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Pirfenidone alleviates concanavalin A-induced liver fibrosis in mice

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ABSTRACT

Aims: Liver fibrosis (LF) is a life-threatening complication of most chronic liver diseases resulting from a variety of injurious agents and hepatotoxic insults. To date, there are no specific therapies for LF, and all the currently available drugs have been developed for other indications. Thus, there is a pressing need to develop new drugs for treatment of LF. Therefore, the current study aimed to elucidate the potential antifibrotic effect of Pirfenidone (PFD) against concanavalin A (ConA)-induced immunological model of liver fibrosis in mice.

Main methods: Hepatic fibrosis was induced in mice by injecting ConA (10 mg/kg/wk./i.v) for 4 weeks. Then, the mice were treated with or without PFD (125 mg/kg/ip/day) for 2 weeks. Hepatic fibrosis was determined by Masson Trichrome staining; Haematoxylin & eosin (H&E) staining, immunohistochemistry staining of type II and IV collagens, and colorimetric assessment of hydroxyproline (HP) content in the liver tissues. In addition, the expression of α-SMA mRNA was determined by real time RT-PCR. The serum levels of TGF-β, TNF-α, TIMP-1 and MMP-2 were measured by ELISA.

Key findings: Treatment with PFD significantly reduced ConA-induced expression of type II and IV collagens, α-SMA mRNA expression, and HP content and decreased inflammatory cells infiltration in hepatic tissues. Furthermore, serum levels of TGF-β, TNF-α, and TIMP-1 were significantly reduced with concomitant increase in MMP-2 expression.

Significance: Treatment with PFD ameliorates concanavalin A-induced hepatic inflammation and fibrosis in mice. Thus, PFD may represent a promising therapeutic option for hepatic fibrosis and its related complications.

1. Introduction

Liver fibrosis (LF) and its life-threatening complications such as cirrhosis and hepatocellular carcinoma are highly prevalent medical conditions with significant mortality and morbidity. LF is regarded as a scarring response to liver damage caused by various etiological factors including viral hepatitis, alcohol abuse, metabolic diseases, and autoimmune diseases [1]. Generally, chronic insults with these etiological factors can cause abnormal proliferation and differentiation of mesenchymal hepatic stellate cells (HSC) into myofibroblasts (MFB) which is the driving force of liver scaring, collagen accumulation, and extracellular matrix (ECM) remodeling associated with the hepatic fibrosis [2,3]. From a clinical perspective, LF caused by different etiological factors may demonstrate similar clinical pictures. Yet, at the molecular level, different signaling pathways are involved in initiation, progression, and resolution of fibrosis caused by different insulting agents [4]. There are currently no FDA-approved pharmacological treatments for advanced LF, and thus development of effective drugs for prevention and/or treatment of liver fibrosis is a pressing medical need.

Experimental models which imitate human liver fibrosis are key components in the development of new pharmacological treatment for liver fibrosis. Currently, there are many rodent models which are commonly used in experimental and preclinical studies of liver fibrosis. Although each of these models is molecularly and functionally distinctive, they limited the clinical relevance to different molecular and clinical subtypes of liver fibrosis [5]. Such discrepancies restricted of the experimental evaluation of novel compounds for treatment of liver fibrosis, as the outcomes is driven by the molecular trigger liver fibrosis [6].

Although several types of inducers were wildly used to mimic autoimmune hepatitis (AIH) and viral hepatitis models; for example: Concanavalin A (ConA), D-galactosamine (GalN) with low dosage of lipopolysaccharides (LPS), and high dosage of LPS [7], ConA-induced liver fibrosis is the most commonly used experimental model for AIH in

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mice [7–9]. It is obvious that the ConA-induced hepatitis model possesses more advantages than the other two; being only one inducer, making it easier to be established compared with the GalN/LPS model. Also with ConA, there is no significant change of the levels of transaminases, which is considered a valid index of the severity of liver injury, in the LPS model. Furthermore, in ConA model, the serum levels of many cytokines are dramatically relevant to inflammatory change, which is favorable for the study of the pathogenic mechanisms of AIH [7]. Other models such as acetaminophen (APAP)-induced liver injury, dietary non-alcoholic steatohepatitis or carbon tetrachloride-induced liver inflammation trigger hepatitis by primary damage to the hepatic parenchymal tissue with subsequent leukocyte activation and secondary immune-mediated damage [9]. In contrast to other experimental models, ConA-induced liver fibrosis is driven by the recruitment and activation of T-cells in the liver [10]. Thus, ConA-induced hepatic fibrosis mimics immune-mediated liver fibrosis in humans caused by AIH, acute viral hepatitis or drug-induced immune activation [9]. Emerging evidence suggests that immune-mediated liver fibrosis and other immune-mediated fibrotic conditions such as idiopathic pulmonary fibrosis (IPF), display a common dysregulated signaling pathways [11,12]. Having similar signaling pathways, it is sensible to consider relevant therapeutic targets for both diseases. Pirfenidone (PFD) is a novel orally bioavailable anti-inflammatory and antifibrotic drug which has been developed as a treatment for IPF. Although the clinical therapeutic effect of PFD in IPFis established [13], its potential therapeutic effects in immune-mediated hepatic fibrosis remain unknown. Thus, we hypothesize that PFD might represent a promising remedy for immune-mediated liver fibrosis which share similar signaling attributes with IPF.

2. Materials and methods

2.1. Chemicals and reagents

ConA was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). PFD was purchased from VEGESNA Laboratories Pvt Ltd (Hyderabad, India). Phosphate buffer saline (PBS) was purchased from El-Gomhouria Co. For Trading Chemicals and Medical Supplies (Cairo, Egypt). Hydroxyprolline Colorimetric Assay Kit (Cat # K555-100) was purchased from Biovision incorporated company (San Francisco, CA, USA). Elisa Kits for TGF-β1 (Cat # MBS175818), TNF-α (Cat # MBS175787), MMP-2 (Cat # MBS722437) and TIMP-1 (Cat # MBS2881293) were purchased from MyBiosource Company (Santiago, MN, USA). BCA protein assay kit (Cat # ab207002) and anti-collagen IV antibody (Cat # ab6586) were purchased from abcam Biochemicals, (Cambridge, MA, USA). Qiagen RNA extraction kit was purchased from Qiagen, Inc. (Germantown, MD, USA). The Reverse Transcription Kit was purchased from Fermentas Biotechnology a subsidiary from Thermo Fisher Scientific (Waltham, MA, USA). Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). Anti-collagen II antibody kit (Cat # A00517) was purchased from Boster Biological technology (Pleasanton, CA, USA). All of the other used chemicals and reagents were of the highest commercially available grade.

2.2. Experimental animals

Male BALB/C Mice (weight, 20–25 g; age, 8–10 weeks) were purchased from the experimental animal facility of The Nile Pharmaceutical Company (Cairo, Egypt). The mice were housed in an animal facility at Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt. The animals were maintained at a temperature of 22 °C and at a lightening (12-h light-dark cycle) control. Only male mice were used for this model, because of the previous report showing that female mice demonstrate a great variation and high mortality rates after ConA treatment [9], the matter that was confirmed also by our preliminary studies. All animals' procedures were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1987) and these procedures were approved by the Institutional Animal Ethics Committee for Al-Azhar University, faculty of pharmacy, Cairo, Egypt.

2.3. Induction of liver fibrosis model and treatment protocol

ConA-induced liver fibrosis was established by intravenous (i.v) injection of ConA into mice as previously described [10,14]. Since previous studies demonstrated that the dose of ConA required to induce liver fibrosis varies considerably depending on the mice species, gender, genetic background and the microbial environment specific for the animal facility barrier [10,14], we conducted a preliminary experiment to establish the appropriate doses and duration of treatment with ConA and PFD (data not shown). Based on our preliminary data, i.v. injection of ConA, at a dose of 10 mg/kg/week for 4 consecutive weeks, followed by intraproteinal (i.p) injection of PFD, at a dose of 125 mg/kg/day for another 2 consecutive weeks, was adopted in the subsequent experiments.

2.4. Design of the work

Fifty male mice were randomly divided into the following four treatment groups:

The first group (control group): the mice (n = 10) were treated with i.v. saline (0.2 ml/week, for 4 consecutive weeks and then with i.p. saline (0.2 ml/day) for another two consecutive weeks. The second group (ConA alone-treated group): the mice (n = 15) were treated with ConA (10 mg/kg/i.v./week for 4 consecutive weeks) and then with i.p. saline (0.2 ml/day) for another 2 consecutive weeks.Third group (Con A + PFD-treated group): the mice (n = 15) were treated ConA (10 mg/kg/i.v./wk. for 4 consecutive weeks) and then with PFD (125 mg/kg/day; i.p.) for another 2 consecutive weeks. The forth group (PFD alone-treated group): the mice (n = 10) were treated with saline (0.2 ml/week, i.v.) for 4 consecutive weeks and then with PFD (125 mg/kg/day, i. p) for another 2 consecutive weeks). The PFD dose used for mice in the current study (125 mg/kg) is less than 30% of the equivalent daily maximum tolerable human PFD dose (423 mg/kg), and less than 90% of the equivalent daily recommended average human PFD dose (140 mg/kg) based on BSA normalization method.

2.5. Histopathological- and immunohistochemical staining

Livers were dissected out from mice of the different groups. Each liver was divided into two halves; one half was fixed in 10% neutral formalin and embedded in paraffin (for immunohistochemical staining and evaluation of liver inflammation/fibrosis) while the second half was stored at −80° c until used for the other biochemical and molecular analysis. For light microscopic analysis of liver histology, the paraffin-embedded liver tissues were sliced into thin sections, and stained with haematoxylin and eosin (H&E) according to the standard protocol [15]. For the assessment of collagen contents in live tissues, liver sections were stained with Masson's trichrome per the standard protocol [15]. For immunohistochemical staining, the slides were stained using anti-collagen II antibody kit (Cat # A00517; Boster Biological technology; Pleasanton, CA, USA) or anti-collagen IV antibody (Cat # ab6586; abcam Biochemicals, Cambridge, MA, USA) to detect collagen II and IV deposition in the liver. The Masson's trichrome stain is used selectively for staining collagen fibers and imparts a blue color to collagen against a red background of hepatocytes and other structures [15]. The stained slides were coded and scored by pathologist who was blinded to the treatment protocol. The acquired microscopic images (× 100-magnification) were initially encoded on 24-bits per pixel on three channels (red, green, and blue), and the colored images were processed using ImageJ 1.46r software. (WyneRasband, National Institutes of Health,

Bethesda, USA) to produce maps showing only the biomarker's stained areas. Quantifications of the samples were made using ImageJ 1.46r software.

2.6. Assessment of the serum levels of the fibrotic marker; hydroxyprolline

Hydroxyprolline (HP), the most common amino acid present in collagen, is a fibrotic marker in liver tissues. The serum level of HP is a reliable biomarker for the assessment of the extend/progression of liver fibrosis. The serum HP levels were determined using Hydroxyprolline Colorimetric Assay Kit (Biovision incorporated company, USA) according to the manufacturer instructions [16].

2.7. ELISA assays

Serum levels of transforming growth factor beta 1 (TGF-β1), tumor necrosis factor-1α (TNF-1α), matrix metalloproteinase-2 (MMP-2) and tissue inhibitors of metalloproteinases (TIMP-1) proteins were measured in plasma samples using the commercially available Elisa Kits (MyBioSource, Inc. San Diego, CA; USA) according to the vender's instructions.

2.8. Determination of α-SMA mRNA in liver tissue by real time RT-PCR

Total RNA was extracted from liver tissues harvested from different treated groups using RNeasy Mini Kit (Qiagen, Inc., Germantown, MD, USA), according to the manufacturer's instructions. To remove any contaminating DNA, the isolated RNA was treated with a DNase I, RNase-free kit (Fermentas, MD, USA). Total RNA (1 μg) from each sample was used for synthesis of cDNA using the AMV Reverse Transcriptase kit (Fermentas, MD, USA). Gene expression was analyzed by quantitative real-time PCR using SYBR Green Real-Time PCR Master Mix. The real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The following primers sequence for mice α-SMA were used: Fwd: 5′-GATAGAACACGGCATCATCACCA-3′, Rev: 5′-GCAGGGACATTGAAG GTCTCAAA-3’

2.9. Statistical analysis

All data are reported as means ± SD. Data were analyzed by one-way analysis of variance (ANOVA) and the least significant difference (LSD) test. Differences between means were considered significant at $P < 0.05$. The experiments were performed at least in duplicate.

3. Results

3.1. Pirfenidone attenuated ConA-induced liver inflammation and fibrosis

Inflammation and fibrosis are tightly linked pathways impacting liver fibrosis, that involves the expression of the inflammatory cytokine,TNF-α which induces liver injury and development of fibrosis [17], along with fibroblast activation by the chemotactic TGF-β [18]. Thus, we investigated the effects of ConA on expression of TNF-1α and whether treatment with PFD modulates this effect. As anticipated, ConA treatment resulted in a robust increase of the serum level of TNF-1α and treatment with PFD significantly ameliorated ConA-induced sharp increase in the serum TNF-1α. Thus, as indicated in Table (1), the serum levels of TNF-1α were 28.55 ± 2.036; 274.1 ± 73.44; 149.7 ± 26.98 and 27.74 ± 2.336 pg/ml in vehicle-treated control group; Con A-treated group, group treated with ConA followed by PFD, and PFD-treated group, respectively, ($P < 0.05$). These data clearly demonstrated that ConA significantly increased the serum level of TNF-1α by about 8-foldas compared with vehicle-treated control or the PFD alone-treated group. Such elevation was significantly decreased when PFD was administered for two weeks by about 0.5 fold compared to the ConA treated group (Fig. 1A).

Table 1
Effect of PFD on inflammation/fibrosis biomarkers in ConA induced liver fibrosis model.

Group	Serum level		
	Inflammation/fibrosis biomarkers		
	TGF-β (pg/ml)	TNF-α (pg/ml)	Hydroxyprolline (ng/ml)
Control	13.95 ± 1.366	28.55 ± 2.036	14.08 ± 2.807
ConA	360.8 ± 124.6 *	274.1 ± 73.44 *	58.83 ± 12.86 *
ConA + PFD	98.20 ± 13.10 #	149.7 ± 26.98 #	22.78 ± 10.33 #
Control + PFD	14.03 ± 1.234	27.74 ± 2.336	12.63 ± 1.382

The illustrated data represents the means ± SD of the serum level of the indicated biomarkers, that were determined as described in methods section (N = 10). Statistical analysis was carried out using one-way ANOVA and the least significant difference (LSD) test; *$P < 0.05$, vs. control untreated animals, # $P < 0.05$, vs. ConA-treated animals.

The Transforming Growth Factor-beta (TGF-β) family plays relevant roles in the development of liver fibrosis via increase the synthesis of ECM proteins including collagens [18]. Hence, we assessed the effects of ConA and/or PFD treatment on the serum level of TGF-β. Similar to its effect on serum level of TNF-1α, treatment with ConA resulted in a significant and drastic increase in the serum level of TGF-β as compared with the vehicle-treated control or PFD alone-treated group. Interestingly, in mice treated with ConA followed by treatment with PFD for 2 consecutive weeks, the serum level of TGF-β was significantly lower compared to the group treated with ConA alone. As demonstrated in table (1), the serum level of TGF-β in vehicle-treated control group, ConA alone-treated group, group treated with ConA followed by PFD treatment for 2 consecutive weeks, and group treated with PFD alone were 13.95 ± 1.366; 360.8 ± 124.6; 98.20 ± 13.10; and 14.03 ± 1.234 pg/ml, respectively, ($P < 0.05$). These results explicitly depict that ConA-induced liver injury is associated with a sharp rise in the serum level of TGF-β (about 25-fold increase) compared to control group. Administration of PFD significantly diminished this effect by about 0.7 fold compared to the ConA-treated group (Fig. 1B).

3.2. Pirfenidone ameliorates ConA-induced increase in serum hydroxyprolline and liver α-Smooth muscle actin

The spectrophotometric assay of hydroxyprolline (HP) is one of the few that allows for the actual quantitation of collagen content and concentration, and therefore, it is considered as a reliable biomarker for the progression of collagen metabolism disorders including liver fibrosis [16]. Thus, we evaluated HP levels in serum from mice treated with ConA and/or PFD to quantitatively assess the extent of liver fibrosis [19]. The results are displayed in table (1) and Fig. 1C, revealed that HP levels in the serum from vehicle-treated control mice; Con A-treated mice; mice treated with ConA followed by PFD; and mice treated with PFD alone were 14.08 ± 2.81; 58.83 ± 12.86; 22.78 ± 10.33; and 12.63 ± 1.382 ng/ml, respectively, ($P < 0.05$). These data point to the sharp rise (~3-fold increase, in the serum level of HP in mice treated with ConA alone as compared to vehicle-treated control group ($P < 0.05$). Interestingly, treatment with PFD, for two weeks, significantly reduced the ConA-induced increase in serum HP by about 0.6 fold (Fig. 1C).

Furthermore, we assessed the effect of ConA and/or PFD on the α-SMA gene expression as reliable marker for hepatic stellate cells (HSCs) activation. Our results showed that mice treated with ConA exhibited higher hepatic α-SMA mRNA levels (by an average of 4-fold) as compared to vehicle-treated control mice ($P < 0.05$). On the other hand, the group of mice injected with PFD after ConA treatments, showed significant inhibition in the Con A-induced induction of α-SMA mRNA

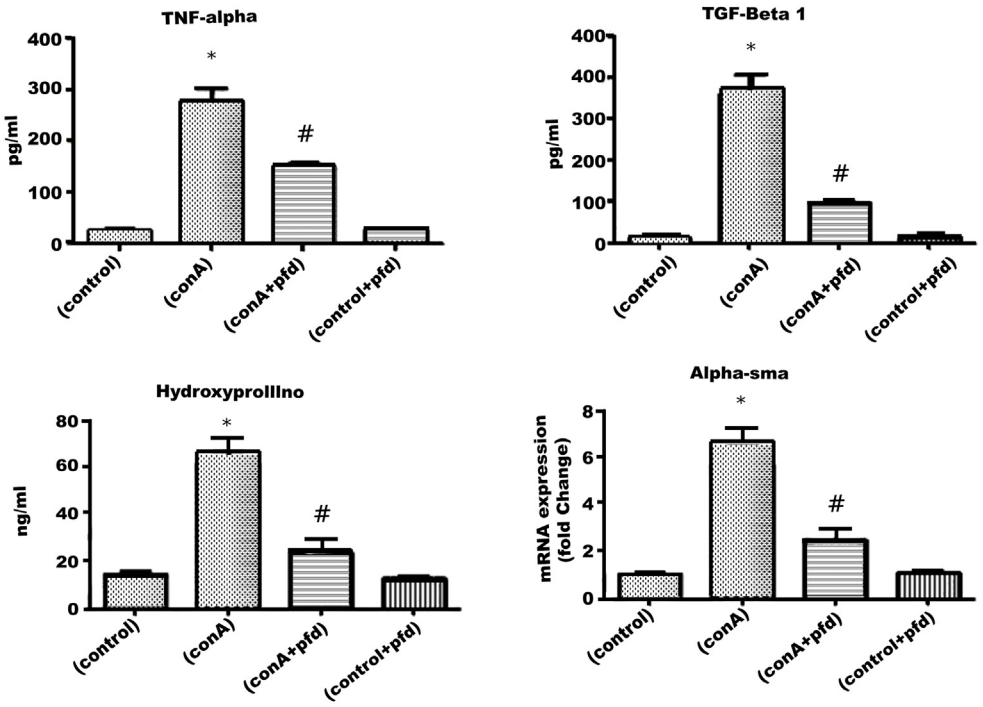

Fig. 1. Effects of PFD on inflammation/fibrosis biomarkers in Con A-induced liver fibrosis model. Serum levels of TNF-α (A), TGF-β (B), hydroxyproline (C) proteins and α-SMA mRNA (D) were determined as described in methods section. Bar charts represents mean ± SD, *P < 0.05, vs. control group, #P < 0.05, vs. ConA group, (N = 10).

Table 2
Modulatory effects of PFD on deposition/degradation of ECM in ConA-induced liver fibrosis model.

	Marker		
Group	HSCs activation/ECM equilibrium		
	Serum level (ng/ml)		liver mRNA level
	TIMP-1	MMP-2	α-SMA
Control	5.383 ± 2.401	25.78 ± 8.736	1.068 ± 0.1410
ConA	55.06 ± 21.41 *	9.471 ± 3.297 *	5.707 ± 2.338 *
ConA + PFD	21.20 ± 7.715 #	19.52 ± 4.673 #	2.509 ± 0.8112 #
Control + PFD	4.543 ± 1.095	24.37 ± 7.224	1.139 ± 0.1673

The illustrated data represents the means ± SD of the serum level of TIMP-1 & MMP-2 along mRNA level of α-SMA in tissues homogenates of liver sections in the indicated groups (N = 10). Statistical analysis was carried out using one-way ANOVA and the least significant difference (LSD) test; *P < 0.05, vs. control untreated animals, # P < 0.05, vs. Con A-treated animals.

expression by about0.5 fold (Fig. 1D, Table 2).

3.3. Pirfenidone ameliorated histopathological/fibrotic modifications in the livers of ConA-treated mice

Haematoxylin–eosin (H&E) staining was used to evaluate the pathologic changes in liver tissues from mice treated with ConA and/or PDF. As indicated in Fig. 2 (A-D), liver tissues from vehicle-treated control mice (A) and mice-treated with PFD alone (B) exhibited normal histological structure of hepatic lobule. Conversely, the liver tissues obtained from ConA-treated mice showed massive inflammatory cells infiltration (C1), sinusoidal leukocytosis (C2), apoptosis of hepatocytes (C3), cholangitis in the portal triad (C4), cystic dilatation of the bile duct with fibroplasia in the portal triad (C5), along with sporadic cell necrosis of some hepatocytes and cytomegally of other hepatocytes (C6). Treatment with PFD remarkably attenuated the severity of ConA-induced pathological changes. The livers tissues from mice treated with ConA followed by PFD showed a few inflammatory cells infiltration (D1), slight Kupffer cells activation (D2), and binucleation of some hepatocytes (D3). Taken together, these findings indicated that treatment with PFD effectively attenuated ConA-induced liver injury in mice.

3.4. Pirfenidone mitigated ConA-mediated pathological accumulation of collagens in hepatic tissues

The cardinal sign of liver fibrosis is the accumulation of excessive amount collagens in the extracellular matrix. Thus, we used Masson's Trichrome stain to detect the extent of liver fibrosis in mice treated with ConA and/or PFD [Fig. 3 (A-D)]. The control group (A) and (saline/PFD) group (B) showed the absence of collagen fibers as indicated by the undetectable Masson trichrome stain. Conversely, evident collagen deposition was observed in liver tissues from ConA-treated mice (C1 & C2). However, the mice in the group treated with ConA/PFD provided liver sections with obvious reduction in the Masson's trichrome staining intensity (D1 & D2) as compared to sections from ConA-treated mice, suggesting the remarkable PFD-mediated attenuation of Con A-induced liver fibrosis.

3.5. Pirfenidone potentiated MMPs, suppressed collagens type II & IV and attenuated ConA-mediated TIMP-1 induction, promoting ECM degradation

Collagen is perhaps the most studied marker that forms the structural basis for much of the ECM of several tissues, including liver. The current study and others demonstrated that ConA activates HSCs and consequently, the upregulation of TIMP-2 and collagens with concomitant inhibition of MMPs [20]. Thus, we studied the effects of treatment with ConA and/or PFD on the expression of collagens (type II & IV), MMP-2, and TIMP-2. As demonstrated in figure (4), liver tissues from vehicle-treated control mice (A) or mice treated with PFD alone (B) showed a negligible expression level of collagen II, suggesting that normal livers don't express appreciable level of collagen type II. Conversely, in liver tissues from ConA-treated mice, there was a strong positive expression of collagen II in the portal triad (C1) and perisinusoidal spaces (C2). On the other hand, liver tissues from mice treated with ConA followed by PFD showed weak to moderate positive expression of collagen II perisinusoidally and around the bile duct (D1 & D2).

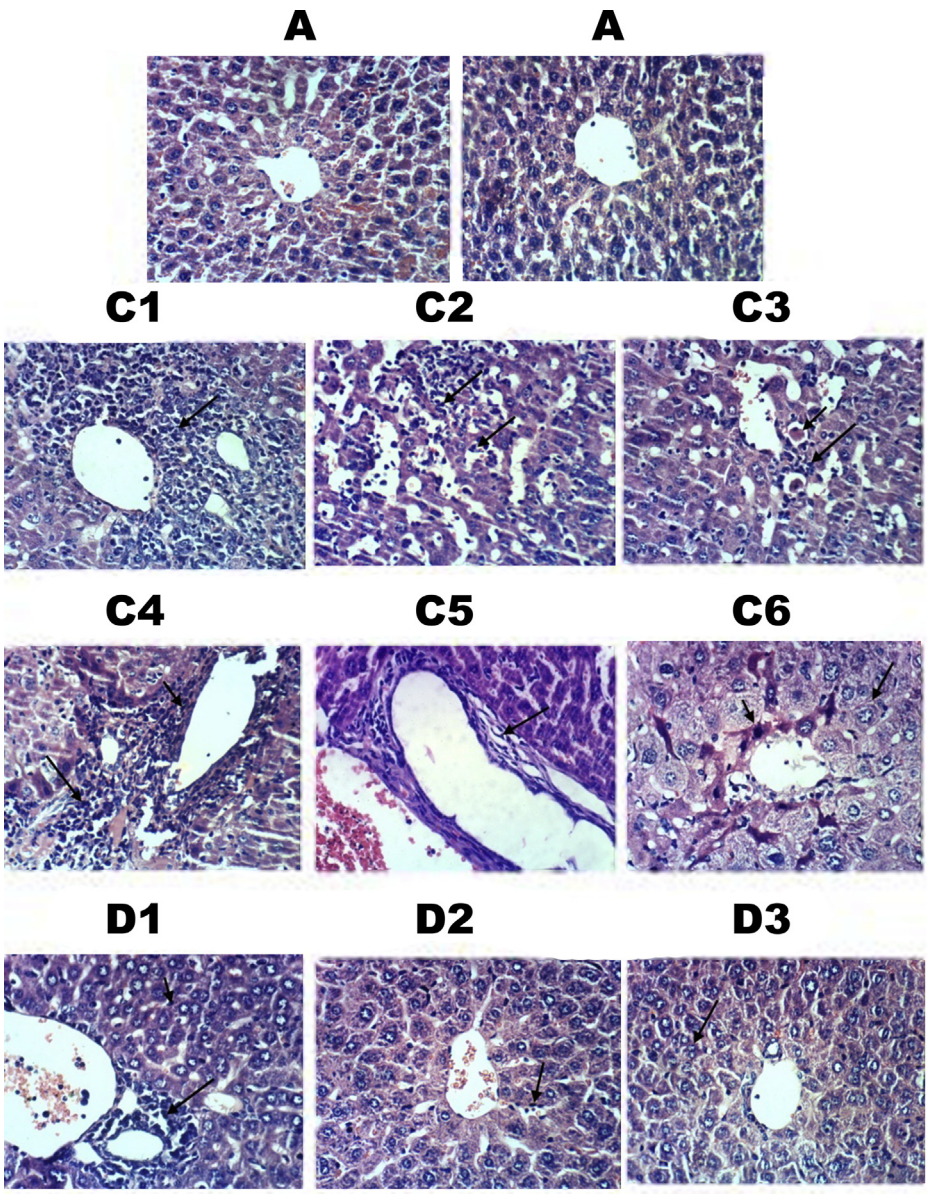

Fig. 2. Microphotomicrographs of liver sections with H&E staining. (A & B) liver sections from Control and (saline/PFD) groups, respectively, showing normal liver lobular architecture. Liver sections in C (1-6) represent ConA group; C1 massive inflammatory cells infiltration in the portal triad, C2 sinusoidal leukocytosis, C3 apoptosis of hepatocytes associated with inflammatory cells infiltration, C4 cholangitis, C5 cystic dilatation of the bile duct and fibroplasia in the portal triad, C6 sporadic cell necrosis and cytomegally of hepatocytes. Liver sections in D (1-3) represent (ConA + PFD) group; D1 few inflammatory cells infiltration in the portal triad, D2 slight Kupffer cells activation, D3 binucleation of hepatocytes. The number of samples examined in each class is 5 (n = 5), and representative images are shown, X 100).

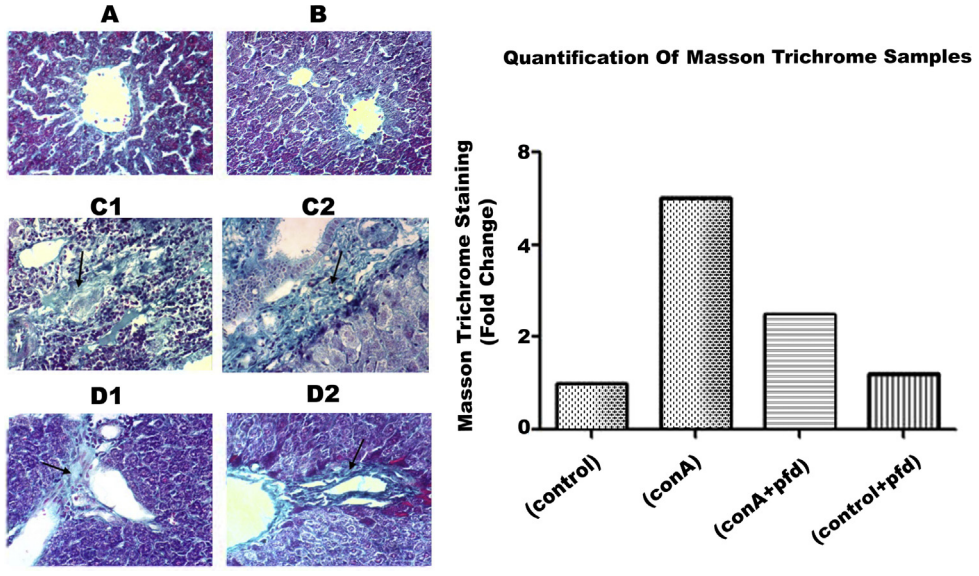

Fig. 3. Microphotomicrographs of liver sections with Masson's trichrome staining (left panel). A & B: liver sections from control and (saline/PFD) groups, respectively, showing no histochemical reaction for collagen fibers. Liver sections in C (1-2) represent sections from ConA group that exhibit strong positive histochemical reaction for collagen fibers. Liver sections in D represent sections from ConA + PFD group with weak (D1) positive histochemical reaction for collagen fibers, and moderate (D2) positive histochemical reaction for collagen fibers. The number of samples examined in each class is 5 (n = 5), and representative images are shown, X 100). Right panel: Bar charts show the fold changes in Masson's trichrome staining reflecting collagens expression; Quantifications were made using ImageJ 1.46r software.

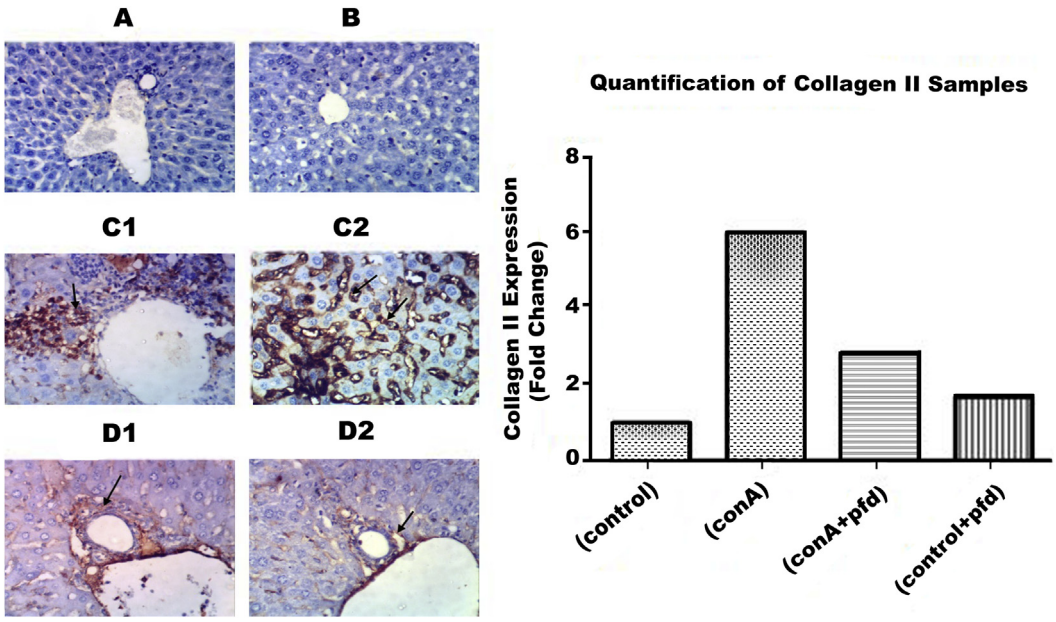

Fig. 4. Microphotomicrographs of liver sections stained for collagen II. Upper left panel (A & B) represents liver sections from control and (saline/PFD) groups, respectively, showing the normal negligible expression of collagen II. The middle left panel represent liver sections from ConA group that exhibit strong positive expression of collagen II in the portal triad (C1) and perisinusoidal (C2). The lower left panel represents the treated group (ConA + PFD) showing weak (D1) to moderate (D2) positive expression of collagen II perisinusoidally and around the bile duct. The number of samples examined in each class is 5 (n = 5), and representative images are shown, X 100. The bar charts in the right panel show the fold changes in collagen II expression; Quantifications were made using ImageJ 1.46r software.

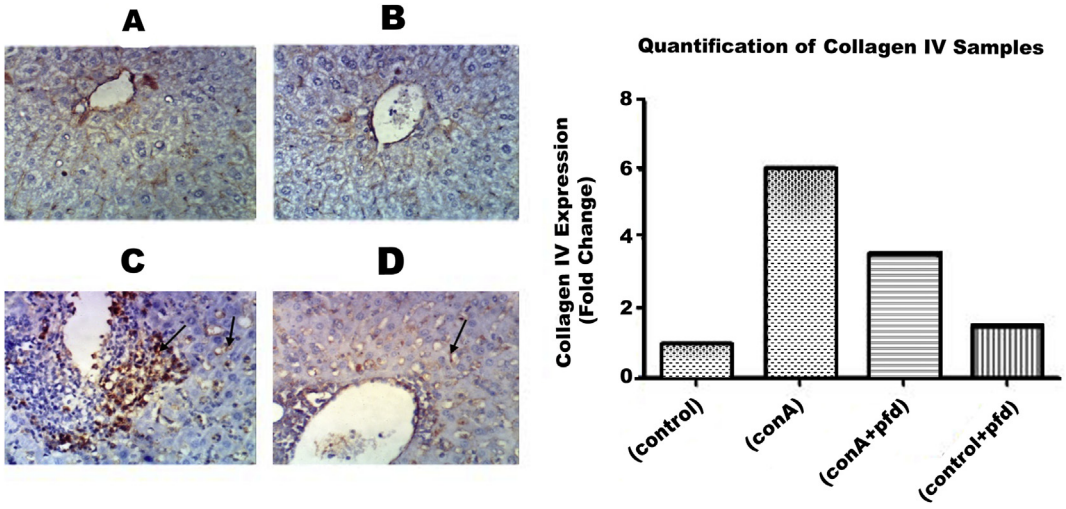

Fig. 5. Microphotomicrographs of liver sections stained for collagen IV (left panel). A & B: sections from control and (saline/PFD) groups, respectively, showing normal expression of collagen IV. C: Section from ConA group that exhibits positive expression of collagen IV in the portal triad and perisinusoidal spaces. D: represents a section from the treated group (Con A + PFD) showing weak positive expression of collagen IV. The number of samples examined in each class is 5 (n = 5), and representative images are shown, X 100. The bar charts in the right panel show the fold changes in collagen IV expression; Quantifications were made using ImageJ 1.46r software.

Comparable alterations in collagen type IV were seen in Fig. 5. Our results showed that there was a basal expression level of collagen type IV (Fig. 5; A & B). In ConA-treatment mice, abundant expression of collagen type IV was observed in the portal triad and perisinusoidal spaces (C). This ConA-induced expression was substantially attenuated by PFD treatment (D).

Since the ECM turnover is largely mediated by the interaction between MMPs and their TIMPs, it is well-established that a correlation and reciprocal influences between MMP and their TIMP determines the combined effect on ECM turnover. Hence, our analysis was extended to explore the effect of ConA and/or PFD treatments on the serum level of MMP-2 and TIMP-1. As demonstrated in (Table 2; Fig. 6A), the treatment with ConA significantly reduced MMP-2 expression. The serum levels of MMP-2 were 25.78 ± 8.74; 9.47 ± 3.3; 19.52 ± 4.67 and 24.37 ± 7.22 ng/ml, in vehicle-treated control mice; ConA-treated mice, mice treated with ConA followed by PFD and PFD-treated mice, respectively. These results clearly demonstrated that ConA treatment resulted in a significant decrease in the serum level of MMP-2 by about 0.6 fold as compared to vehicle treated control mice ($P < 0.05$), while in sera from mice treatment with PFD for two weeks after ConA treatment, the MMP-2 level was increased by about one fold compared to the ConA treated mice (Fig. 6A).

On the other hand, treatments with ConA and/or PFD conversely alter the expression of TIMP-1. As shown in (Table 2; Fig. 6B), ConA

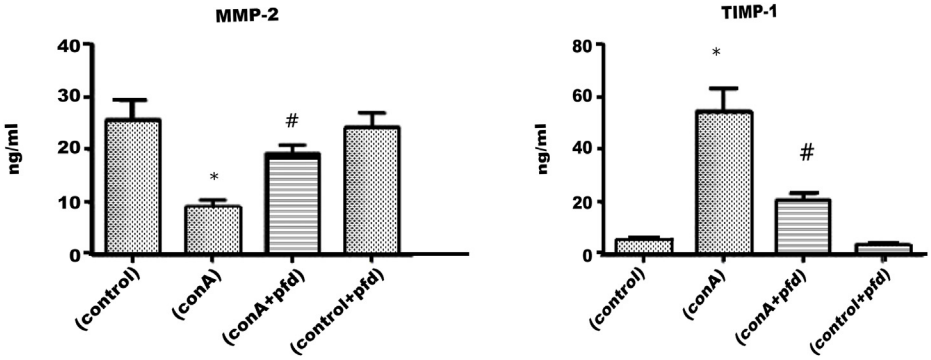

Fig. 6. Modulatory effects of PFD on ECM degradation in ConA-induced liver fibrosis model. Serum levels of the indicated biomarkers (A & B) were determined as described in methods section. Bar charts represents mean ± SD, *P < 0.05, vs. control group, #P < 0.05, vs. ConA group, (N = 10).

treatment induced a sharp rise in the mean serum level of TIMP-1 (about nine-fold) compared to control group. Such rise was markedly decreased by about 0.6 fold after treatment with PFD for two weeks after ConA, compared to the group of mice treated with ConA alone.

4. Discussion

Chronic viral hepatitis and autoimmune hepatitis are chronic hepatic inflammatory diseases that lead to the progression of hepatic inflammation to fibrosis and cirrhosis. Fibrosis is characterized by the excessive deposition of ECM in- and around injured tissues. If unresolved, fibrosis can lead to permanent scarring, organ failure, and, eventually, death [1,2,21]. Liver fibrosis can progress into advanced stage, cirrhosis, which is characterized by a severe distortion of the delicate hepatic vascular architecture, the shunting of the blood supply away from hepatocytes and the resultant functional liver failure. Therefore, it is important to evaluate the degree of hepatic fibrosis when diagnosing and treating such condition. The currently used remedies for chronic liver inflammation are not designed to be antifibrotic, but they depend on eliminating the etiological agents or damping the immune response which can halt the fibrotic process [22]. The present study served as a trial to investigate the possible antifibrotic effect of Pirfenidone (PFD) in ConA induced liver fibrosis model. ConA-induced fibrosis provides a suitable model for directly investigating the mechanisms and possible methods of treatments of T-cells-mediated hepatitis [14]. Nevertheless, the dose of ConA necessary for triggering liver inflammation and fibrosis varies considerably depending on the genetic background of the used animals and also the microbial content specific for the animal facility barrier level [14]. Also the duration and number of injections required for induction of fibrosis vary between previous studies [14,23–26]. The standard operating procedure (SOP) comprises a detailed protocol for the ConA application, including preparation of ConA working solution, handling of the animals, choice of the appropriate conditions and endpoints, as well as efficient dose-finding [14]. Accordingly, based on our several preliminary studies in our lab to identify the dose and treatment time-schedule suitable for the strain adopted in the current study, ConA was given to male mice in a dose of 10 mg/kg/week by i.v. route for 4 weeks. Although female mice may show a higher susceptibility to ConA, the study was limited to male mice because of the great variation in the disease outcome associated with female mice [14]. Our therapeutic regimen was based on administration of PFD in a dose of 125 mg/kg/day that was optimal for providing antifibrotic effect against ConA without induction of CNS depression or mortality. Previous toxicity studies of single oral dose PFD were conducted in SW & B6C3F1 mice and other species. A dose of 1,000 mg/kg was the maximum non-lethal dose and the LD_{50} in B6C3F1 mice and SW mice, respectively (https://www.tga.gov.au/sites/default/files/auspar-pirfenidone-160809.pdf). Mortality was preceded by hypoactivity, ataxia, salivation, ptosis, lacrimation, abnormal gait and abnormal respiration. In addition, in FDA pharmacological reviews for PFD: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2014/022535Orig1s000PharmR.pdf); the minimum lethal dose was 792-2000 mg/kg in different mice strains and the LD_{50} for males was 1026.8 mg/kg. The side effects noticed in our preliminary studies were hypoactivity, abnormal gait, and reduced activity. In our current study, PFD at a dose of 125 mg/kg, did not cause any mortality and was associated with minimal side effects. From the clinical point of view, the recommended average dose of pirfenidone (Esbriet® tablets) in treatment of IPF is 801 mg/day, while the maximum tolerable dose is 2403 mg/day (equivalent to 11.4 mg/kg & 34.3 mg/kg, respectively, for 70 kg individual) (https://www.esbriethcp.com/safety-tolerability-dosing/dosing.html). Based on body surface area (BSA) normalization method for human/animals drug doses conversion schedule, the mouse dose used in the current study (125 mg/kg) is less than 30% of the equivalent daily maximum tolerable human PFD dose (423 mg/kg), and less than 90% of the equivalent daily recommended average human PFD dose (140 mg/kg). Importantly, hepatotoxicity due to PFD is rare and generally manifests as a mild rise in serum aminotransferases [27].

Hepatocytes death, inflammation, and liver fibrosis (with fibroblast activation) are the hallmarks of chronic liver disease. TNF-α is an inflammatory cytokine involved in sustained liver inflammation that leads to liver fibrosis [17]. Because tissue fibrogenesis is a complex process that has several cascades, involving inflammatory cell infiltration, apoptotic cell death, ECM expansion, and fibroblast activation, we analyzed the expression profile of different serum biomarkers that are widely used as sensitive markers for hepatic fibrosis such as TGF-β, TNF-α [1,28,29], TIMPs & MMPs [20,30]. Importantly, fibroblasts contribute to tissue fibrosis in multiple ways, including activation of the chemotactic TGF-β, secreting excess collagen and other ECM proteins [31]. Numerous studies have identified activated myofibroblasts as the common cellular element ultimately responsible for the replacement of normal tissues with nonfunctional fibrotic tissue, with critical signaling cascades, initiated primarily by TGF-β [32]. In addition, several chemokines that are released by diverse infiltrating cells modulate the inflammatory reaction and contribute to the progression of HSCs activation and the fibrotic insult, demonstrating the complexity of the disease process [5]. It is also worthy to mention that the correlations between hepatic levels of HP, an amino acid residue found only in collagen and elastin, and hepatic fibrosis, was established in several studies [16,33]. Interestingly, serum HP level provides a valuable information about the biochemical and pathologic events of hepatic fibrosis and reduce the need for liver biopsy [19,34]. Furthermore, liver fibrogenesis involves the activation of the quiescent HSCs into an activated myofibroblasts that are characterized by over expressing α-SMA [35], making it a good indicator for early stages of hepatic fibrosis, and another reliable marker of HSCs activation which precedes fibrous tissues collagens deposition [36]. In the present study, induction of

fibrosis after ConA administration was confirmed by observing a significant elevation in the serum levels of TNF-α, TGF-β, HP protein levels and hepatic α-SMA mRNA contents along with the increase in the levels of collagens in the fibrotic liver samples in the ConA group. PFD treatment, on the other hand, decreased such elevations to a great extent which reflects the inhibitory effect on HSCs activation and, in turn, reflects the anti-fibrotic effect of this drug.

The histological findings of liver sections (H&E and MTC staining) in the current study showed similar patterns of the resultant degree of fibrotic reactions and antifibrotic outcomes with respect of the used treatment regimens. The present study showed that injection of ConA resulted in massive inflammatory cells infiltration, significant accumulation of collagen fibers with a classical appearance of fibrotic response. On the other hand, the treated group (ConA + PFD) showed only slight Kupffer cells activation, binucleation of hepatocytes and weak to moderate positive histochemical reaction for collagen fibers which may favor regeneration of liver with decrease in the inflammatory cells infiltration, representing the antifibrotic effect of PFD. Importantly, previous studies show that during progression from normal liver to cirrhosis, total collagen increases nearly 10-fold with an abnormal increase in fibril-forming collagen and other ECM molecules [37,38]. Activated HSCs, portal fibroblasts, and myofibroblasts of bone marrow origin have been identified as major collagen-producing cells in such injured livers [1]. In fact, collagens' elevations during fibrogenesis shows distinct patterns, such that expression of certain collagens may predominate in early phase of fibrosis and other collagens may be more prominent in late fibrosis [37]. In particular, collagens III, IV, V, and VI all showed statistically significant increases from early to late stages [37]. To date, although no studies have investigated the expression of collagen type II in fibrotic livers, our work demonstrated dramatic differences in the levels of collagen II between normal- and fibrotic liver sections. In spite of negative expression of collagen II in normal livers as shown by immunohistochemistry staining (Fig. 4A & B), levels of collagen II markedly increased after ConA treatment (Fig. 4C) in correlation with induction of other fibrotic markers, being one of our interesting findings in the present study. Such elevation was markedly decreased after treatment with PFD (Fig. 4D). As expected, similar manner of the increase of the expression of collagen type IV was observed in the present study in samples of ConA-treated group of mice (Fig. 5C), with a marked decrease in PFD-treated one (Fig. 5D).

It should be noted that pathogenesis of liver fibrosis orchestrates the complex interplay of various hepatic cells including hepatocytes, Kupffer cells and HSCs [17]. Activated HSCs not only play a major role in the deposition of excess ECM proteins, including fibrillar collagens but also regulate matrix degradation in the liver through a combination of inhibiting the active matrix-degrading enzymes, MMPs and increasing TIMPs. It was previously shown that inhibition of matrix degradation mediated by a change in HSC-expression of TIMPs relative to MMPs, such as interstitial collagenase, may contribute to progression of liver fibrosis [20]. Up-regulation of TIMP-1 during hepatic fibrogenesis promoting fibrosis in the injured liver by inhibition of MMPs-mediated degradation of ECM, causing hepatic fibrogenesis to progress more rapidly [39]. The implication of the alteration in the extent/pattern of matrix degradation led to a conclusion that liver fibrosis is potentially reversible and that ECM deposition could be reversed if the TIMP-MMP balance is altered to favor ECM degradation [3]. In our study, serum levels of MMP-2 and TIMP-1 were inversely proportional to each other. ConA was found to elevate serum level of TIMP-1 and decrease MMP-2 level which favors ECM accumulation. However, the group that further treated with PFD demonstrated that TIMP-1 levels were greatly decreased with a significant increase in MMP-2 levels which reversed of the fibrosis and reflect the antifibrotic effect of PFD. These observations have led to the suggestion that the anti-fibrotic effects of PFD might be also mediated via inhibition of HSC-expression of TIMPs along with promoting of matrix degradation mediated by MMPs, to offer limitation of liver fibrosis progression. Overall, the pattern of elevation/decrease of the investigated collagens in this study was parallel to the elevation/decrease in serum fibrotic biomarkers.

5. Conclusion

This study provides evidence that PFD promoted the resolution of hepatic fibrosis through decreasing HSCs activation, decreasing TGF-β, TNF-α, HP and α-SMA levels, altering TIMP-MMP balance to favor ECM degradation in liver fibrosis which resulted in decreasing collagen content. Further experimental & clinical investigations are required to confirm the therapeutic effects of PFD in liver fibrosis.

5.1. Limitations of study

The concepts presented in the current study are based on experimental rodent model of ConA-induced hepatic fibrosis. Thus, it is now important to extend the model to human study, especially in patients with IPF and hepatic fibrosis co-morbidities. Ultimately, it will also be important to translate these observations into improved therapy for patients with hepatic fibrosis. This will involve the demonstration of the therapeutic efficacy and safety of PFD in hepatic fibrosis. In addition, we acknowledge that, the molecular mechanism underlying the therapeutic effects of PFD in ConA-induced hepatic fibrosis in rodent model may be distinct from that in patients with immune-mediated hepatic fibrosis.

Declaration of competing interest

The authors declare no competing interests.

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