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Pirfenidone protects against paraquat-induced lung injury and fibrosis in mice by modulation of inflammation, oxidative stress, and gene expression

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ABSTRACT

In this study we investigated the protective effects and possible mechanisms of pirfenidone (PF) in paraquat (PQ)-induced lung injury and fibrosis in mice. Lung injury was induced by injection of PQ (20 mg/kg). Thereafter, mice orally received water and PF (100 and 200 mg/kg) for four weeks. After 28 days, the inflammation and fibrosis were determined in the lungs by analysis of histopathology, bronchoalveolar lavage fluid (BALF) cell count, lung wet/dry weight ratio, hydroxyproline content, and oxidative stress biomarkers. Expression of several genes involved in fibrogenesis and modulation of reactive oxygen species (ROS) production, such as TGF-β1, α-SMA, collagen Iα and IV, NOX1, NOX4, iNOS, and GPX1 were determined using RT-qPCR. PF significantly decreased the lung fibrosis and edema, inflammatory cells infiltration, TGF-β1 concentration, and amount of hydroxyproline in the lung tissue. PF dose-dependently improved the expression level of the studied genes to the near normal. Decreasing of lung lipid peroxidation and catalase activity, and increasing of SOD activity in the treated mice were significant compared to the control group. Pirfenidone ameliorate paraquat induced lung injury and fibrosis partly through inhibition of inflammation and oxidative stress, and downregulation of genes encoding for profibrotic cytokines and enzymatic systems for ROS production.

1. Introduction

Paraquat poisoning is the most common cause of fatal herbicide intoxication and most cases result from deliberate ingestion. It has been observed in patients who ingest the pesticide either accidentally or intentionally as a suicide attempt (Dinis-Oliveira et al., 2008). PQ has detrimental effects on many organs namely, the central nervous system, gastrointestinal tract, kidney, liver, and heart. Pulmonary toxicity is the most common complication in treatment of paraquat poisoning (Gawarammana and Buckley, 2011). It has been shown that generation of redox cycling and free radicals is the main culprit in progressive inflammatory responses and pulmonary fibrotic process in lungs (Blanco-Ayala et al., 2014; Cheresh et al., 2013; Dinis-Oliveira et al., 2008; Mohammadi-Bardbori and Ghazi-Khansari, 2008).

Growing strategies for decreasing the toxicity of PQ have been proposed, including prevention of absorption, hemoperfusion, prevention of accumulation in the lungs, scavenging oxygen free radicals and prevention of lung fibrosis (Blanco-Ayala et al., 2014; Dinis-Oliveira et al., 2008; Gawarammana and Buckley, 2011). However these interventions have no confirmed effects for improving survival (Loveman et al., 2014). Among the therapeutic drugs available for pulmonary fibrosis, pirfenidone has opened a new promising window for both patients and physicians, suggesting that we could be able to modify and improve the prognosis for this devastating disease in the future (Rasooli et al., 2017b; Selvaggio and Noble, 2016). Many cases of idiopathic pulmonary fibrosis (IPF) have been improved by pirfenidone (Cottin

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and Maher, 2015; Hilberg et al., 2012; Inomata et al., 2014; Noble et al., 2016) and it may be effective against PQ induced lung fibrosis (Rasooli et al., 2017b; Seifirad et al., 2012).

Pirfenidone (PF) [5-methyl-1-phenyl-2-(1H)- pyridone] as an agent that had antifibrotic, analgesic, antipyretic and anti-inflammatory actions, is an orally available synthetic small molecule which is easily absorbed from the gastrointestinal tract after oral administration and is able to move through cell membranes without requiring a receptor (Selvaggio and Noble, 2016). The exact cellular mechanism whereby PF modulates fibrogenesis is still unclear in details, but its effects are attributed to antioxidant, anti-transforming growth factor-beta (*anti*-TGF-β) anti-tumor necrosis factor (TNF)-alpha, upregulation of RGS2, and antiplatelet derived growth factor (Iyer et al., 1999; Macias-Barragan et al., 2010; Nakazato et al., 2002; Salazar-Montes et al., 2008; Selvaggio and Noble, 2016; Xie et al., 2016). Although multiple different mechanisms of action of PF in IPF have been suggested, studies on PF and novel analogues are continued and further work in this field will possibly contribute to a better understanding of the mechanisms behind IPF and treatment.

Considering the possible antifibrotic effects of PF in PQ-induced lung fibrosis (Rasooli et al., 2017a, 2017b; Seifirad et al., 2012), in the present study we hypothesized that PF elicits its pharmacological effects by modulating the expression of some genes contributed to the enzymatic production of reactive oxygen species (ROS) and fibrogenesis, and oxidative stress inhibition. To test this hypothesis, we administered PF to PQ treated mice and examined the effect of PF on morphological changes, wet/dry weight ratio, differential cell count in bronchoalveolar lavage fluid (BALF), hydroxyproline content, and tissue oxidative stress parameters in lung tissue. In addition, expression of several genes contributed in fibrogenesis (TGF-β1, α- SMA, collagen Iα and IV) and ROS production (NOX1, NOX4, iNOS, GPX1) was determined in the lung tissues.

2. Materials and methods

2.1. Chemicals

Pirfenidone was obtained from Intermune Company (United States). Paraquat, hydroxyproline, 4-dimethylaminobenzaldehyde, chloramine T and malondialdehyde (MDA), and all the reagent for histological staining were purchased from Sigma–Aldrich Chemical Co. (USA). TRIzol® RNA isolation reagent and HyperScript™ first strand cDNA synthesis kit were purchased from Invitrogen (Germany). SYBR Green Master Mix was obtained from Takara (Japan). TGF-β1 kit was purchased from Bioassay Technology Laboratory (China).

2.2. Animals

Male NMRI mice, weighing 18–25 g, were obtained from Neuroscience Research Center, Kerman University of Medical Sciences and housed in normal laboratory conditions at 21 ± 2 °C under a 12 h/12 h light–dark cycle. All the mice had free access to water and standard laboratory food and were kept in standard cages. All the animals were treated humanely according to the guideline approved by the Animal Experimentation Ethics Committee of Kerman Neuroscience Research Center (EC/KNRC/90).

2.3. Paraquat-induced pulmonary fibrosis

Mice were randomly divided into five groups (except for BALF and lung wet/dry weight ratio experiments) eight mice in each: PQ group (PQ 20 mg/kg), PQ + water group (10 ml/kg as vehicle), PF100 group (PQ + pirfenidone 100 mg/kg), PF200 group (PQ + pirfenidone 200 mg/kg), and control group (normal animals without any treatment). For BALF analysis and lung wet/dry weight ratio experiments, four groups of 14 mice in each were given no treatment (as control), PQ (PQ + water), PQ + PF100, and PQ + PF200. Pulmonary fibrosis was induced by intraperitoneal (i.p.) injection of PQ at the dose of 20 mg/kg body weight. The dosage of PQ was based on our preliminary experiments showing induction of lung injury with no mortality

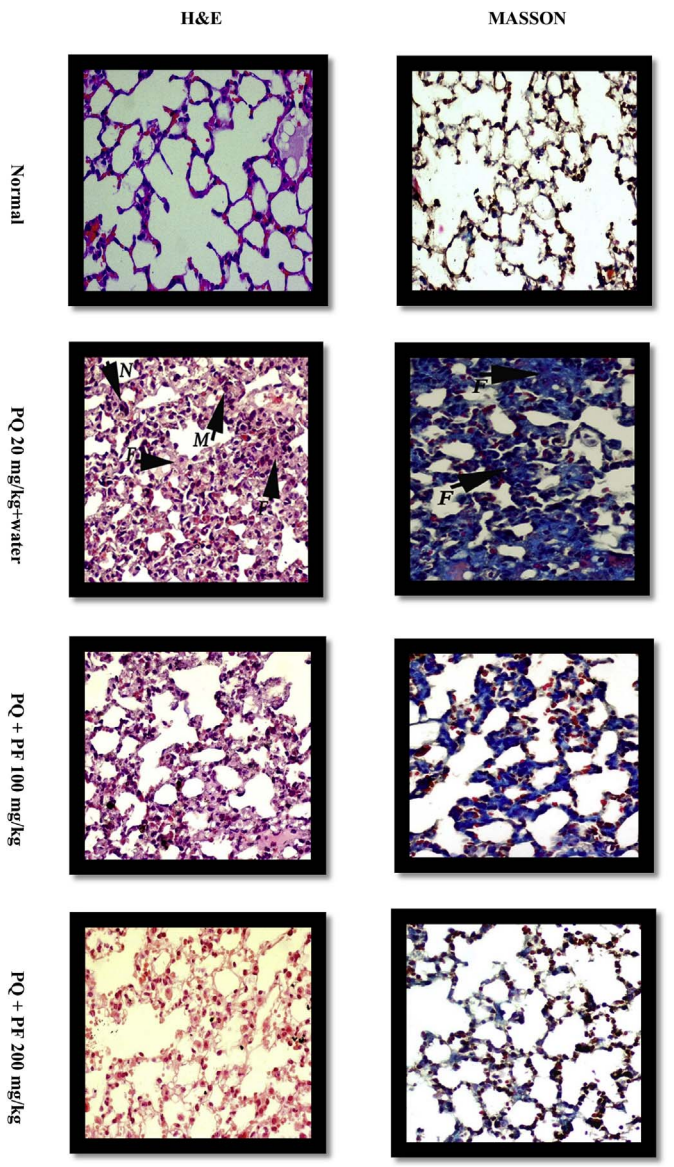

Fig. 1. Histopathological changes in the lungs of the mice in PQ-induced lung fibrosis. Lung fibrosis was induced in the lungs of the mice by injection of PQ (20 mg/kg, i.p.). They were then treated with PF 100 or 200 mg/kg/day for 28 days by oral gavage. The morphopathological changes (400×) in the lung tissues were analyzed by hematoxylin and eosin (H&E), and Masson's trichrome staining.
PQ: paraquat; PF: pirfenidone, M: Macrophage; N: Neutrophil; F: Fibrosis.

Table 1
Histopathological lesions of the lung tissue subsequent to therapeutic effects of PF.

Histological features	Control	PQ + water	PQ + PF100	PQ + PF200
Alveolar hemorrhage	absent	focal (mild)	mild	absent
Alveolar macrophages	present	increase	present	present
Interstitial inflammation	absent	moderate	mild	absent
Peribronchial fibrosis	absent	present	absent	absent
Interstitial fibrosis	absent	severe	moderate	mild

Mice treated with 100 and 200 mg/kg PF for 28 days after i.p. injection of single dose of PQ.
PQ: paraquat 20 mg/kg; PF: pirfenidone.

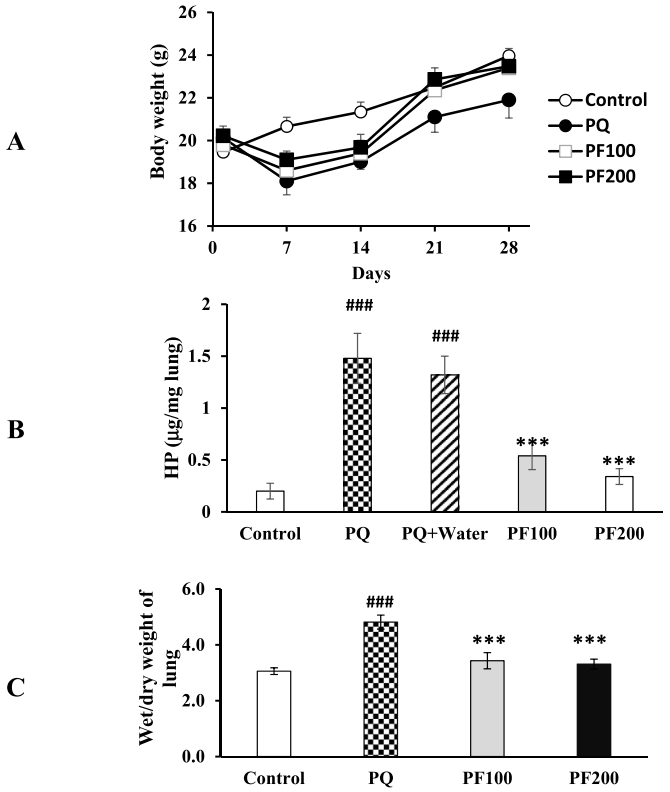

Fig. 2. Effect of PF on body weight, lung collagen contents, and lung wet/dry weight ratio in PQ-induced lung fibrosis. The doses of 100 and 200 mg/kg of PF and 10 ml/kg water were administered orally to the mice for 28 days after i.p. injection of PQ (20 mg/kg). Control mice did not receive any treatment. The values are the means of 8 replicates ± SD.
Control: (no treatment); PQ: paraquat; PF: pirfenidone; HP: hydroxyproline.
***p < .001 in comparison with the PQ group.
###p < .001 in comparison with the control group.

(Pourgholamhossein et al., 2016). PF was dissolved in water and administered by oral gavage for 28 consecutive days started one day after PQ injection.

2.4. Sample collection and analytical procedures

At the end of the treatment period (day 28), the mice were anesthetized by injecting 20% Ketamine/Xylazine (i.p., 1ml/100 g body weight). Lung promptly removed and divided into two halves. The right lung snap frozen in liquid nitrogen and stored at −70 °C for analysis of oxidative stress, gene expression and hydroxyproline content. The left lung was immersed in 10% buffered formalin for histopathological examination. BALFs were collected from 6 mice by intratracheal instillation and draining of 0.5 ml normal saline at 37 °C. Eight mice used for determination of wet/dry weight lung ratio.

2.5. Histopathological analysis

The lungs were fixed, paraffin embedded, sectioned at 5 µm and finally stained by hematoxylin and eosine for microscopic examination of morphological changes. The slides were evaluated by a blinded pathologist for the degree of inflammation on a subjective scale of 0, 1, 2, and 3 corresponding to none, mild, moderate, or severe inflammation, respectively (Kwak et al., 2003; Sur et al., 1999). The sections were also subjected to Masson's trichrome staining to identify collagen fibers. Briefly, the sections were incubated with hematoxylin for 6 min to stain the nuclei and then with Ponceau-Acid Fuchsin solution (%0.7 w/v ponceau, %0.3 w/v fuchsin acid, 1% v/v glacial acetic acid, for 1 min to stain the cytoplasm). After washing with 0.2% glacial acetic acid, the sections were incubated with 1% phosphomolybdic acid for 5 min to de-stain the connective tissue, and the collagen fibers were stained by aniline blue (2% w/v aniline blue + 2% v/v glacial acetic acid) for 5 min. The sections were dehydrated, mounted, and observed at 100× and 400× magnifications. The degree of fibrosis was evaluated using the criteria defined by Aschroft et al. (Ashcroft et al., 1988).

2.6. Lung wet weight/dry weight ratio

For determination of pulmonary edema, the wet/dry (W/D) ratio of the lungs were calculated by measuring and dividing the weight of lung tissues at the end of experiments and the weight of dried lungs at 60 °C for 72 h.

2.7. Differential cell count in BALF

The differential leukocytes counts were determined in the collected BALF. Collected BALF samples were centrifuged at 1500 rpm for 15 min at 4 °C, the cell-free supernatants were stored in −80 °C for the detection of TGF-β1 concentrations, and the cell pellet was re-suspended in 0.2 ml PBS. All the cell suspension was made into the smear on a glass slide. The cell smear was stained with Wright-Giemsa solution and the total and differential leukocyte counts were determined under 400 × magnification.

2.8. TGF-β1 concentration in BALF

TGF-β1 concentration in BALF was measured using enzyme-linked immunosorbent assay (ELISA) kit. Specific primary antibody against TGF-β1 was coated on wells. TGF-β1 was sandwiched between primary and secondary HRP-coated antibody, then color progress within the 10 min was assessed by ELISA reader (450 nm, reference wavelength 630 nm).

2.9. Determination of oxidative stress parameters

Preliminary preparation of lung tissue samples for biochemical evaluation was done with 0.1 M Tris–HCl buffer (pH 7.4) at 4 °C using a tissue homogenizer. The lipid peroxidation level was measured according to the method of Ohkawa et al. (1979). The lipid peroxides including malondialdehyde (MDA) in the lung tissue was measured using thiobarbituric acid at 532 nm. Different concentrations of MDA prepared as a standard solution. The activities of superoxide dismutase (SOD) was measured according to the method of Beyer and Fridovich (1987) and catalase (CAT) activity was determined using the method of Aebi (1984)

2.10. Measurement of hydroxyproline

Hydroxyproline, as a measure of collagen deposited in the lung tissue and fibrosis, was assessed using Reddy and Enwemeka's (Kesava Reddy and Enwemeka, 1996) method with minor modification (Ghazi-Khansari et al., 2007; Pourgholamhossein et al., 2016). Briefly, after homogenization of dried lung tissue and determination of protein concentration in the supernatant, hydroxyproline in the supernatant was hydrolyzed for 45min in NaOH 2N and oxidized with 1.4% chloramine T (Sigma, USA), and was formed reddish purple complex with 1 M Ehrlich's reagent (4-dimethylaminobenzaldehyde, Sigma, USA) and the chromophores were developed at 65 °C for 20 min. The chromophore was measured at 550 nm by an ELISA microplate reader.

2.11. Determination of gene expression in the lung

The level of genes in the tissue homogenate was determined with Real-time RT-PCR. Total RNA was extracted from pulmonary tissues using a RiboEx™ kit according to the manufacturer's protocol. Samples

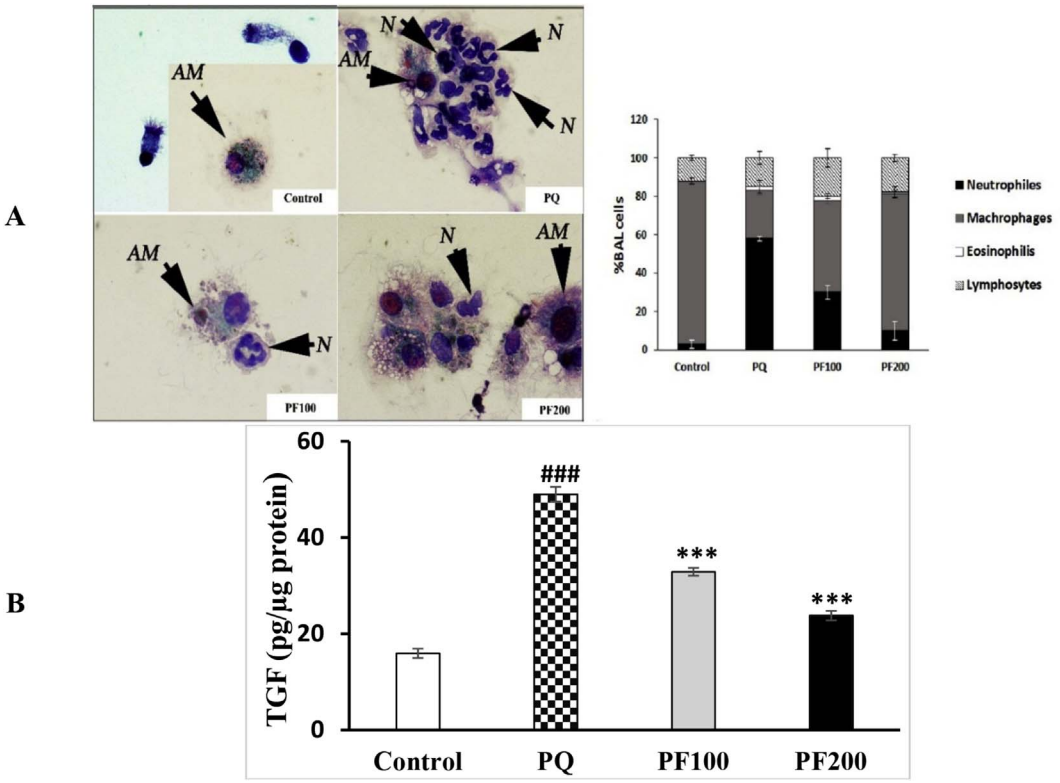

Fig. 3. Effect of PF on inflammation in PQ-induced lung fibrosis. The doses of 100 and 200 mg/kg of PF and 10 ml/kg water were administered orally to the mice for 28 days after i.p. injection of PQ (20 mg/kg). Control mice did not receive any treatment. The percentage of macrophages, neutrophils, eosinophils, and lymphocytes (A) and concentration of TGF-β1 cytokine (B) were determined in the BALF at day 28. Values are the means of 6 replicates ± SD.
Control: (no treatment); BALF: bronchoalveolar lavage fluid; PQ: paraquat; PF: pirfenidone; AM: alveolar macrophage; N: neutrophil.
***p < .001 in comparison with the PQ group.
###p < .001 in comparison with the control group.

(2 µg RNA) were reverse-transcribed using a HyperScript™ first strand cDNA synthesis kit. Synthesized cDNA was used in real-time RT-PCR (lightcycler® 96 Roche, Germany) experiments using SYBR GREEN Supermix and analyzed with lightcycler® 96 software. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, actin cDNA was used as an internal standard. Primers were designed using the Primer 3 website. The primers used were: alpha-smooth muscle actin (α-SMA): forward-5′-TGA CGC TGA AGT ATC CGA TAG A-3′, reverse-5′-CGA AGC TCG TTA TAG AAA GAG TGG-3′; collagen type 1 (Col Ia): forward-5′-CTG CTG GCA AAG ATG GAG A-3′, reverse-5′-ACC AGG AAG ACC CTG GAA TC-3′; collagen type 4 (Col IVa): forward-5′-AGC TGC TAA AGG TGA CAT TCC T-3′, reverse-5′-GGA GGC CCA GGT ACT CCT-3′; SOD1: forward-5′-GTG ATT GGG ATT GCG CAG TA-3′, reverse-5′-TGG TTT GAG GGT AGC AGA TGA GT-3′; NOX1: forward-5′-AAT GCC CAG GAT CGA GGT-3′, reverse-5′-GAT GGA AGC AAA GGG AGT GA-3′; NOX4: forward-5′-TTG CCT GGA AGA ACC CAA GT-3′, reverse-5′- TCC GCA CAA TAA AGG CAC AA-3′; iNOS: forward-5′-CAG CTG GGC TGT ACA AAC CTT-3′, reverse-5′-CAT TGG AAG TGA AGC GGT TCG-3′; GPX1: forward-5′-CGC TTT CGT ACC ATC GAC ATC-3′, reverse-5′-GGG CCG CCT TAG GAG TTG-3′, and GAPDH: forward-5′- TCA AGA AGG TGG TGA AGC AGG-3′, reverse-5′- CAC CAC CCT GTT GCT GTA G-3′.

2.12. Statistical analysis

The quantitative data were presented as mean ± S.D. Differences between the means were analyzed using one-way analysis of variance (ANOVA) followed with Tukey HSD post-hoc test. For all the experiments, $p < .05$ was considered as significance level. The data were analyzed using SPSS 18.0.

3. Results

3.1. Histopathology

The histopathological changes in the treated and control groups have been shown in Fig. 1 and Table 1. The histological evaluation of lung sections four weeks after the PQ injection revealed evidence of obvious interstitial inflammation, macrophages infiltration, local alveolar hemorrhage, hemosiderin deposit, peribronchial fibrosis, and severe interstitial fibrosis. Treatment with both concentrations of PF (100 and 200 mg/kg) caused a noticeable dose dependent alleviation in pathological lung lesions produced by PQ. The sections of the control group showed structural integrity without inflammation or fibrosis development.

3.2. Effects of PF on the body weight, lung collagen contents, and lung wet weight/dry weight (W/D) ratio in PQ-induced lung fibrosis

PQ significantly decreased mice body weight one week after injection. Treatment with PF returned the body weight to the level of control mice (Fig. 2A). Hydroxyproline content in the lung of PQ-treated mice significantly increased compared with the control group. Administration of PF (100 and 200 mg/kg) reduced the content of hydroxyproline in the lung tissue in a dose-dependent manner (Fig. 2B). Treatment with PF (100 and 200 mg/kg) significantly reduced the increased lung W/D weight ratio in the mice treated with PQ. These findings were consistent with the histological results.

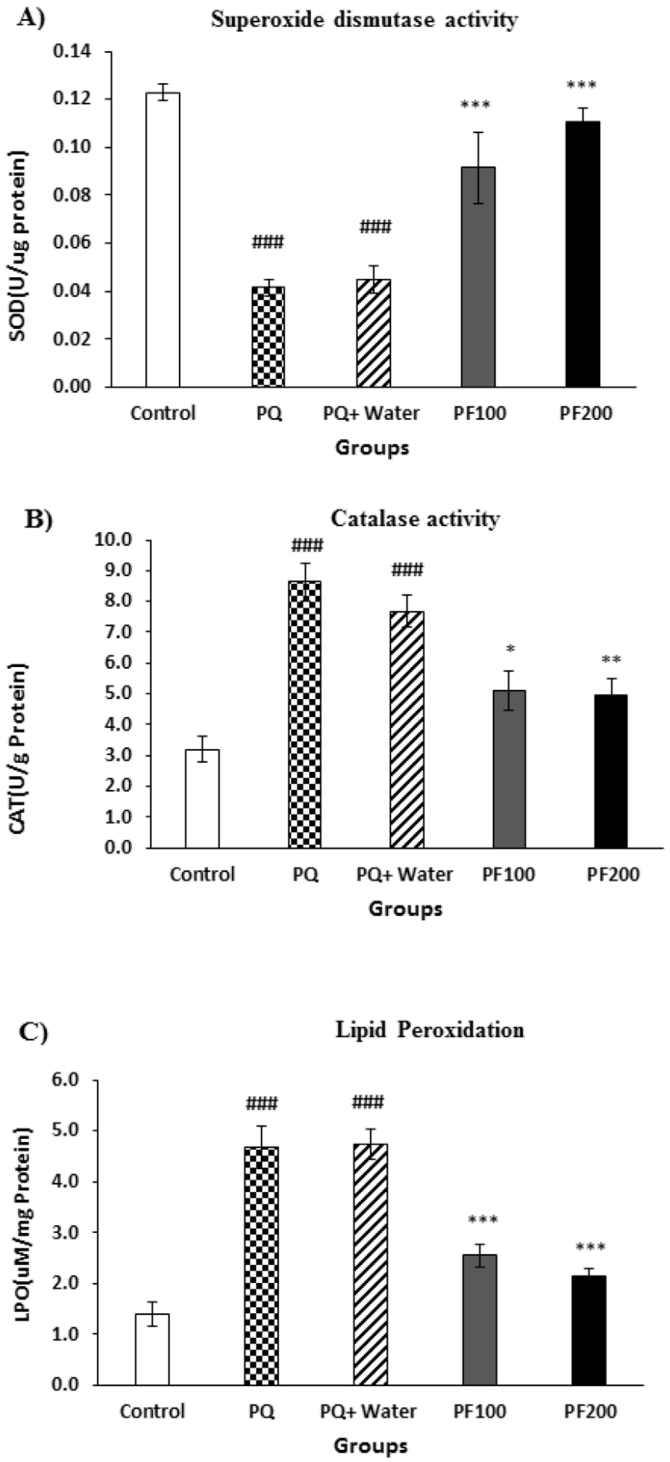

Fig. 4. Effect of PF on the lung oxidative stress parameters in PQ-induced lung fibrosis. The doses of 100 and 200 mg/kg of PF and 10 ml/kg water were administered orally to the mice for 28 days after i.p. injection of PQ (20 mg/kg). Control mice did not receive any treatment. The oxidative stress parameters including MDA levels (A), SOD (B) and CAT (C) activities were determined in the lung tissues of the treated mice at day 28. Values are the means of 8 replicates ± SD.
Control: (no treatment); PQ: paraquat; PF: pirfenidone.
*$p < .05$, **$p < .01$, and ***$p < .001$ in comparison with the PQ group.
###$p < .001$ in comparison with the control group.

3.3. Cell differential and TGF-β1 in BALF

There was a significant increase in inflammatory cells infiltration, particularly neutrophils, and TGF-β1 concentration in BALF in the PQ-treated mice. Treatment with PF dose-dependently decreased the percentage of neutrophils and TGF-β1 concentration in BALF (Fig. 3).

3.4. Effect of PF on the oxidative stress parameters in PQ-induced lung fibrosis

The levels of lipid peroxidation and the antioxidant activities of SOD and CAT were depicted in the lung tissue of the control and experimental mice (Fig. 4). A significant rise in the levels of lipid peroxidation and activity of CAT in the lung tissue was observed in the PQ treated animals ($p < .001$). This effect was accompanied by a decrease in the enzymatic activity of SOD. The administration of PF significantly decreased lipid peroxidation in a dose dependent manner and restored the CAT and SOD activity to near normal ($p < .01$).

3.5. Gene expression

The mRNA expression of TGFβ-1, collagen1a1, collagen 4a1, α-SMA, NOX1, NOX4, and iNOS were significantly increased in the PQ group compared with that of the control group. In a dose dependent manner, the mRNA expressions of these genes were decreased in PF treated mice compared with the PQ group. However, expression of GPX1 gene decreased by PQ and treatment with PF increased GPX1 expression. These results suggest that PF could inhibit the activation of ROS producing and profibrotic genes and ECM deposition (Fig. 5).

4. Discussion

As a continuous study on the therapeutic mechanisms of PF against lung fibrosis (Rasooli et al., 2017a, 2017b), we evaluated some criteria of lung tissues in a PQ-induced lung fibrosis in order to gain insight into the treatment of this devastating disease. We found that the administration of PF effectively attenuated PQ-induced lung injury and fibrosis including destruction of the structure of the lung alveoli and inflammation. The increased TGF-β1, collagen-I, IV, α-SMA, NOX1, NOX4, and iNOS genes expression and the decreased GPX1 gene expression induced by PQ were significantly inhibited by PF. Meanwhile, PF reduced the W/D weight ratio, hydroxyproline content, CAT activity and MDA level, and increased activity of SOD in the fibrotic lungs. Taking together, our results suggest that PF can ameliorate lung fibrosis induced by PQ.

Great strides have been made in understanding of the pathophysiology behind fibrosis and its treatment. It is found that the basis of fibrosis treatment is breaking of three strong barriers: oxidative stress, inflammation, and fibrogenesis, which are all manifested in the present model of PQ induced pulmonary fibrosis. Increase in W/D weight ratio, and TGF-β1 concentration and cell count in BALF show that PQ increase lung inflammation and treatment with PF inhibit this process. These findings confirm the previous reports regarding anti-inflammatory effects of PF in lung (Bayhan et al., 2016; Iyer et al., 2000; Liu et al., 2005; Schaefer et al., 2011). It is shown pirfenidone inhibits production of inflammatory mediator TNF-α at the translational level (Nakazato et al., 2002). Prevention of PQ-induced lung injury by immunomodulators such as FTY720 and cyclophosphamide has also been reported (Choi et al., 2013; Gawarammana and Buckley, 2011; Qian et al., 2014).

This study showed an overall oxidative stress in lung tissue of PQ treated mice which was including a significant rise in the levels of lipid peroxidation, activity of CAT, and decrease in the enzymatic activities of SOD. This was more confirmed by increase in the expression of iNOS, NOX1, NOX4 genes which belong to the ROS-production enzymatic systems and decrease in expression of GPX1 antioxidant gene. The administration of PF significantly decreased lipid peroxidation in a dose dependent manner and restored the SOD and CAT activity to near normal. Meanwhile, expression of iNOS, NOX1, and NOX4 genes which are contributed in ROS producing decreased. Oxidative stress due to

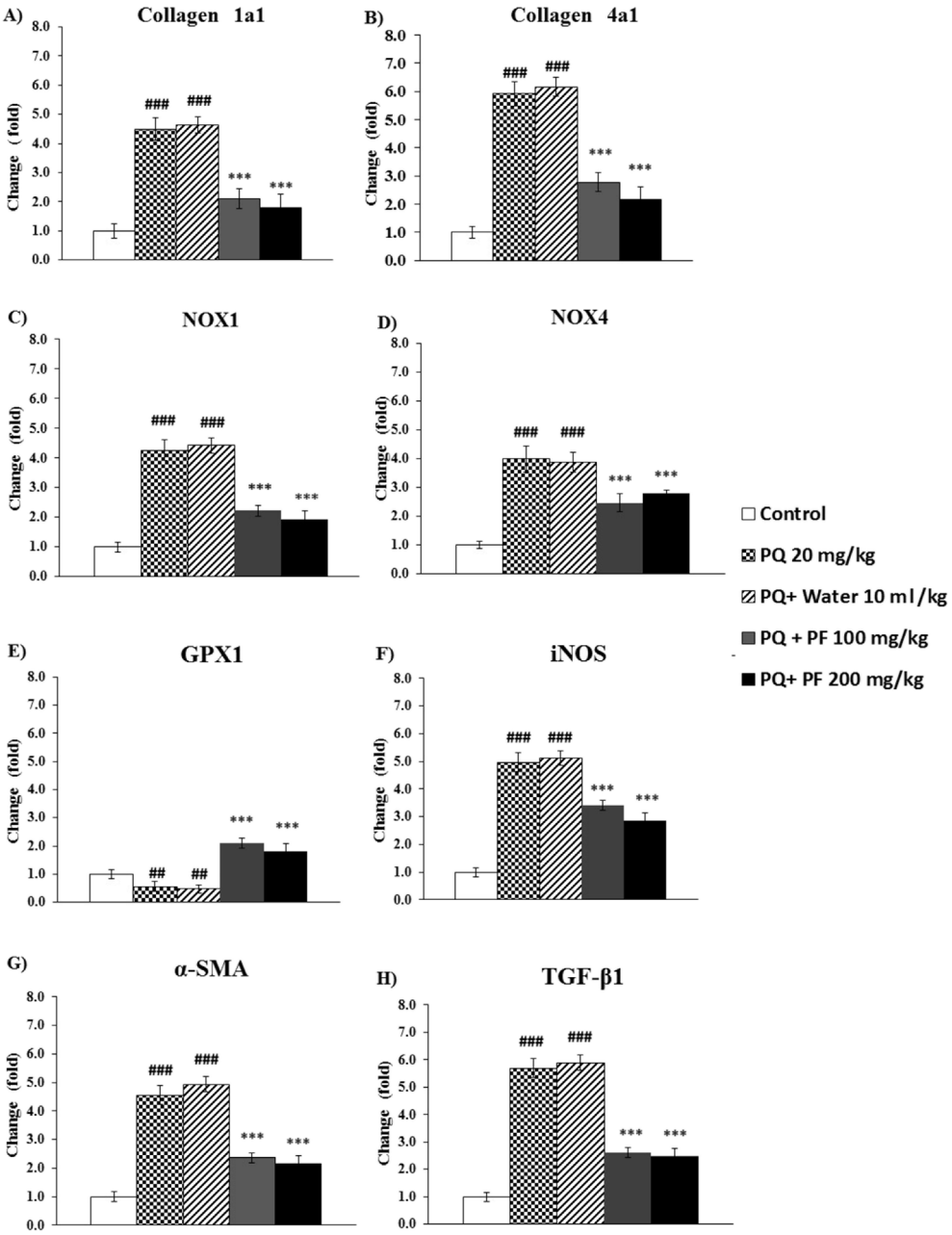

Fig. 5. Effect of PF on the expression of several fibrotic and oxidative stress related genes in PQ-induced lung fibrosis. The doses of 100 and 200 mg/kg of PF and 10 ml/kg water were administered orally to the mice for 28 days after i.p. injection of PQ (20 mg/kg). The expressions of α-SMA, TGF-β1, collagen 1a1, collagen 4a1, NOX1, NOX4, iNOS, SOD, and GPX-1 mRNA were determined in the lung of the mice by real-time RT-PCR. The data are means ± SD (two replicates in each assay) for 8 mice.
PQ: paraquat; PF: pirfenidone.
***p < .001 in comparison with the PQ group.
###p < .001 in comparison with the control group.

PQ-induced ROS generation is considered as the foundational mechanism by which pulmonary damage is induced in this model (Blanco-Ayala et al., 2014; Cheresh et al., 2013; Dou et al., 2015; Hong et al., 2015; Kliment and Oury, 2010; Toygar et al., 2015). The main suggested sources of PQ-induced ROS production are PQ metabolism in microsomal enzyme systems and mitochondria (Blanco-Ayala et al., 2014; Castello et al., 2007; Mohammadi-Bardbori and Ghazi-Khansari, 2008), and activation of NADPH oxidase (NOX), especially NADPH oxidase-4 (NOX4), in inflammatory and lung target cells (Cheresh et al., 2013; Ghatak et al., 2017; Wang et al., 2014). NOXs are a family of flavoenzymes that they are solely responsible for primary ROS (O^2. or H_2O_2) production (Teixeira et al., 2017). Inducible nitric oxide synthase (iNOS) is also highly expressed in alveolar macrophages and epithelial cells and is involved in lung inflammation. iNOS can induce ROS production in lung under stress conditions (Naura et al., 2010; Yan et al., 2017; Zhao et al., 2009). These results indicate that reduction in ROS formation and oxidative stress might be as mechanisms by which the antifibrotic effect of the PF are mediated. These results are in consistent with those reported the anti-oxidative stress properties of PF (Macias-Barragan et al., 2010; Oku et al., 2008; Salazar-Montes et al., 2008; Schaefer et al., 2011).

Consistent with lipid peroxidation and antioxidant findings, histological analysis in lung tissue revealed mild to moderate injury in PF received groups and severe injury in PQ group, which confirmed the antifibrotic effects of PF. Seifirad et al. (2012) has also reported such effects in PQ fibrosis model (Seifirad et al., 2012). Moreover, PF treatment inhibited the hydroxyproline and TGF-β1 content, and expression of some fibrogenic genes such as TGF-β1, α-SMA, collagen I and collagen IV in a dose dependent manner which indicates attenuation of lung injury after PF treatment. TGF-β1 is the pivotal mediator of fibrogenesis and is well-known to stimulate the collagen production. Inhibition of TGF-β1 gene expression and production is also reported for pirfenidone in bleomycin model of lung fibrosis in hamster (Iyer et al., 1999). It is been shown that ROS not only apply their destructive effects through attack the cell membrane and organelles, but also activate latent TGF-β1 (Liu and Desai, 2015).

There is substantial evidence for a relationship between TGF-β1 and NADPH oxidase (NOX)-dependent redox signaling in the profibrotic responses (Amara et al., 2010; Ghatak et al., 2017; Jiang et al., 2014). TGF-β1 can up-regulate NOX4 gene expression which results in increased ROS production, while NOX-dependent redox signaling may in turn regulate TGF-β/Smad signaling in a feed-forward manner (Jiang et al., 2014; Teixeira et al., 2017). The seven isoforms of NOXs exist and most of them express in fibroblasts and contributed in pathogenesis of lung fibrosis. NOX1 and 4 are focused in many diseases, in particular inflammatory and fibrotic diseases (Teixeira et al., 2017). NOX4 expression has increased in lung fibrosis (Amara et al., 2010; Ghatak et al., 2017; Hecker et al., 2014). Type I collagen (COL 1) is the major fibrous collagen synthesized by fibroblasts in the repair process and it has direct proportion with TGF-β1 levels (Todd et al., 2012; Wynn, 2011). It means when TGF-β up-regulated, excessive fibrosis results from increased collagen deposition. As expected here this effect was antagonized by both concentrations of PF (100 and 200 mg/kg). This finding is consistent with the *in vitro* effect of pirfenidone on the A549 cell line (Hisatomi et al., 2012).

Epithelial mesenchymal transition (EMT) is believed to have crucial role in the fibrosis process through converting epithelial cells to mesenchyml (Chapman, 2011; Wynn, 2011). During this transition process, epithelial cells loss their intrinsic formation and performance, and acquire mesenchymal characteristics. It is well established that the myofibroblasts are the key source of ECM. Advent of fibroblasts is accompanied by stimulation of α-SMA actin expression (Gu et al., 2007; Todd et al., 2012; Wynn, 2011; Zhang et al., 1996). The interaction of fibroblast cells with the surrounding collagen fibrils results in a dense and compact formation of the ECM. Ultra structural and histochemical studies have shown that the numbers of myofibroblasts, characterized by α-SMA actin expression, increased progressively in the lung fibrosis and that they represent the cell type most responsible for the increase in lung type collagen expression. Thus, these cells also appear to play an important role in the pathogenesis of pulmonary fibrosis, and their presence may contribute to increased extracellular matrix deposition and contractility of lung tissue in this disease. In the current study, the administration of PF blocked TGF-β1-augmented expression of α-SMA and collagen. As anticipated, the data were consistent with the results of hydroxyproline content and histopathological finding.

In conclusion, we have demonstrated that administration of PF mitigates PQ-mediated lung injury and fibrosis in a dose-dependent manner. This protective effect of PF may be due to the inhibition of inflammation and oxidative stress, and down-regulation of genes encoding for profibrotic cytokines and enzymatic systems for ROS production. However, further study is needed to investigate the many other molecular pathways involved in anti-fibrotic effect of PF.

Disclosure statement

These data have not been submitted or published elsewhere.

Conflicts of interest

The author(s) declare that they have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

FP: Participated in the design of the study, treatment and maintenance of animals, and doing the experiments. MSF: Participated in the design of the study. RR: Drafted the manuscript. MGK: Conceived the study, prepared some materials for the study. MP: Participated in the analysis of data. LP: Participated in qRT-PCR experiments. MI: Interpreted the histopathological data. HRP: Performed the BALF experiments. MRH: Participated in the design of the study and revised the manuscript. AM: Designed the study, analyzed the data and wrote the manuscript.

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