group (0.15 ml/kg), and three PFD-treated groups (20, 40, and 80 mg/kg p.o., respectively). Arterial blood gases, lung wet/dry weight ratio, and postmortem histological changes were determined 0.5, 1, 2, 6, and 24 h after OA challenge. Electron spin resonance spectroscopy was used for free radical detection and measurement. Experiments were examined based on the orthogonal test L4 (4²) setting two factors (PFD dose and PFD valid time) with four different levels. The results of the orthogonal test showed that the sequence of effect of PFD was 0.5 h (oxygen radicals), 1 h (histological changes), 2 h (lung edema), and 6 h (partial pressure of oxygen) after OA challenge, and 40 mg/kg 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 the orthogonal test will be a powerful method to help obtain the optimum experimental conditions with PFD in ALI in the future.

Acute respiratory distress syndrome (ARDS) is a complex syndrome of noncardiogenic edematous acute lung injury characterized by diffuse pulmonary infiltration, increased pulmonary capillary permeability, and severe hypoxemia (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 neutrophil aggregation and activation, release of proteases and lipid mediators, production of free radicals and cytokines, etc. (Repine, 1992). OA-induced lung injury is well known as a model of ARDS (Leeman, 1991). Although 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 (Tampo et al., 1999; Zhang et al., 2000; Yang et al., 2003). The NADPH oxidase-dependent pathway from activated polymorphonuclear leukocytes and endothelial cells (Sanders et al., 1999) contributes 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 neutrophil 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 an 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

ABBREVIATIONS: ARDS, acute respiratory distress syndrome; ALI, acute lung injury; OA, oleic acid; ROS, reactive oxygen species; ESR, electron spin resonance; PFD, 5-methyl-L-phenyl-2-(1H)-pyridone, pirfenidone; PBN, α-phenyl-N-tet-butyl nitrone, PaW₂, partial pressure of carbon dioxide; PaO₂, partial pressure of oxygen.
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 opening 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, although 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, the early course of the disease. PFD [5-methyl-L-phenyl-2-(1H)-pyridone, Deskar; Tocris Cookson Inc., Ellisville, MO], a new experimental drug, was chosen as a therapeutic agent for ALI and ARDS in this study which has shown that PFD could attenuate the severity of experimental ALI by lipopolysaccharide challenge (Cain et al., 1998; Corbel et al., 2001; Oku et al., 2002; Spond et al., 2003) and bleomycin induction (Iyer et al., 1995, 1998, 2000; Schegle et al., 1997; Mansoor et al., 1999).

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.

Materials 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 (250–280 g) 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 Inc.) was dissolved in a 0.4% methylcellulose vehicle solution (0.4% hydroxypropyl methylcellulose in H₂O; Dow Corning, Midland, MI). α-Phenyl-N-tert-butylnitronine (PBN) was purchased from Sigma-Aldrich (St. Louis, MO). OA was purchased from Golden Dragon Chemical Co. (Beijing, China). Diethylenetriaminepentaacetic acid and ethyl acetate were the products of Beijing Chemical Agent Manufactury (Beijing, China), which were at the analytical reagent level.

Study Design. The protocol was divided into five time points (0.5, 1, 2, 6, and 24 h) after the injection of OA. At each time point, there were five groups (five rats per group): 1) control group only received 0.4% methylcellulose vehicle solution, 2) ARDS group administered OA (0.15 ml/kg i.v.) and 0.4% methylcellulose vehicle solution, and 3) three PFD-treated groups (20, 40, or 80 mg/kg p.o., respectively).

![Fig. 1. The effect of PFD on PO₂ with oleic acid induced acute lung injury in rats. Sprague-Dawley rats were randomized into five groups (n = 5 per group): normal control group, oleic acid group (0.15 ml/kg), and three PFD-treated groups (20, 40, or 80 mg/kg p.o., respectively). Oleic acid-induced time points are 0.5, 1, 2, 6, and 24 h, 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 40 mg/kg PFD group.](https://jpet.aspetjournals.org/ asset_upload/1555869746.png)
The animal model of ARDS was verified by blood gas analysis and pathological examination of the lung.

After being anesthetized by inhalation of isoflurane, rats from the ARDS group and PFD-treated groups were injected OA via tail vein, whereas control rats were only administered the same volume of saline intravenously. The rats of the PFD-treated groups were immediately given PFD once (20, 40, or 80 mg/kg p.o.) after OA injection, whereas control rats and ARDS rats 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 (1.0 g/kg). After receiving 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 a Ciba Corning-170 blood gas analyzer (Ciba Corning, Canada).

Lung Wet/Dry Ratio. After the experimental 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 72 h to examine the lung wet/dry weight ratio.

Lung Histopathology. Lung sections from the right lower lobe were fixed into 10% buffered formalin for 24 h. Lung tissues were embedded in paraffin and 4-μm 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 homogenized with 100 mM PBN as a spin trap and 2 mM diethylenetriaminepentaacetic acid. An X-band ESR spectrometer (Varian E-109E; Varian, Inc., Palo Alto, CA) was used to detect the levels of free radicals in lung tissues directly with measurement conditions as: X-band, central magnetic field (3445 G), scan width (200 G), microwave power (20 mW), frequency (100 kHz), modulation amplitude (2.5 G), and time constant (0.128 s) at 37°C (Capani et al., 2001). Before measurement, ESR spectrometer was standardized with diphenyl-picri-hydrazyl to make sure that the equipment was in the same condition in every time experiment.

Statistical Analyses. Statistical analyses were performed by SPSS 11.5 software, and the results were presented as mean ± S.D. Continuous parametric data were subjected to analysis of variance followed by the Student-Newman-Keuls post hoc test for between-groups difference. A Kruskal-Wallis test was used to detect the lung injury score differences among groups. A p value ≤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 (42) (Wang et al., 2004) setting two factors (PFD dose and PFD valid time) with four different levels of each (Table 1).

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 80 mg/kg PFD group died, respectively, 24 h after injection. At 6 h, 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. Figure 1 showed that hypoxemia was much more evident in the ARDS group than in the control group after injection of OA, especially 2 h after OA injection.
injection. The changes of PO₂ corresponding with the 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. PO₂ in the PFD groups, especially the 40 mg/kg group, was significantly more elevated than in the OA group (Fig. 2). There was no difference in effect on ALI among three PFD-treated groups, except for the 80 mg/kg group 1 h after OA challenge. PaCO₂ and pH did not change among different groups at each time point (PaCO₂ 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 in the control group. The summit of lung wet/dry weight ratio in the OA group was found 1 h after OA injection (Fig. 3). Lung wet/dry weight ratio in the PFD groups, especially the 40 mg/kg group, was significantly lower than that in the OA group (Fig. 4) at all time points.

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<th>Level</th>
<th>PFD Dose (mg/kg)</th>
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**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 Figs. 7 and 8. The peak height of spectrum represented relative concentration of oxygen radicals. The result showed that no signal of oxygen radicals could be seen in normal lung tissues. The significant release of oxygen radicals was measured 1 and 6 h after intravascular injection of OA. Free radical levels in the OA group enhanced considerably over the control group at each time point. Free radicals in the PFD-treated groups (especially the 40 mg/kg group) were sharply lower than those in the OA group at all time points (Fig. 9). There was no difference in effect on ALI among PFD-treated groups, except for the 80 mg/kg group 24 h after OA challenge.

**Lung Histopathology.** The differences were illustrated in histological sections (Supplemental Data). 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 0.5 h after injection of OA and were most serious 24 h after OA challenge. In PFD groups (especially the 40 mg/kg group), these changes and pathologic score (Figs. 5 and 6) were far less marked than those in the OA group. There was no difference in effect on ALI among the three-dose PFD-treated groups.

**TABLE 1**

Factors and levels of orthogonal design L⁴(4²) with pirfenidone

Experiments were examined based on the orthogonal test L⁴(4²) setting two factors (PFD dose and PFD valid time) by four different levels for obtaining the optimum conditions.

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<th>Level</th>
<th>PFD Dose (mg/kg)</th>
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Fig. 3. 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 five groups (five rats per group): normal control group, oleic acid group (0.15 ml/kg), three PFD-treated groups (20, 40, or 80 mg/kg p.o., respectively). Oleic acid-induced time points are 0.5, 1, 2, 6, and 24 h, 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.
Fig. 5. The effect of PFD on the pathologic score with oleic acid-induced acute lung injury in rats. Sprague-Dawley rats were randomized into five groups (five rats per group): normal control group, oleic acid group (0.15 ml/kg), and three PFD-treated groups (20, 40, or 80 mg/kg p.o., respectively). Oleic acid-induced time points are 0.5, 1, 2, 6, and 24 h, 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.

Fig. 4. Comparison of lung wet/dry weight ratio among the oleic acid group, the 40 mg/kg PFD group, and the control group. The best experimental dose of PFD was 40 mg/kg b.wt. ***, p < 0.01 versus the control group. ###, p < 0.01 versus the oleic acid group.
Orthogonal Test. Tables 2 and 3 showed that the best experimental pharmacological dose of PFD was 40 mg/kg b.wt. The time sequence of the most outstanding pharmacological effects of PFD was reducing free radical contents in lung tissues at 0.5 h, improving pathological changes at 1 h, alleviating lung edema at 2 h, and raising PaO2 levels 6 h after OA challenge.

Discussion

It is well known that ARDS is a serious disease with a high mortality, although extensive supportive treatments were given. Because ROS is 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 (Morcillo et al., 1999; MacNee, 2001). Therefore, we examined the utility of PFD to inhibit oxygen radicals in an OA-induced ARDS model. Meanwhile, we first used ESR spectroscopy in ARDS to measure ROS directly in OA-induced rat models 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-hydroperoxide), 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 1 and 6 h after OA exposure provides strong evidence that oxidative stress could be involved in lung injury during OA-induced ARDS. The comparison between ESR spectra and blood gas analysis results (PO2) indicates that the change of ROS was earlier than that of pathophysiology because the concentration of ROS increased significantly 1 h after OA exposure and began to decrease at 2 h, whereas the reduction of PO2 started from 2 h. Nevertheless, our results by ESR are a confirmation of previous studies (Tampo et al., 1999; Zhang et al., 2000; Yang et al., 2003) which support the critical role of free radicals in OA-induced lung injury.

The data from this study demonstrates that PFD reduces the severity of experimental lung injury by several physiological gravimetric and histological criteria. We propose that

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it was due to the association between PFD and the decrease of oxygen radicals in this study. First, we directly found that PFD decreased oxygen radicals in ALI by an X-band ESR spectrometer. The free radicals in PFD-treated groups, especially the 40 mg/kg group, were sharply lower than that in the OA group at all time points. Second, previous reports (Iyer et al., 1998; Misra and Rabideau, 2000) show PFD suppresses free radicals through directly scavenging and indirectly modulating activities of pro-oxidant and antioxidant enzymes. Third, the sequence of effect of PFD is 0.5 h (oxygen radicals), 1 h (histology), 2 h (lung edema), and 6 h (PO₂) after OA challenge by the orthogonal test, which indicates the decrease of the 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 (Tampo et al., 1999; Zhang et al., 2000; Yang et al., 2003). 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 pro-oxidant and antioxidant enzymes. PFD can alleviate 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 β1 levels (Corbel et al., 2001) because it is reported (Arsalane et al., 1997) that transforming growth factor 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). 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-κB 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 cell recruitment and activation in the lung, lung parenchymal cell 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-κB (Tsuchiya et al., 2004) and nuclear factor-κB induced several cytokines such as tumor necrosis factor-α relevant to ARDS pathology (Iyer et al., 2000; Corbel et al., 2001; Oku et al., 2002; Spond et al., 2003), 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 (10 and 30 mg/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 (30 mg/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 80 mg/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 these groups, the dose of PFD was in the effective

### Table 3

Variance analysis of orthogonal test

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<td>A2:B2</td>
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<td>PO2</td>
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**Fig. 8.** The effect of PFD on oleic acid induced acute lung injury in rats. Sprague-Dawley rats were randomized into five groups (five rats per group): normal control group, oleic acid group (0.15 ml/kg), three PFD-treated groups according to the doses divided (20, 40, or 80 mg/kg p.o., respectively). Oleic acid-induced time points are 0.5, 1, 2, 6, and 24 h, 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 40 mg/kg PFD group.
dose range. PFD in the 80 mg/kg group, however, had slightly protective effects on ALI except PO2 1 h and oxygen radicals 24 h after OA challenge are near 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. 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 the above conclusion that the best experimental dose of PFD is 40 mg/kg b.wt. Because PaCO2 and PH did not change among PFD-treated groups, there are no changes in blood acid base status. Similar changes in PaCO2 and PH have been observed in patients and rats after PFD treatment (Mansoor et al., 1999; Nagai et al., 2002). Our results in rats suggest PFD also has no other adverse effects within the dose range observed that was consistent with the previous reports in animals and humans (Mansoor et al., 1999; Gahl et al., 2002; Giri et al., 2002; Nagai 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 (Giri et al., 2002; Nagai et al., 2002). We think the findings that a rat in the 80 mg/kg PFD group died 24 h 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 effects.

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 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. Because 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 in which the treatment with PFD at the early phase of ALI may help prevent lung tissue impedance and ameliorate symptoms of lung edema and hypoxemia. The treatment also can avoid the side effects related to prolonged and high doses of steroids. Although these initial results were obtained from limited data, the results of this study show that an 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.

Acknowledgments

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