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Original article

Protective role of pirfenidone against experimentally-induced pancreatitis

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ABSTRACT

Background: Pirfenidone (PFD) is an orally active antifibrotic agent that has anti-inflammatory activity in diverse animal models. Its effect against acute pancreatitis (AP) has not been elucidated. Hence, the present investigation was carried out to assess the potential protective role of PFD against L-arginine-induced AP in mice.

Methods: AP was induced in adult male Swiss albino mice *via* intraperitoneal injections of L-arginine (4 g/kg, twice each 1 h apart). PFD (250 mg/kg, orally) was administered one day before and on the day of L-arginine challenge. Twenty-four hours after L-arginine injection, the severity of AP was evaluated using biochemical and histological analyses. Indices of oxidative stress, inflammation and apoptosis were evaluated using ELISA and immunohistochemistry (IHC).

Results: PFD suppressed the development of L-arginine-induced AP as revealed by the improvement of histopathological lesions of pancreatic specimen and the significant reduction of serum amylase and lipase levels. Notably, PFD reduced the lipid peroxidation and enhanced the antioxidants such as reduced glutathione (GSH) and superoxide dismutase (SOD) in pancreatic tissue. Importantly, PFD suppressed AP-associated elevation of inflammatory cytokines along with depression of nuclear factor kappa-B (NF-κB) immuno-expression in pancreatic tissue. Lastly, PFD efficiently ameliorated AP-induced elevation of the pro-apoptotic protein (Bax) and increased AP-induced reduction of the anti-apoptotic protein (Bcl2).

Conclusions: PFD protected against L-arginine-induced AP in mice through anti-oxidative, anti-inflammatory and anti-apoptotic properties.

Introduction

Acute pancreatitis (AP) is a pathological inflammatory condition of pancreas that varies from mild to severe. Most patients with AP exhibit mild pancreatic injury with no complications while about 20% suffer from life-threatening diseases with multiple organ dysfunctions and high rate of morbidity [1]. So far, the current management of AP includes supportive management and treatment of the developed complications and thus, searching for effective therapeutics is mandatory [2]. Many investigations were performed to elucidate the exact mechanisms that regulate the development and severity of AP. However, the precise events underlying the pathogenesis of AP within pancreatic acinar cells are still unclear. Inflammatory cytokines have been reported to play a central role in the induction and modulation of AP [3,4]. Earlier studies have reported the elevation of serum tumor necrosis factor-α (TNF-α) and interleukins (ILs) in AP patients [5]. Moreover, the activation of nuclear factor-kappa B (NF-κB) with subsequent recruitment of neutrophils and release of inflammatory cytokines has crucial roles in AP [6,7]. In this regard, searching for new drugs that target the key components of the immune system, as neutrophils, and suppress the inflammatory response seems to be the most promising approach to protect against AP.

Pirfenidone (PFD) is a synthetic pyridone derivative which has evident anti-inflammatory and anti-fibrotic activities in experiments and clinical trials [8–10]. It has been approved since 2011 for the treatment of idiopathic pulmonary fibrosis [11,12]. Additionally, numerous clinical reports have revealed the safety and efficacy

Abbreviations: ANOVA, one-way analysis of variance; AP, acute pancreatitis; H&E, hematoxylin-eosin; GSH, reduced glutathione; IHC, immunohistochemical; IL-6, interleukin-6; MDA, malondialdehyde; NF-κB, nuclear factor-kappa B; PFD, pirfenidone; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α.

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of PFD in other diseases such as liver fibrosis, chronic hepatitis C, myelofibrosis, and fibrotic renal disorders [13–16]. To date, intense efforts have been done to clarify the exact underlying mechanisms of anti-fibrotic effects of PFD. Studies have shown that PFD can inhibit both production and activity of transforming growth factor-β (TGF-β). Other studies suggested that the antifibrotic effect of PFD might be attributed to its ability to ameliorate the transcription of TGF-β along with procollagen I and III genes in lung and liver fibrosis models [15,17,18]. Moreover, PFD exhibited potent efficacy to stop the progression of fibrosis *via* its ability to induce the matrix metalloproteinases and decrease the transcription of tissue inhibitors of metalloproteinase. In addition to its anti-fibrotic activity, the anti-inflammatory activity of PFD is well documented in several experimental studies. PFD showed potent hepatoprotective effects against D-galactosamine/lipopolysaccharide-induced hepatic injury [19] as well as concanavalin-A induced autoimmune hepatitis [20]. The anti-inflammatory activity of PFD was attributed to its capability to suppress the production of inflammatory cytokines such as TNF-α, ILs and nitric oxide [21,22]. PFD can also modulate nuclear factor-erythroid-related factor 2 pathway which enhances its antioxidant capacity [23]. Recently, PFD was found to impair Th2 differentiation and ameliorate Th2 profibrogenic response in liver fibrosis *via* inhibition of p38 mitogen activated protein kinase [24]. Till now, no studies have evaluated the effects of PFD against pancreatitis. Therefore, the target of our study was to test the efficacy of PFD to protect against experimentally induced pancreatitis and explore the possible pathways.

Materials and methods

Chemicals

PFD was bought from Tocris Bioscience (Bristol, UK) and suspended in 0.5% carboxymethylcellulose. L-Arginine hydrochloride was purchased from Sigma Aldrich (St. Lois, MO, USA) and prepared as 8% solution in normal saline (pH 7.0). Other reagents and biochemicals were of highest analytical grades.

Animals and experimental design

Male Swiss albino mice, weighing 18–25 g, were left to acclimatize for 1 week before starting the experimental work. Animals had free access to standard laboratory food and water under standard conditions of humidity and temperature. All animal related procedures were approved by the Research Ethics Committee of Faculty of Pharmacy, Mansoura University which follows "Principles of Laboratory Animals Care" (National Institute of Health).

Mice were randomly assigned into three experimental groups, each of eight mice. The first one served as control group where mice received intraperitoneal injection of saline two times (1 h apart). The second one was AP group where mice were injected with L-arginine (4 g/kg, *ip*, twice each 1 h apart) [25]. The third one was AP + PFD group where mice received PFD (250 mg/kg, orally once a day) one day before and on the day of AP-induction. Previous studies reporting the protective anti-inflammatory activities of PFD were taken into consideration during the selection of PFD dose [20,26]

Twenty-four hours after administration of L-arginine, mice were anesthetized and humanely sacrificed by cervical dislocation. Blood samples were collected to measure serum amylase and lipase activities. Pancreas from each mouse was rapidly isolated, weighed and divided into two parts. On pancreatic part was fixed in 10% neutral buffered formaldehyde and submitted for histological assessment. The other part of pancreas was used to prepare tissue homogenate (10%w/v) using ice-cold phosphate-buffered saline (PBS; 10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl; pH 7.4).

Histological examination

Fixed pancreatic tissues were embedded in paraffin and sectioned at 5-μm thickness. Specimens were stained by hematoxylin-eosin (H&E) and examined in random order without knowing the animal or the group. The severity of pancreatic lesions was evaluated and graded according to a semiquantitative score ranking from 0 to 3 depending on the degree and extent of histopathological damage.

Amylase estimation

Amylase activity was measured by UV–vis double-beam spectrophotometer (Labomed, USA) using kinetic assay kit (Spectrum, Egypt) based on the manufacturer's procedures. In brief, serum was incubated for 1 min at 37 °C with amylase reagent and absorbance was measured at 405 nm every minute for the subsequent 4 min. The mean change in absorbance per minute was used to determine amylase activity.

Lipase estimation

Lipase activity was measured using spectrophotometric kinetic assay kit (Spectrum, Egypt) according to the manufacturer's instructions. Absorbance was measured at 580 nm every minute for 2 min. The changes in absorbance were used to calculate lipase activity.

Immunohistochemical estimation of nuclear factor-κB (NF-κB) p65 in pancreatic tissue

Pancreas specimen was exposed to immunohistochemical staining of NF-κB p65. Briefly, sections were dewaxed, hydrated, and immersed in an antigen retrieval. Incubation with NF-κB p65 primary antibody for 1 h (Thermo Fisher Scientific, Waltham, MA, USA; dilution 1:100) was done after treatment with hydrogen peroxide and protein block. Specimens were incubated with secondary antibody (EnVision + System HRP; Dako) for 30 min at room temperature. 3,3′-diamino benzidine was used for visualization then post-fixation was performed. Sections were then counterstained using Mayer's hematoxylin. The results of NF-κB immunostaining were expressed as the percentage of immunopositive cells across 10 high power fields.

Estimation of parameters of oxidative stress and antioxidant status

Supernatants of pancreatic homogenates were used for the estimation of malondialdehyde (MDA), reduced glutathione (GSH) and superoxide dismutase (SOD). The MDA content was used as index of lipid peroxidation and was determined as described previously [27]. In brief, MDA reacts with thiobarbituric acid to form a pink chromogen which was measured spectrophotometrically at 532 nm. Tetramethoxypropane was used as an external standard. MDA level was expressed as nmol/g wet tissue.

GSH was determined according to the method described by Ellman [28]. The method is based on the reduction of Ellman's reagent by (—SH) groups of GSH to produce a yellow-product which was measured spectrophotometrically at 412 nm. GSH concentration was expressed as μmol/g wet tissue.

SOD activity was determined based on inhibition of autooxidation of pyrogallol as described by Marklund [29]. Briefly, the reaction mixture (pyrogallol in 10 mM HCl and Tris buffer; pH 7.8) was added to samples at 25 °C. Absorbance change at 420 nm was recorded for 3 min at 1-min interval. One unit of enzyme activity is defined as 50% inhibition of pyrogallol autooxidation under the assay conditions. The SOD activity was expressed as Unit/g wet tissue.

Cytokines estimation

Levels of NF-κB, TNF-α and IL-6 in the supernatants of pancreatic tissues were determined using ELISA kits according to manufacturer's protocol (CusaBio, USA; R&D Systems, USA; MyBioSource Inc., USA respectively).

Estimation of apoptosis markers

The apoptotic marker, Bax (Bcl-2 Associated X Protein) and the anti-apoptotic marker (Bcl2) were determined in the supernatants of pancreatic tissues using commercial kits according to manufacturer's instructions (MyBioSource Inc., USA).

Statistical analysis

One way analysis of variance (ANOVA) followed by Tukey's Kramer Multiple Comparison Test were used for data analysis. Non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison post-test were used for analysis of histopathological scores. Represented data are means ± SEM; $p < 0.05$ was considered as significant.

Results

Effect of PFD on the histopathology of pancreas in L-arginine-induced AP

As shown in Fig. 1, L-arginine administration resulted in acute pancreatic injury in the form of cellular necrosis, marked perivascular edema, cell vacuolization and infiltration of inflammatory cells into the pancreatic tissue. PFD treatment significantly ameliorated these pathological changes and improved the histopathological picture of the pancreas.

Effect of PFD on amylase and lipase activities in L-arginine-induced AP

Elevation of serum amylase and lipase activities clearly reflected pancreatic acinar cell injury. As shown in Fig. 2A and B, L-arginine

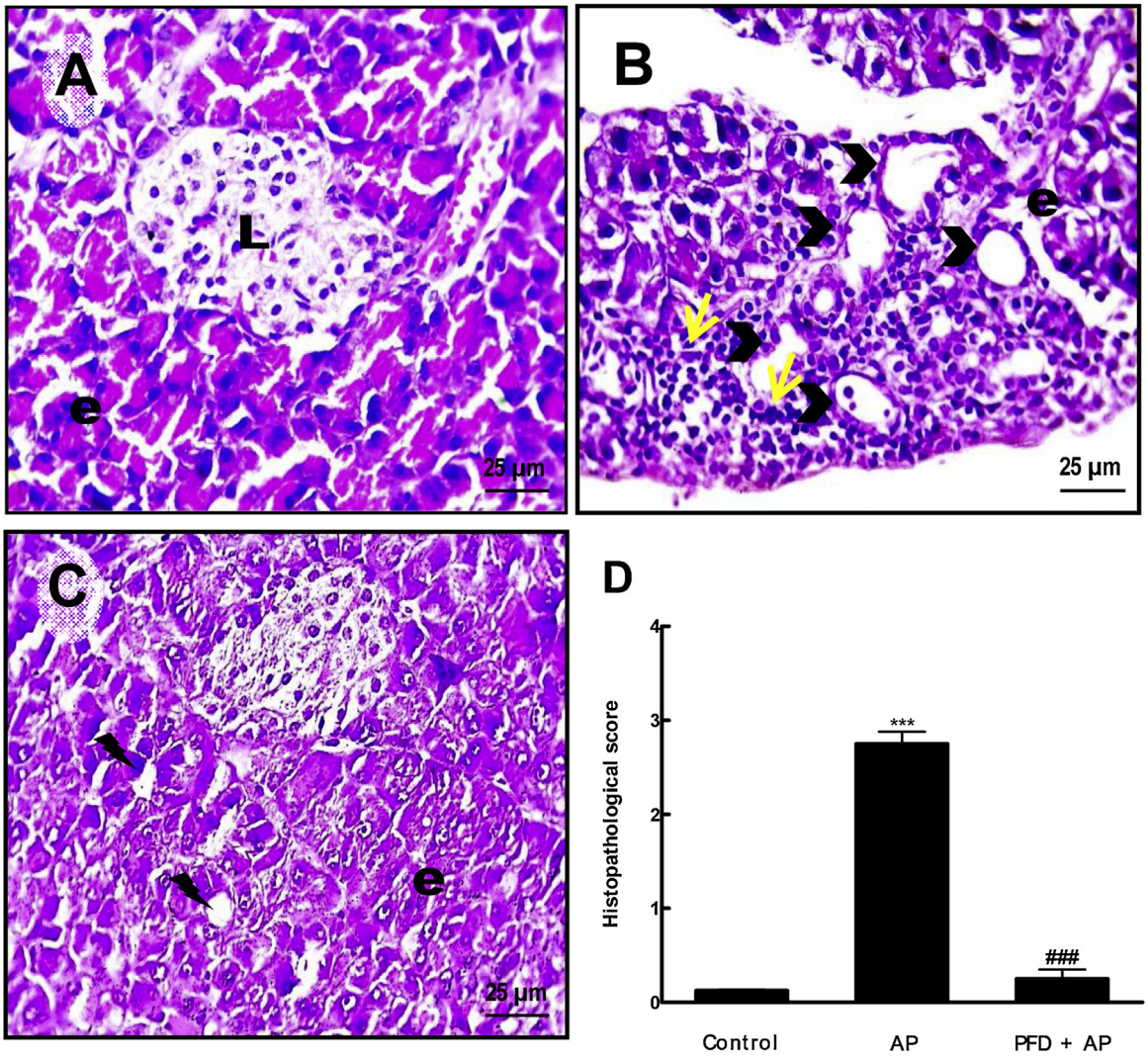

Fig. 1. Pirfenidone (PFD) attenuated the pancreatic lesions in L-arginine-induced acute pancreatitis (AP) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.). Microscopic pictures of the pancreatic tissues among different groups (H&E, 400×). (A) Control: Specimen showed normal histology of exocrine acini (e) and islet of Langerhans (L) (B) AP: Pancreatic tissue showed significant disruption of normal architecture with marked necrotic β cells of islet of Langerhans (arrowheads) and infiltration of inflammatory cells between acini (yellow arrows), (C) PFD + AP: Specimen of pancreatic tissues showed marked improvement of the pancreatic lesions except for few individual acinar cell vacuolization (lightning bolt), (D) Semiquantitative analysis of the pancreatic lesions. AP was induced by twice intraperitoneal injections of L-arginine (4 g/kg, each 1 h apart). Mice received PFD (250 mg/kg, orally once a day) one day before and on the day of AP-induction. Data are mean ± SEM (n = 8). $^{***}p <$ 0.001 *vs.* the control; $^{###}p < 0.001$ *vs.* the AP group (Kruskal-Wallis test followed by Dunn's multiple comparison).

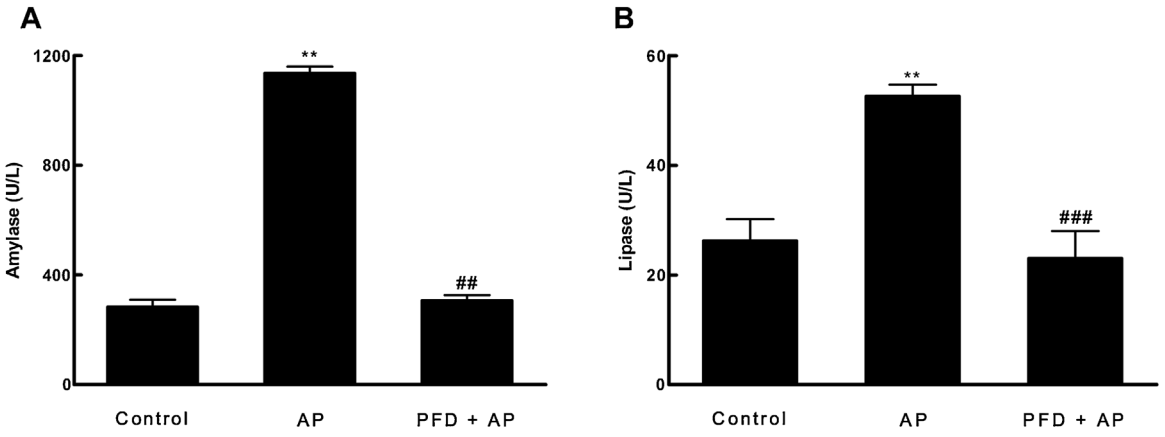

Fig. 2. Pirfenidone (PFD) ameliorated high serum levels of amylase and lipase levels in L-arginine-induced acute pancreatitis (AP). AP was induced by twice intraperitoneal injections of L-arginine (4 g/kg, each 1 h apart). Mice received PFD (250 mg/kg, orally once a day) one day before and on the day of AP-induction. Data are mean ± SEM (n = 8). $^{**}p < 0.01$ *vs.* the control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* the AP group (ANOVA followed by Tukey-Kramer multiple comparison).

administration induced a significant elevation of amylase and lipase activities compared to control animals. On the other hand, PFD reduced the serum concentration of these enzymes compared to the AP group.

Effect of PFD on oxidative stress parameters in L-arginine-induced AP

L-arginine injection caused elevation of oxidative stress as evidenced by significant high level of MDA in AP group compared

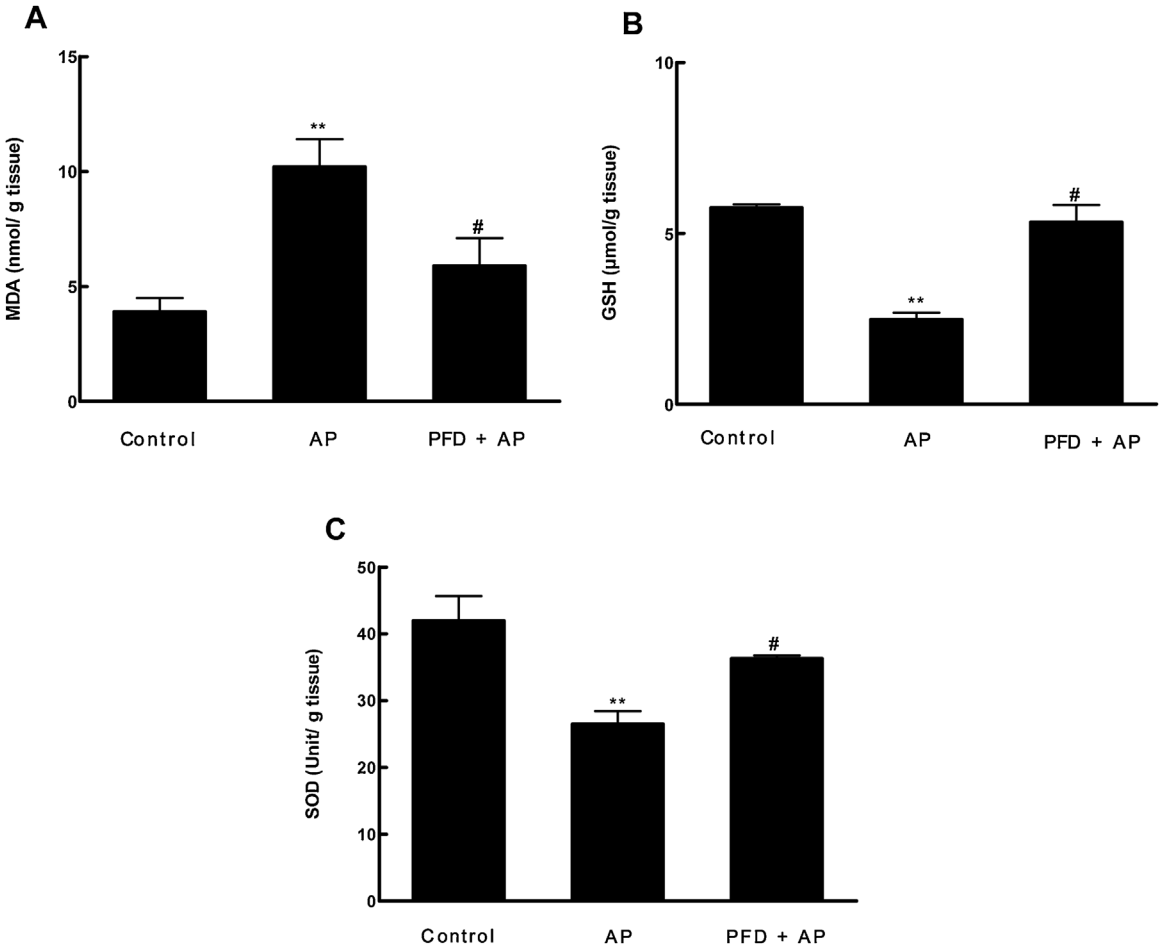

Fig. 3. Pirfenidone (PFD) attenuated oxidative damage and enhanced antioxidant parameters in L-arginine-induced acute pancreatitis (AP). A) Malondialdehyde (MDA) B) Reduced glutathione (GSH) C) Superoxide dismutase (SOD). AP was induced by twice intraperitoneal injections of L-arginine (4 g/kg, each 1 h apart). Mice received PFD (250 mg/kg, orally once a day) one day before and on the day of AP-induction. Data are mean ± SEM (n = 8). $^{**}p < 0.01$ *vs.* the control; $^{\#}p < 0.05$ *vs.* the AP group (ANOVA followed by Tukey-Kramer multiple comparison).

to the control group (Fig. 3A). Furthermore, L-arginine significantly decreased GSH content and SOD activity (Fig. 3B and C). However, PDF treatment significantly ameliorated MDA level simultaneously with significant increase in GSH and SOD levels compared to AP group.

Effect of PFD on inflammatory cytokines in L-arginine-induced AP

As shown in Fig. 4, L-arginine induced a significant increase in the immuno-expression and the level of NF-κB in the pancreatic tissue as compared to control group. Additionally, L-arginine elevated the levels of TNF-α and IL-6 in the pancreatic tissue as compared to the control mice. PFD treatment showed efficacy to suppress the immuno-expression of NF-κB and to decrease the elevated levels of NF-κB and the inflammatory cytokines (TNF-α and IL-6) as compared to AP group.

Effect of PFD on apoptosis in L-arginine-induced AP

L-arginine injection increased the levels of pro-apoptotic protein, Bax and decreased the level of anti-apoptotic protein, Bcl2 as compared to control group (Fig. 5). PFD administration resulted in amelioration of the elevated level of Bax simultaneously with increase in the level of Bcl2 as compared to AP group.

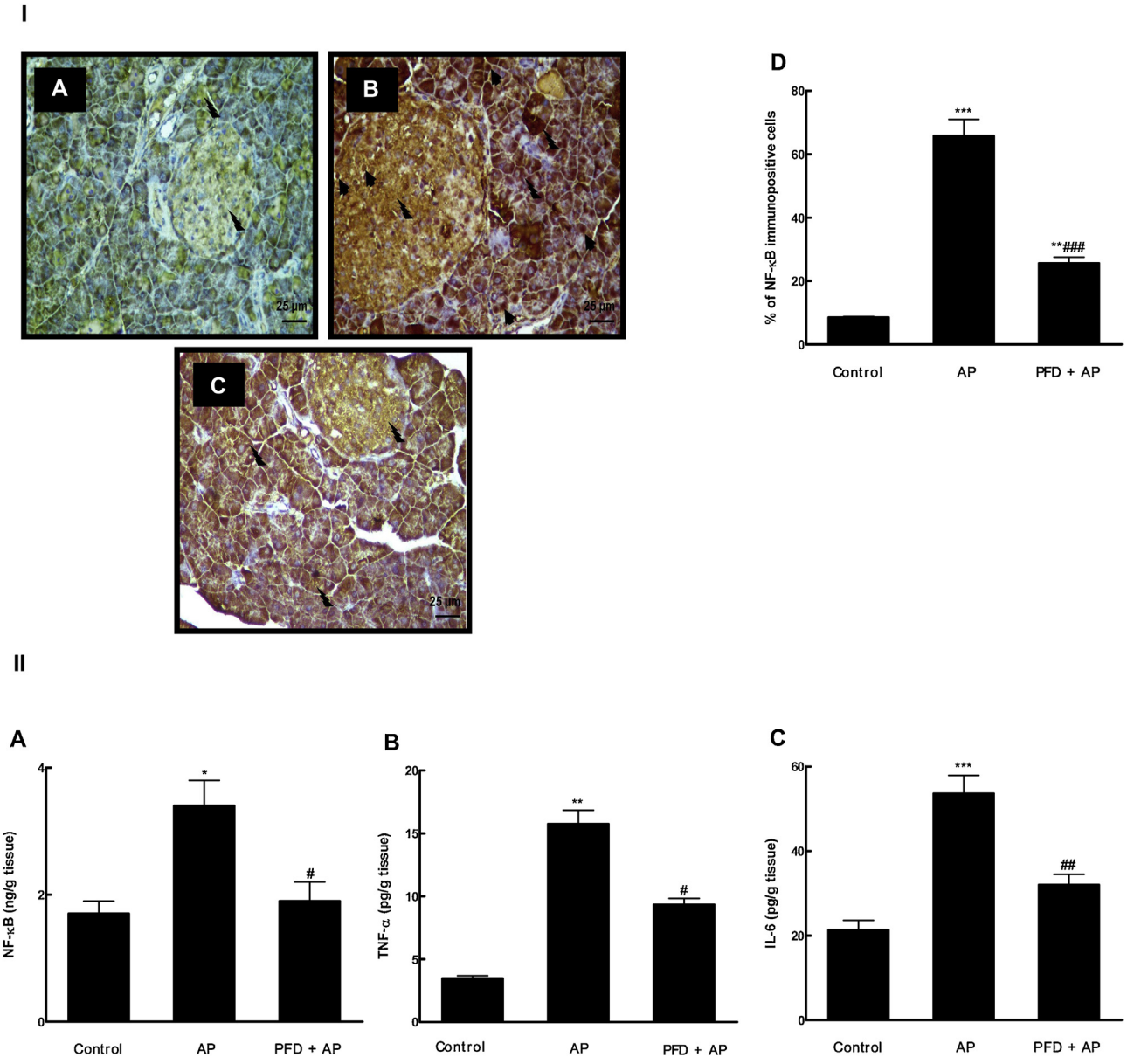

Fig. 4. Pirfenidone (PFD) suppressed the activation of nuclear factor-κB (NF-κB) and decreased the levels of inflammatory cytokines in L-arginine-induced acute pancreatitis (AP). I. Representative NF-κB immunostaining of pancreatic sections (×400) showing (A) Normal pancreatic control with minimal yellowish cytoplasmic staining (lightning bolt) both in exocrine acinar cells and endocrine Islet of Langerhans cells (B) Liver specimen of AP group showed marked nuclear (arrow head) and cytoplasmic (lightning bolt) brown staining both in the acinar and the Islet of Langerhans cells (C) PFD + AP group showed mild cytoplasmic brownish staining (D) Semi-quantitative analysis of NF-κB immunostaining results in pancreatic tissues expressed as % of NF-κB immunopositive cells. II. Levels of (A) NF-κB; (B) Tumor necrosis factor-alpha (TNF-α); (C) Interleukine-6 (IL-6). AP was induced by twice intraperitoneal injections of L-arginine (4 g/kg, each 1 h apart). Mice received PFD (250 mg/kg, orally once a day) one day before and on the day of AP-induction. Data are mean ± SEM (n = 8). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* the control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* the AP group (ANOVA followed by Tukey-Kramer multiple comparison).

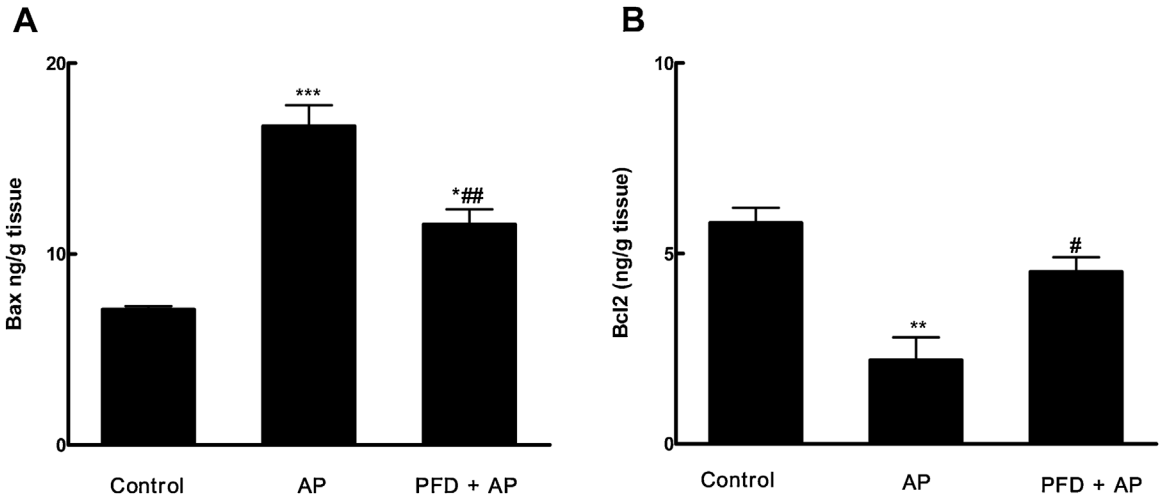

Fig. 5. Pirfenidone (PFD) decreased the levels of the pro-apoptotic marker (Bax) and the anti-apoptotic marker (Bcl2) in L-arginine-induced acute pancreatitis (AP). AP was induced by twice intraperitoneal injections of L-arginine (4 g/kg, each 1 h apart). Mice received PFD (250 mg/kg, orally once a day) one day before and on the day of AP-induction. Data are mean ± SEM (n = 8). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* the control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* the AP group (ANOVA followed by Tukey-Kramer multiple comparison).

Discussion

AP is an inflammatory life-threatening condition with high mortality rate and there is no satisfactory therapy till now. PFD is a potent anti-fibrotic agent that has been proven to modulate multiple signaling pathways, interact with various proteins and modify their expression and activity including transcription factors, inflammatory mediators and enzymes. Up to our knowledge, this is the first study which demonstrates the protective effects of PFD against L-arginine-induced AP.

Several reports have shown that AP is characterized by degenerative changes of the pancreas which may be in the form of necrosis, inflammation, edema and vacuolization. Leukocytes infiltration, mainly neutrophils, is crucial in the pathogenesis of AP as they produce pro-inflammatory cytokines and proteases [30]. Previous investigations have cleared that L-arginine can be used to establish a model of AP in rodents. L-arginine is a natural amino acid that causes partial inhibition of L-arginase enzyme which converts L-arginine to L-ornithine and urea. L-Arginine is precursor of nitric oxide and nitrate which induce oxidative stress [31]. It is believed that initiation of AP may result from induction of nitric oxide synthase by L-arginine which causes interaction of nitric oxide and superoxide radicals leading to generation of peroxynitrite radicals that are responsible for cell damage [32]. In consistence with previous investigations, our data demonstrated the efficiency of L-arginine in the establishment of AP model with characteristic neutrophil infiltration into pancreatic tissue [25,31]. Histopathological results revealed pancreatic damage in the form of disruption of histo-architecture, necrosis and neutrophils infiltration in L-arginine group. Importantly, PFD group ameliorated the histopathological lesions of pancreas and decreased the histopathological damage score.

Under normal conditions, amylase and lipase concentrations in serum are low. Upon pancreatic dysfunction as in case of AP or pancreatic adenocarcinoma, acinar cells may undergo destruction, leading to the release of pancreatic enzymes including amylase and lipase into serum. Thus, measurement of serum concentrations of pancreatic amylase and lipase indicates AP [33]. Our results have shown that intraperitoneal administration of L-arginine induced significant elevation of amylase and lipase activities. PFD treatment returned the elevated levels of amylase and lipase to normal. Meanwhile, PFD conferred protection against L-arginine-induced pancreatitis.

Oxidative stress is greatly implicated in the pathogenesis of AP as verified by both experimental and clinical studies [34]. Parameters of oxidative stress as superoxide radicals, MDA and lipid peroxide levels are significantly higher in the blood of patients and animals with AP and these reflect the degree of AP severity [35,36]. Previously, it was shown that pancreatic GSH is markedly depleted in case of AP simultaneously with the elevation of MDA [37]. Previous investigations have considered the depletion of GSH in the pancreatic tissue as a hallmark during the initial phase of AP. Studies have suggested that GSH depletion could induce activation of digestive enzymes in the acinar cells leading to enlargement of inflammatory response [36]. Hence, agents that enhanced GSH content exhibited beneficial effects in AP. Results of the present study were in the same line of the previous ones. L-Arginine administration induced significant increase in lipid peroxidation along with depressed antioxidant parameters as GSH and SOD. However, PFD treatment successfully counteracted the deleterious effects of L-arginine-induced oxidative stress in the pancreatic tissue. Previously, PFD was found to be a free radical scavenger and an inhibitor of NADPH-dependent microsomal lipid peroxidation [38]. Furthermore, the ability of PFD to reduce oxidative burden have been noticed before in the hepatic tissue during cirrhosis and hepatitis [9,20]. Hence, we can presume that PFD conferred protection against L-arginine-induced AP through anti-oxidative pathway.

NF-κB is a transcription factor that has crucial role in multiple biological processes including the development, immune response, cell growth and survival. Under normal physiological circumstances, NF-κB is present in the cytosol in an inactive form as it is sequestered by the inhibitor I-κB. Upon activation of NF-κB, I-κB is phosphorylated and NF-κB is released and translocated into the nucleus. Then it stimulates the transcription of multiple inflammatory genes including TNF-α. Activation of NF-κB can be considered as a key process in the pathogenesis of AP [39,40]. The results of the present study indicated that L-arginine induced elevation of NF-κB and its downstream inflammatory cascade, TNF-α and IL-6. However, PFD administration counteracted the activation of NF-κB and subsequently decreased the levels of TNF-α and IL-6. The inhibitory effect of PFD against NF-κB activation has been shown in autoimmune hepatitis [20]. Additionally, the anti-inflammatory activity of PFD was clearly evident in murine endotoxic shock model [41].

Many reports tried to explore the role of apoptosis in the modulation of AP and how it can affect its severity. Earlier studies have shown the role of NF-κB activation and TNF-α transcription in the signal transduction pathways leading to apoptosis [42]. In addition, the role of inflammatory mediators specifically, TNF-α, in the induction of apoptosis in pancreas was demonstrated [43]. More recent, increased mRNA and protein expression of pro-apoptotic gene, Bax, was observed in AP which led to acinar cell apoptosis [44]. Pancreatic acinar cell apoptosis is the predominant cause of cell death in AP [45]. L-Arginine-induced AP has been associated with apoptosis of acinar cells. Consistent with previous investigations, L-arginine-induced AP was characterized by increased levels of Bax and decreased Bcl2. A large body of evidence suggests the hypothesis that apoptosis inhibition might be beneficial in the treatment of AP [46–48]. Results of the current study demonstrated that PFD treatment remarkably counteracted AP-associated apoptosis as it significantly enhanced the level of Bcl2 while it decreased the level of Bax. This may support the idea that PFD could exert its beneficial effect in AP partially through inhibiting the apoptosis of acinar cells during AP.

In conclusion, our study provided evidence of the protective role of PFD against L-arginine-induced AP which counted on its anti-oxidative, anti-inflammatory, and anti-apoptotic effects. Hence, PFD might be promising agent in clinical treatment of AP.

Conflict of interest

None.

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Authors contributions

All authors equally contributed to all aspects of the study.

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