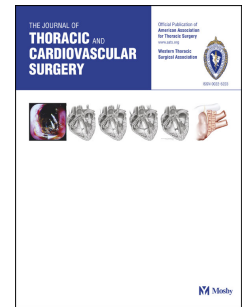


Accepted Manuscript

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PII: S0022-5223(18)32493-0

DOI: [10.1016/j.jtcvs.2018.08.098](https://doi.org/10.1016/j.jtcvs.2018.08.098)

Reference: YMTC 13446

To appear in: *The Journal of Thoracic and Cardiovascular Surgery*

Received Date: 7 May 2018

Revised Date: 27 August 2018

Accepted Date: 29 August 2018

Please cite this article as: Saito M, Chen-Yoshikawa TF, Suetsugu K, Okabe R, Takahagi A, Masuda S, Date H, Pirfenidone alleviates lung ischemia-reperfusion injury in a rat model, *The Journal of Thoracic and Cardiovascular Surgery* (2018), doi: <https://doi.org/10.1016/j.jtcvs.2018.08.098>.

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Pirfenidone alleviates lung ischemia-reperfusion injury in a rat model

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Conflict of interest: Pirfenidone was provided by Shionogi & Co., Ltd.

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Word Count: (3382/3500)

Abbreviations: IRI (ischemia-reperfusion injury), PFD (pirfenidone), WI (warm ischemia), W/D ratio (wet to dry weight ratio), PGD (primary graft dysfunction), IPF (idiopathic pulmonary fibrosis), FiO₂ (fraction of inspiratory oxygen), ssDNA (single strand DNA), HPFs (high power fields), PCR (polymerase chain reaction), TNF- α (tumor necrosis factor-alpha), mRNA (messenger RNA), IL-1 β (interleukin-1 beta), MPO (myeloperoxidase), NF- κ B (nuclear factor-kappa B), cDNA (complementary DNA), RQ (relative quantity), GAPDH (glyceraldehyde 3-phosphate dehydrogenase), UPLC-MS (ultra performance liquid chromatography-mass spectrometry)

Central picture

Pirfenidone alleviates lung ischemia-reperfusion injury in a rat model.

Central message

Pirfenidone attenuated deterioration of pulmonary function by suppressing inflammation in lung ischemia-reperfusion injury.

Perspective message

36 Administration of pirfenidone before ischemia-reperfusion injury alleviated lung edema
37 and inflammation in the acute phase. Pirfenidone may be useful as a new option for
38 treatment of lung IRI.

Abstract

Objective: Lung ischemia-reperfusion injury (IRI) is one of the complications after lung transplantation resulting in morbidity and mortality. Pirfenidone (PFD), an anti-fibrotic agent for the treatment of idiopathic pulmonary fibrosis, is reported to have cytoprotective properties in various disease models. The purpose of this study was to investigate the effect of PFD on lung IRI.

Methods: Male Lewis rats (260-290g) were divided into 3 groups: sham group (n=5), warm ischemia (WI) group (n=10), and WI plus PFD (WI+PFD) group (n=10). The sham group underwent 210 minutes of perfusion without ischemia. The WI and WI+PFD groups underwent 90 minutes of warm ischemia and 120 minutes of reperfusion. In the WI+PFD group, PFD (300mg/kg) was administered orally by gavage 30 minutes prior to ischemia. After reperfusion, arterial blood gas analysis, lung mechanics, lung wet-to-dry weight (W/D) ratio, and histological findings were obtained. The gene expression of pro-inflammatory cytokines in lung tissue was measured by quantitative reverse transcription polymerase chain reaction.

Results: Compared to the WI group, the WI+PFD group had significantly better dynamic pulmonary compliance ($p<0.01$) and oxygenation levels ($p<0.05$). W/D ratio was lower in the WI+PFD group ($p<0.05$). Histological analysis showed that the

WI+PFD group had reduced perivascular edema and neutrophil infiltration. The expression of TNF- α mRNA was decreased in the WI+PFD group ($p<0.05$).

Conclusion: Our results revealed that in a rat hilar clamp model, PFD alleviated lung ischemia-reperfusion through anti-inflammatory effects.

(231/250 words)

Introduction

Lung ischemia-reperfusion injury (IRI) is characterized by nonspecific alveolar damage, lung edema, and hypoxemia that can occur in several clinical situations, such as lung transplantation,¹⁻³ pulmonary embolism,⁴ cardiopulmonary bypass cardiac surgery,⁵ single lung ventilation,⁶ and high volume resuscitation.⁷ Particularly in lung transplantation, IRI is the main cause of primary graft dysfunction (PGD), which is a major cause of mortality and morbidity in the post-operative period.⁸ Furthermore, lung IRI is associated with an increased risk of chronic lung allograft dysfunction.⁹ Therefore, it is important to alleviate lung IRI to improve the survival rate after lung transplantation.

Pirfenidone (PFD) is an anti-fibrotic agent used for idiopathic pulmonary fibrosis (IPF) and IPF is one of the diseases for which lung transplantation is performed.¹⁰ Thus, PFD is often taken orally in pre-transplant patients with severe IPF. The potential anti-inflammatory role of PFD has been well documented in various studies.^{11, 12} In addition, it has been already reported that PFD attenuated IRI in other organs (small intestine¹³ and liver¹⁴). However, the effect of PFD on lung IRI is unknown. The purpose of this study was to investigate the effect of PFD on lung IRI in a rat model.

Materials and Methods

Animals

All experimental protocols received approval from the Ethics Committee of the Graduate School of Medicine at Kyoto University.

We used male Lewis rats, 11 weeks-old, weighing 260-290g (Japan SLC, Hamamatsu, Japan).

Chemicals

PFD for administration to the animal was provided by Shionogi & Co., Ltd. (Osaka, Japan).

PFD for concentration measurement was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan) and carbamazepine was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals were of the highest purity available.

This study consisted of 2 experiments (Experiment 1 and Experiment 2). Experiment 1 was to investigate the function and inflammatory change in the lung after ischemia-reperfusion injury. Experiment 2 was to investigate concentrations of PFD in blood and lung tissue.

Experiment 1

Rat left hilar clamp model

Rats were randomly assigned to 3 groups: sham group (n=5), warm ischemia (WI) group (n=10), and WI plus PFD (WI+PFD) group (n=10). PFD (300 mg/kg) dissolved with 1ml of 0.5% carboxymethyl cellulose was administered orally by gavage in the WI+PFD group, and 1 ml of 0.5% carboxymethyl cellulose was administered in both the sham group and the WI group.

All rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (120 mg/kg). Tracheotomy and mechanical ventilation were performed with a tidal volume of 10 ml/kg, a respiratory rate of 70 breaths/min, a positive end-expiratory pressure of 2 cmH₂O and a fraction of inspiratory oxygen (FiO₂) of 1.0. Heparin (50 IU) was injected through the jugular vein, then a left thoracotomy at the fifth intercostal space was performed.

The sham group underwent 210 min of perfusion without ischemia. In the WI groups, the left pulmonary hilum was occluded with a vascular clamp to induce warm ischemia 30 minutes after PFD or vehicle administration. During clamping of the left hilum, the tidal volume was adjusted to 6 ml/kg. After 90 minutes of ischemia, the clamp was

removed to begin reperfusion for 120 minutes and the tidal volume recovered to 10

ml/kg.

Lung mechanics measurements

After 120 minutes of reperfusion, a median sternotomy was performed. The right pulmonary hilum was occluded with a vascular clamp, and the left lung was ventilated with a tidal volume of 5 ml/kg for five minutes. Then, an arterial blood sample was obtained through the ascending aorta. After that, the rat was connected to a flexiVent (SCIREQ, Montreal, Quebec, Canada) to measure the left pulmonary function. We measured the compliance, mean airway pressure, and peak airway pressure by alternating perturbations of the single forced oscillation families in a closely spaced manner (SnapShot).

Lung wet-to-dry weight ratio

After measurement of pulmonary function, the left lung was harvested to obtain tissue samples, and divided into three parts.

The upper part was used to calculate the lung wet-to-dry weight (W/D) ratio. The wet weight was measured soon after harvesting, and the dry weight was measured after 24 hours at 100 °C. The ratio was calculated as wet weight divided by dry weight.

Pathological evaluation

The middle part of the left lung was fixed in 10% formalin and stained with hematoxylin-eosin. Naphtol AS-D chloroacetate esterase staining was used to count neutrophil infiltration into the perivascular area, as previously reported.¹⁵ For immunohistochemical staining to detect the apoptotic cells, the Avidin-Biotin complex method was used. After deparaffinization and blocking with normal bovine serum, the section was incubated with a primary antibody (anti-single strand DNA (ssDNA) antibody) and then with a biotin labeled secondary antibody in a standard manner. The number of red blood cell was expressed by the average number in 5 randomly chosen hi-power fields (HPFs) per section at a magnification of 400. The number of neutrophils was expressed by the average number in 5 randomly chosen HPFs per section at a magnification of 400x. The number of ssDNA positive cells, as apoptotic cells, was expressed by the average number in 5 randomly chosen fields at a magnification of 200x. Three separate investigators evaluated in a blinded manner. The degrees of

bleeding and pulmonary edema were divided into 4 grades ranging from - to +++, and the three values were averaged.

Quantitative real-time polymerase chain reaction (PCR)

The lower part of the left lung was obtained for quantitative real time PCR to evaluate gene expression of tumor necrosis factor alpha messenger RNA (TNF- α mRNA), interleukin-1beta mRNA (IL-1 β mRNA), myeloperoxidase mRNA (MPO mRNA), and nuclear factor kappa B subunit 1 mRNA (NF- κ B mRNA). The tissue was stabilized using RNeasy Lysis Buffer (QIAGEN, Hilden, Germany) and RNA was extracted using an RNeasy Plus Mini Kit® (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. The total RNA concentration was determined by spectrophotometer and the quality was evaluated with a 260/280 nm ratio (1.8-2.0). Then, the total RNA was reverse transcribed to complementary DNA (cDNA) using Ready-To-Go You-Prime First-Strand Beads® (GE Healthcare, Pittsburgh, PA). Each cDNA was diluted to 10 μ g/ μ l, and 2 μ l of cDNA was mixed with the Thunderbird® probe qPCR Mix (Toyobo, Osaka, Japan) and TaqMan Gene Expression Assay probe set (Thermo Fisher Scientific, Waltham, MA). The PCR was performed on the StepOnePlus™ Real-time PCR system (Thermo Fisher Scientific, Waltham, MA). The relative quantity (RQ) was calculated

using the comparative $\Delta\Delta\text{CT}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene and the samples of the sham group were used as controls. Analysis of each sample was run in triplicate. The probes used for PCR were as follows: GAPDH (NM_017008), TNF- α mRNA (NM_012675), IL-1 β mRNA (NM_031512), MPO mRNA (NM_001107036), NF- κ B mRNA (NM_1276711).

Experiment 2

Concentration of PFD in rat hilar clamp model

PFD administration (300 mg/kg), 90 minutes clamping of the left hilum, and 120 minutes of reperfusion were performed as described in experiment 1 (n=10).

To confirm the concentration of PFD in the model, the ultra-performance liquid chromatography mass spectrometry (UPLC-MS) method was used. In this experiment, a blood sample was collected through the inferior vena cava, and the lungs were harvested at 30 minutes after PFD administration and 120 minutes after reperfusion (n=5, in each condition).

Lung weight was measured with an analytical balance (Shimadzu Corp., Kyoto, Japan) before concentration measurement. Next, the lungs were added saline to make total 10

times volumes and homogenized with a Polytron PT 1300 D (KINEMATICA AG,

Luzern, Switzerland).

Fifty microliters of collected plasma and the lung homogenized samples followed by the

addition of 100 μ L of methanol with the internal standard (IS) working solution

(carbamazepine 500 ng/mL) were used for measurement. After vortex-mixing for 1 min,

the sample was centrifuged at 13,000 rpm for 8 mins. The supernatant was injected into

an ACQUITY UPLC system (Waters Corp., Milford, MA, USA) for analysis. The

standards were prepared in the same way.

The calibration curves were constructed in the concentration range of 5-2,000 ng/mL

and samples exceeding 2,000 ng/mL were measured again with the appropriate dilution.

Chromatographic separation was achieved on an ACQUITY UPLC system with an

Acquity BEH C18 column, 2.1 mm $\times$ 50 mm, 1.7 μ m particle size column (Waters

Corp., Milford, MA, USA) maintained at 30°C. A gradient program was employed with

the mobile phase combining solvent A (0.1% formic acid in water) and solvent B

(methanol) as follows: 30-90% B (0-1.9 min), 90-30% B (1.9-2.0 min). A 1-minute

period of re-equilibration was allowed before the next injection. The flow rate was 0.4

mL/min. PFD and the IS were detected with an ACQUITY QDa Detector (Waters Corp.,

Milford, MA, USA) using positive ion electrospray ionization. The detected m/z was

186.2 for PFD and 237.1 for carbamazepine (IS). Data analyses were performed with

EmpowerTM3 software (Waters Corp., Milford, MA, USA).

Statistical analysis

All values are presented as the mean $\pm$ the standard error. Analyses were performed

using JMP pro 12.2.0 software (SAS Institute, Cary, NC). One-way analysis of variance

(ANOVA) was used to compare the means of the three groups and a two-tailed

Dunnett's t-test was then performed to determine difference. We also performed

sensitivity analysis using Kruskal-Wallis test to make sure the results of ANOVA. The

correlations between plasma concentration and lung tissue concentration of PFD were

calculated with the Pearson product moment correlation analysis. A p-value < 0.05 was

considered statistically significant.

Results

Pulmonary function of the left lung

The results of the lung function analyses are shown in Fig. 1.

Dynamic pulmonary compliance was significantly higher in the WI+PFD group than that in the WI group ($p<0.01$, Fig. 1A). Airway pressure was lower in the WI+PFD group ($p<0.05$, Fig. 1B-C). The WI+PFD group had better oxygenation levels ($p<0.05$, Fig 1D).

Histological findings

The left lungs harvested from the sham group showed almost normal histology. In contrast, those from the WI group showed severe perivascular edema, alveolar wall swelling and hemorrhage into the alveolar area. These findings were ameliorated in the WI+PFD group, and the W/D ratio was significantly lower in the WI+PFD group compared to the WI group ($p<0.05$, Fig. 2A-D). We measured the perivascular cuff area in histologic sections of lungs isolated from five sham (15 vessels), 10 WI (30 vessels), and 10 WI+PFD (30vessels) animals. We calculated the index of perivascular cuff area/vessel area to eliminate variations due to vessel size. The index was significant lower in the WI+PFD group compared to the WI group ($p<0.01$, Fig 2E).

The number of red blood cells in alveolar space was expressed by the average number in 5 randomly chosen high-power fields (HPFs) per section at a magnification of 400 using dynamic cell count (BZ- II analyzer, Keyence, Japan). The number was significant lower in the WI+PFD group compared to the WI group ($p<0.05$, Fig 2F).

The number of neutrophil infiltrations into the perivascular area was markedly increased in the WI group as compared to the sham group. Neutrophil infiltration was significantly lower in the WI+PFD group compared to the WI group ($p<0.05$, Fig. 3).

Immunostaining for ssDNA showed the number of apoptotic cells was lower in the WI+PFD group compared to the WI group ($p<0.05$, Fig. 4).

Gene expression of TNF- α , IL-1 β , and NF- κ B in the lung tissue

Gene expression of TNF- α mRNA was significantly reduced in the WI+PFD group compared to the WI group ($p<0.05$, Fig. 5A). Although not significant, the expression of IL-1 β mRNA and MPO mRNA were lower in the WI+PFD group ($p=0.15$, $p=0.19$, respectively. Fig. 5B, 5C).

In the WI group, the expression of NF- κ B mRNA was increased by 1.7-fold compared to the sham group. However, in the WI+PFD group, the expression was only increased

by 1.2-fold compared to the sham group, and there was a significant difference between

the WI+PFD group and the WI group ($p<0.05$, Fig. 5-D).

Concentration of PFD in plasma and lung tissue

The PFD concentrations in plasma (μ g/ml), right lung (μ g/lung g), and left lung (μ g/lung g) at 30 minutes after administration were 32.9 ± 10.8 , 34.0 ± 10.5 , and 30.8 ± 8.60 , respectively. At 120 minutes after reperfusion, they were 19.0 ± 4.32 , 17.3 ± 3.90 , 16.6 ± 3.02 , respectively. The bilateral lung concentrations were strongly correlated with plasma concentrations (right lung, $r=0.99$, $p<0.01$; left lung, $r=0.98$, $p<0.01$).

Comment

In the present study, we demonstrated that administration of PFD in lung IRI improved pulmonary function, oxygenation level, pulmonary edema, and neutrophil infiltration. Furthermore, PFD decreased the number of apoptotic cells and pro-inflammatory cytokines in lung tissue.

There have been some reports that PFD suppresses fibrosis due to rejection and inflammatory change in the chronic or subacute phase after lung transplantation,¹⁶⁻¹⁸ but there are no reports of the acute phase, especially within 72 hours, after transplantation.

In this study, it was shown that administration of PFD was effective prior to the onset of lung IRI, which is the main cause of acute lung injury after lung transplantation. Liu H reported that PFD inhibit lung fibrosis through decreasing TGF- β and arginase activity at 21 days after lung transplantation, but they did not evaluate acute lung injury.¹⁸ In our study, we focused on lung IRI with administration of PFD. The results clearly showed that the lung IRI was alleviated from the acute phase by PFD through inhibition of TNF- α and NF- κ B.

In clinical practice PFD is used for the treatment of IPF. Some studies have reported that it is possible to reduce the incidence of acute exacerbation of IPF associated with surgery by orally administering PFD before and after surgery.^{19, 20}

The molecular target of PFD has yet to be identified. Previous reports have suggested direct inhibition of TNF- α and reduction of inflammatory cytokines via the NF- κ B pathway. NF- κ B is known as a crucial transcription factor of the inflammatory response. Oxidation by reactive oxygen species causes the release of the inhibitory subunits of the NF- κ B inhibitor and activation of NF- κ B, which generate pro-inflammatory signaling.²¹ In this study, PFD decreased NF- κ B mRNA in the lung tissue as previously reported and it had a protective effect on acute lung injury. Moreover, NF- κ B and TNF- α have an effect on cell survival.^{22, 23} The present study showed that PFD significantly decreased both of these and reduced apoptotic cells in the immunohistochemical staining of ssDNA on lung IRI. The staining with anti-ssDNA antibody was a specific and sensitive procedure for the detection of apoptotic cells compared to TUNEL staining.²⁴ In experiment 2, our results demonstrated that administration of PFD orally by gavage in a rat IRI model increased concentration of PFD in blood and lung tissue within the experimental period.²⁵⁻²⁷ Several limitations of this study should be acknowledged. Pirfenidone had a protective effect on lung IRI in a rat hilar clamp model, but our findings cannot be directly applied to human lung transplantation at the present stage. We chose the hilar-clamp model in this study for two reasons. First, IRI occurs in many clinical settings and a

clamp/vascular occlusion model has been utilized in the study of IRI in other organs such as kidney, liver and intestine.^{13, 14, 28-30} Second, the hilar clamp model is a simpler and more consistently reproducible procedure when compared to a transplant model. For a proof-of-concept experiment examining PFD, the clamp model was employed to limit variability in IRI.

However, the hilar-clamp model differs from clinical lung transplantation in three aspects. The first is the difference in ischemic condition. In this experiment, reperfusion occurs after warm ischemia alone. In contrast, lung transplantation involves a period of cold ischemia followed by warm ischemia of the graft. Second, in clinical lung transplantation, there is no PFD present in the allograft prior to implantation during cold ischemia. Recipients taking PFD pre-transplant will have lower circulating levels of the drug given that the last dose might be nearly 24 hours prior to transplant. And so the reperfused donor lung will be exposed to zero to low doses of recipient-derived PFD. In this study, it cannot be distinguished whether PFD in the donor lung is effective during ischemia or PFD in the blood and/or donor lung is effective for the reperfusion. Furthermore, in this study, we had only set one dose (300 mg/kg) for the PFD group, but by using pre-surgical administration and titration of dosage, we could get useful information for clinical use in lung transplantation. In addition, because it is assumed

that in lung transplantation it will only be administered to the recipient, it is necessary to

consider using the transplant model with administration of PFD only in the recipient.

Third, the response of the immune system of rats is different from that of humans. We

need to confirm our findings in other animal models before applying these results to

humans.

This was an observational study of the effects of PFD and we did not clarify the drug's

mechanism. Further investigation will be necessary to define the mechanism associated

with the protective effect of pirfenidone.

In conclusion, PFD administration before ischemia attenuated lung IRI through

inhibition of pulmonary edema, production of pro-inflammatory cytokines, and

apoptosis in a rat model. PFD may be useful as a new option for treatment of lung IRI.

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423

Figure 1

Pulmonary function of the left lung. (A) Dynamic pulmonary compliance. (B) Mean airway pressure. (C) Peak airway pressure. (D) Arterial partial pressure of oxygen. All parameters were significantly improved in the WI+PFD group compared to the WI group.

Sham: sham group; WI: warm ischemia group; WI+PFD: warm ischemia plus pirfenidone group. Bars and error bars show mean and standard error of mean; * $p < 0.05$, ** $p < 0.01$.

Figure 2

Hematoxylin and eosin staining and lung wet-to-dry weight (W/D) ratio

Sham group. (B) WI group. (C) WI+PFD group. (D) W/D ratio. (E) The index of perivascular cuff area/vessel area. (F) The number of red blood cells in the alveolar space per high-power field (HPF).

Arrows indicate perivascular edema.

Bars and error bars show mean and standard error of mean; * $p < 0.05$, ** $p < 0.01$

Figure 3

Naphtol AS-D chloroacetate esterase staining to detect neutrophils.

(A) Sham group. (B) WI group. (C) WI+PFD group. (D) The number of neutrophils in the perivascular area per high-power field (HPF).

Neutrophil infiltration (arrows) was significantly lower in the WI+PFD group compared to the WI group. Bars and error bars show mean and standard error of mean; * $p < 0.05$

Figure 4

Immunostaining for ssDNA to detect apoptotic cells.

(A) Sham group. (B) WI group. (C) WI+PFD group. (D) The number of ssDNA positive cells per high-power field (HPF).

The number of ssDNA positive cells (arrow) was lower in the WI+PFD group compared to the WI group. Bars and error bars show mean and standard error of mean; * $p < 0.05$

Figure 5

Gene expression in the left lung. (A) TNF- α mRNA. (B) IL-1 β mRNA. (C) MPO mRNA (D) NF- κ B mRNA.

Gene expression of TNF- α and NF- κ B mRNA were significantly reduced in the WI+PFD group compared to the WI group.

Bars and error bars show mean and standard error of mean; * $p < 0.05$

Figure 6

Graphical abstract

Administration of PFD in lung ischemia-reperfusion injury improved pulmonary function and decreased pro-inflammatory cytokines in lung tissue.

WI; warm ischemia, PFD; pirfenidone, TNF- α ; tumor necrosis factor-alpha, mRNA: messenger RNA



