Study the Potential Prophylactic Effect of Azilsartan and Pirfenidone on Amiodarone-Induced Lung Fibrosis in Rats

Abstract:

Background: Lung fibrosis is a progressive interstitial disease characterized by epithelial injury and excessive extracellular matrix deposition. Its pathogenesis involves oxidative stress, inflammation, fibroblast activation, and TGF-β1 signaling. Despite antifibrotic therapies, effective prevention of early fibrotic remodeling remains limited, necessitating novel or combined prophylactic approaches. **Aim:** This study aimed to evaluate the prophylactic effects of pirfenidone and azilsartan against amiodarone-induced lung fibrosis in rats, and to assess whether their combined use provides superior protection by targeting multiple pathological pathways. **Methods:** Forty-two rats were randomized into six groups: normal control, DMSO control, amiodarone fibrotic, pirfenidone prophylactic, azilsartan prophylactic, and combined pirfenidone + azilsartan. Pulmonary fibrosis was induced by oral amiodarone for 4 weeks. Pirfenidone and azilsartan were administered orally, alone or combined, over the same period. Outcomes included biochemical markers (MPO, hydroxyproline, TGF-β1, and p53) and histopathology with Ashcroft scoring, analyzed using ANOVA with post-hoc tests. **Results:** Amiodarone administration induced significant ($p < 0.05$) pulmonary injury, evidenced by increased levels of myeloperoxidase, hydroxyproline, transforming growth factor-β1, and p53 compared with controls, reflecting inflammation, collagen deposition, profibrotic signaling, and apoptosis. These changes were associated with marked histopathological alterations. Prophylactic treatment with pirfenidone or azilsartan significantly ($p < 0.05$) improved these parameters, indicating reduced inflammation and fibrosis. The combination therapy produced the greatest reductions in biochemical markers, the lowest Ashcroft score, and near-complete preservation of lung structure compared with either monotherapy. **Conclusion:** Pirfenidone and azilsartan exhibit significant prophylactic effects against amiodarone-induced lung fibrosis. Combination therapy provides the greatest protection, suggesting synergistic modulation.

Keywords: Amiodarone; Pulmonary fibrosis; Pirfenidone; Azilsartan; Rat model.

1) Introduction

Amiodarone is a widely prescribed class III antiarrhythmic agent, known for its efficacy in the management of supraventricular and ventricular arrhythmias; however, its therapeutic use is limited by the risk of severe pulmonary toxicity [1]. Amiodarone-induced pulmonary fibrosis constitutes the most serious manifestation of this toxicity, characterized by chronic inflammation, oxidative injury, alveolar epithelial disruption, and progressive extracellular matrix deposition, leading to irreversible architectural distortion [2, 3]. Experimental and clinical data suggest that Amiodarone promotes mitochondrial dysfunction, excessive reactive oxygen species generation, lipid peroxidation, apoptosis, and activation of profibrotic cytokines—particularly transforming growth factor-β1 (TGF-β1) [4]. These processes converge to drive fibroblast proliferation, myofibroblast transformation, collagen accumulation, and structural remodeling, which is reminiscent of human idiopathic pulmonary fibrosis [5].

Despite increasing recognition of the pathological mechanisms underlying amiodarone-induced pulmonary fibrosis, effective prophylactic therapies remain unavailable [6]. This unmet clinical need has stimulated growing interest in repurposing antifibrotic and anti-inflammatory agents to mitigate early pathological events before irreversible damage occurs [5].

Pirfenidone, an FDA-approved antifibrotic agent for idiopathic pulmonary fibrosis, exerts multifaceted protective actions, including suppression of TGF-β1 signaling, attenuation of fibroblast proliferation, inhibition of collagen synthesis, and reduction of inflammatory

cytokines [7]. Its antioxidant actions further contribute to the attenuation of oxidative-stress–mediated epithelial injury [8, 9]. These pharmacological properties position pirfenidone as a promising candidate for counteracting the early fibrotic cascade triggered by amiodarone administration [7].

Azilsartan, a potent angiotensin II type 1 receptor (AT1R) blocker, has demonstrated antioxidant, anti-inflammatory, antiapoptotic, and tissue-protective actions beyond its antihypertensive effects [10-12]. AT1R activation is known to stimulate TGF-β1 production, NADPH oxidase activity, fibroblast migration, and extracellular matrix accumulation. Therefore, suppression of AT1R signaling may provide important upstream protection against the molecular triggers of Amiodarone-induced pulmonary fibrosis [13]. Emerging studies further demonstrate that ARBs attenuate lung fibrosis by modulating oxidative stress, cytokine release, and the epithelial–mesenchymal transition [14].

Given that amiodarone-induced fibrosis involves multiple interacting pathways—including oxidative stress, inflammation, apoptosis, epithelial injury, and TGF-β1–mediated fibrogenesis—combining pirfenidone and azilsartan may offer superior protection by simultaneously modulating several molecular targets. No prior experimental work has evaluated the prophylactic efficacy of combining these two agents against amiodarone-induced pulmonary fibrosis.

Therefore, the present study was designed to evaluate the potential prophylactic effects of pirfenidone and azilsartan—individually and in combination—against amiodarone-induced lung fibrosis in rats, and to determine whether dual targeting of inflammatory, oxidative, and profibrotic pathways confers enhanced protection compared with monotherapy.

Aim of the Work

To evaluate the potential prophylactic effectiveness of pirfenidone and azilsartan, individually and in combination, in preventing or attenuating amiodarone-induced lung fibrosis in a rat model through assessment of biochemical, histopathological, and fibrotic changes.

1) Materials and Methods

2.1 Animals and Ethical Approval

This experimental controlled animal study involved forty-two healthy adult male Sprague–Dawley rats, weighing between 150–250 g. The study commenced on March 15, 2024, and spanned a total duration of 28 days (4 weeks).

Upon arrival, the animals were acclimatized for one week under standard laboratory conditions. The rats were housed in well-ventilated cages, with 7 rats per cage, maintaining a 12-h light/dark cycle at a controlled temperature of 28–30 °C. They were provided with ad libitum access to standard chow and tap water.

All experimental procedures were conducted in strict accordance with the guidelines of the Ethics Committee of Scientific Research, Faculty of Medicine, Benha University {Approval Code: M.S.9.4.2023}.Trained technicians closely monitored the animals throughout the study period. At the conclusion of the experiment, the rats were anesthetized for sample collection and humanely euthanized via incineration.

2.2 Chemicals and Reagents

Amiodarone hydrochloride (Sanofi-Aventis, France), Pirfenidone (Cipla Ltd., India), Azilsartan medoxomil (Takeda Pharmaceuticals, Switzerland), Dimethyl sulfoxide (DMSO), and normal saline (EL-Gomhouria Co., Egypt), Rat Hydroxyproline, HYP ELISA Kit (BT Laboratory, China), Myeloperoxidase (MPO) (Rat) ELISA Kit (BioVision Inc., USA), Rat TP53 (Tumor Protein p53) ELISA Kit, Catalog No. NBP2-75359 (Novus Biologicals, USA), and Rat TGF-β1 (Transforming Growth Factor Beta 1) ELISA Kit, Catalogue No. ER1378

(FineTest, China). All drugs were freshly prepared in 0.5% DMSO for oral gavage administration.

2.3 Induction of Lung Fibrosis

Amiodarone (40 mg/kg/day) was administered to rats on a daily basis for a duration of 4 weeks [5].

2.4 Drug solvent

Amiodarone, pirfenidone and azilsartan were dissolved in dimethyl sulfoxide (DMSO) and given orally.

2.5 Experimental Design

Rats were randomly assigned to six groups (n = 7 each):

G1 – normal control: received standard chow and water only.

G2 – DMSO control: received 0.5% DMSO orally for 4 weeks.

G3 – non-prophylactic pulmonary fibrosis group: received amiodarone 40 mg/kg/day orally for 4 weeks [5].

G4 – pirfenidone prophylactic group: received amiodarone + pirfenidone 200 mg/kg/day for 4 weeks [8].

G5 – azilsartan prophylactic group: received amiodarone + azilsartan 4 mg/kg/day for 4 weeks [15].

G6 – pirfenidone & azilsartan prophylactic group: received amiodarone + pirfenidone + azilsartan daily for 4 weeks. All treatments were administered by oral gastric gavage.

2.6 Sample Collection

Twenty-four hours following the final dose rats were anesthetized with urethane (ethyl carbamate; 1.4 g/kg, i.p.)[16], and then sacrificed for sample collection. blood samples were collected from the heart, lung tissues were rapidly excised. The hilum was ligated, and lungs were divided for: Histopathology (fixed in 10% neutral-buffered formalin), while the others were used for lung tissue homogenate preparation.

2.7 Biochemical Assays

Lung tissue homogenates and serum samples were used to determine: TGF-β1 (ELISA)(5), Hydroxyproline (ELISA; collagen deposition marker) [17], p53 (Sandwich ELISA) [18] , Myeloperoxidase (MPO) (serum ELISA; inflammation/oxidative stress)[19] Assay procedures were performed according to the manufacturer's instructions.

2.7 Histopathological Examination

Paraffin-embedded lung sections were stained with: Hematoxylin and Eosin (H&E) for inflammation and architecture [18] and Masson's Trichrome for collagen deposition [17].

Fibrosis severity was scored using the Ashcroft scale (0–8), evaluating 10–20 microscopic fields per section. The mean fibrosis score per rat was used for analysis [20].

2.8 Statistical Analysis

Data were analyzed using SPSS (version 24). Results were expressed as mean ± SD. Normality was assessed using Kolmogorov–Smirnov and Shapiro–Wilk tests. Intergroup comparisons were performed using one-way ANOVA, followed by appropriate post-hoc tests. Significance thresholds: $p > 0.05$, not significant, $p < 0.05$: significant

3) Results

3.1 Biochemical Markers

Myeloperoxidase (MPO) and hydroxyproline (HPO)

MPO and HPO levels as presented in **table (1)** were significantly ($p < 0.05$) elevated in non-prophylactic pulmonary fibrosis group compared with all other groups. Pirfenidone prophylactic, and azilsartan prophylactic pulmonary fibrosis group, significantly ($p < 0.05$) reduced MPO and HPO compared to non-prophylactic pulmonary fibrosis group, but levels remained higher than in controls. Pirfenidone & Azilsartan prophylactic pulmonary fibrosis group produced the most significant reduction, with MPO and HPO values statistically insignificant ($P>0.05$) compared to control groups.

TGF-β1 and p53

TGF-β1 and p53 levels as presented in **table (1)** were significantly ($p < 0.05$) elevated in non-prophylactic pulmonary fibrosis group relative to all other groups. Pirfenidone prophylactic, azilsartan prophylactic pulmonary fibrosis group and pirfenidone & azilsartan prophylactic pulmonary fibrosis group, significantly ($p < 0.05$) reduced TGF-β1 and p53 levels compared to non-prophylactic pulmonary fibrosis group, but remained significantly ($p < 0.05$) higher than control values.

Table (1): Effect of pirfenidone, azilsartan, and their combination for four weeks on (MPO, TGFB1, HPO, P53) in experimentally induced lung fibrosis by amiodarone in rats:

SD: standard deviation

Groups / Parameters	G1 (n=7)	G2 (n=7)	G3 (n=7)	G4 (n=7)	G5 (n=7)	G6 (n=7)
	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD
MPO (ng/ml)	2.448±0.209	2.474±0.180 **c d e**	7.340±1.025 **a b d e f**	3.702±0.122 **a b c f**	3.274±0.354 **a b c f**	2.566±0.16 **c d e**
TGFβ1 (pg/mg protein)	44.031±6.059	46.791±5.77 **c d e f**	233.40±39.79 **a b d e f**	147.717±14.70 **a b c f**	127.97±14.52 **a b c f**	79.81±12.97 **a b c d e**
Hydroxyproline (ng/mg protein)	1.183±0.202	1.134±0.196 **c d e**	3.401±0.437 **a b d e f**	2.333±0.396 **a b c f**	2.339±0.0933 **a b c f**	1.328±0.213 **c d e**
P53 (pg/mg protein)	70.963±11.098	66.197±9.51 **c d e f**	231.09±25.67 **a b d e f**	145.357±15.36 **a b c f**	141.87±27.46 **a b c f**	91.414±13.5 **a b c d e**

Data was represented as means±SD, n= number of rats/group.

G1: normal control group. G2: DMSO control group. G3: Non-prophylactic Pulmonary fibrosis group G4: Pirfenidone prophylactic pulmonary fibrosis groupG5: Azilsartan prophylactic pulmonary fibrosis groupG6: Pirfenidone & Azilsartan prophylactic pulmonary fibrosis group **a**: Significant versus normal control (G1). **b**: Significant versus DMSO control group (G2). **c**: Significant versus Non-prophylactic Pulmonary fibrosis group (G3). **d**: Significant versus Pirfenidone prophylactic pulmonary fibrosis group (G4). **e**: Significant versus Azilsartan prophylactic pulmonary fibrosis group (G5). **f**: Significant versus Pirfenidone & Azilsartan prophylactic pulmonary fibrosis group (G6).

3.2 Histopathological Findings

H&E-stained lung sections, **as shown in figure (1)** showed normal architecture in both normal and DMSO control groups, with open alveoli, thin septa, and intact bronchioles. The non-prophylactic pulmonary fibrosis group demonstrated severe pathological changes, including inflammation, alveolar destruction, septal thickening, hemorrhage, fibrosis, and

honeycombing. In contrast, the pirfenidone and azilsartan prophylactic groups showed marked preservation of lung structure with only mild interstitial changes. Notably, the combination group exhibited superior protection, with nearly normal lung architecture, uniformly open alveoli, minimal inflammation, and absence of fibrotic or vascular alterations.

H&E Staining

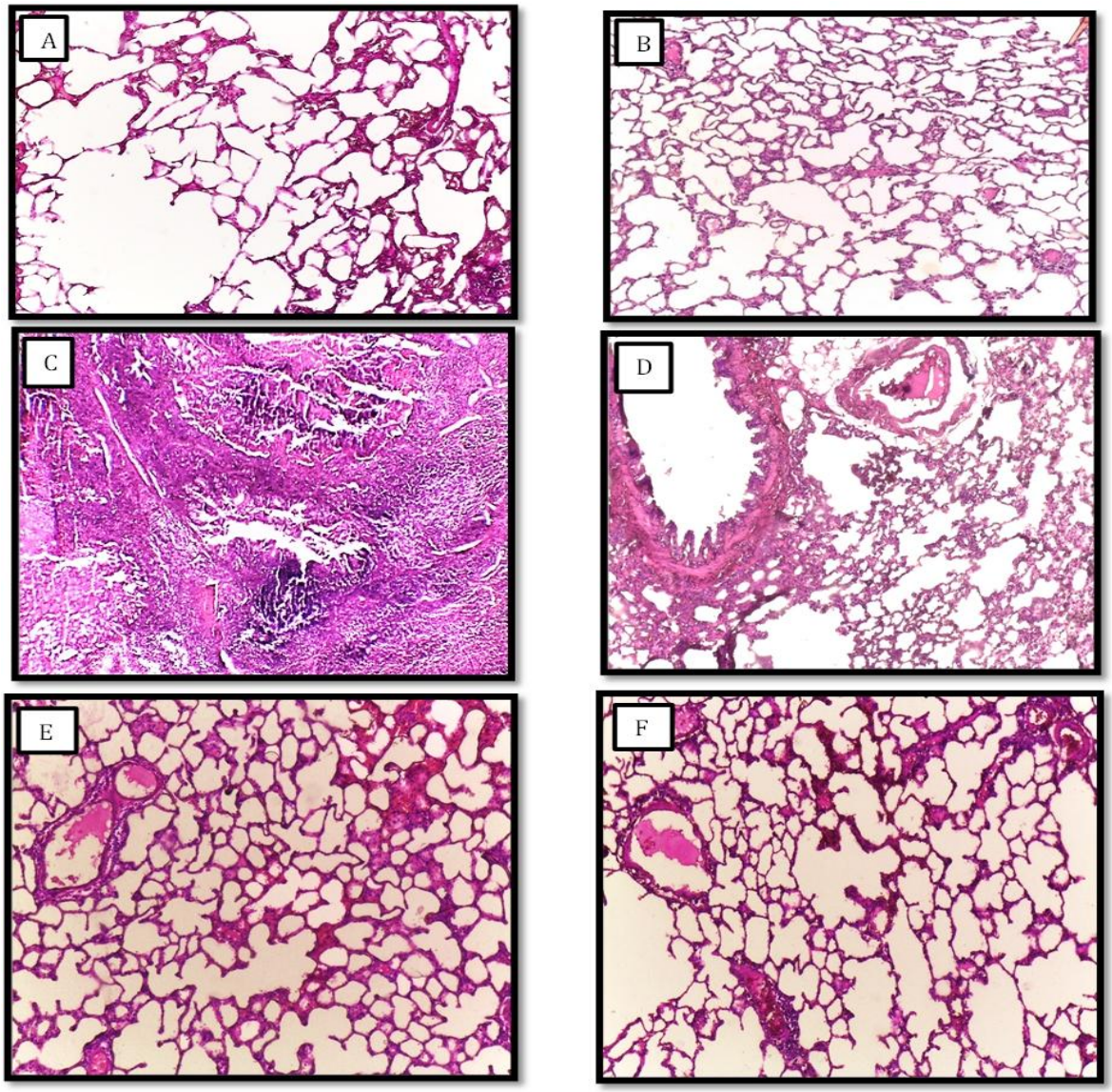

Figure (1): Photomicrographs of lung sections (H&E, magnification x200): (A)normal control group, (B) DMSO control, (C) non prophylactic pulmonary fibrosis group, (D) pirfenidone prophylactic pulmonary fibrosis group, (E) azilsartan prophylactic pulmonary fibrosis group, (F) Pirfenidone and azilsartan prophylactic pulmonary fibrosis group .

3.4 Ashcroft Fibrosis Score

Ashcroft's scoring, as presented **in table (2) and figure (2)** showed no significant difference between the normal and DMSO control groups. Amiodarone in the non-prophylactic pulmonary fibrosis group led to a significant increase in the fibrosis score compared with controls. Both pirfenidone and azilsartan prophylactic groups significantly reduced the score compared with the non-prophylactic group, although values remained higher than controls, with no significant difference between the two treatments. Notably, the combination therapy

produced a marked reduction in fibrosis, restoring scores to near-normal levels with no significant difference from control groups.

Table (2): Effect of pirfenidone, azilsartan, and their combination for four weeks on Ashcroft scoring in experimentally induced lung fibrosis by amiodarone in rats:

Groups / Parameters	G1 (n=7)	G2 (n=7)	G3 (n=7)	G4 (n=7)	G5 (n=7)	G6 (n=7)
	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD
Ashcroft Score (0-8)	0.0±0.0	0.0±0.0	6.857±0.900 abdef	3.857±0.900 abcf	4.000±0.816 abcf	0.571±0.535 cde

SD: standard deviation

Data was represented as means±SD, n= number of rats/group.

G1: normal control group. G2: DMSO control group. G3: Non-prophylactic Pulmonary fibrosis group G4: Pirfenidone prophylactic pulmonary fibrosis groupG5: Azilsartan prophylactic pulmonary fibrosis groupG6: Pirfenidone & Azilsartan prophylactic pulmonary fibrosis group **a**: Significant versus normal control (G1). **b**: Significant versus DMSO control group (G2).**c**:Significant versus Non-prophylactic Pulmonary fibrosis group (G3). **d**: Significant versus Pirfenidone prophylactic pulmonary fibrosis group (G4). **e**: Significant versus Azilsartan prophylactic pulmonary fibrosis group (G5). **f**: Significant versus Pirfenidone & Azilsartan prophylactic pulmonary fibrosis group (G6).

***Each group contain 7 rats.**

Histogram showing the effect of pirfenidone, azilsartan, and their combination on Ashcroft score in amiodarone-induced pulmonary fibrosis in rats.

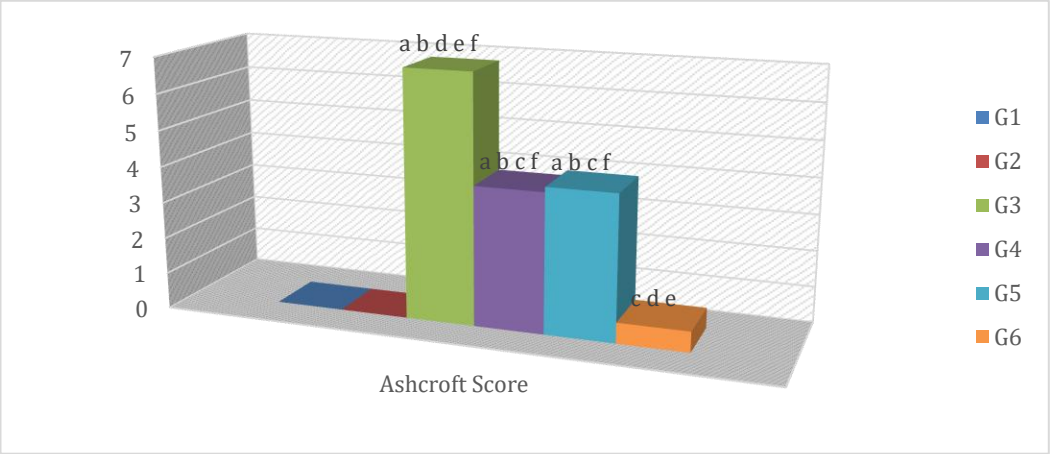

Figure (2): Histological evaluation using Ashcroft

3.5 Masson's Trichrome Staining

Masson's trichrome staining, as presented in **figure (3)** revealed a normal distribution of collagen fibers in the normal control and DMSO control groups, localized around bronchioles, within alveolar sacs, and along interalveolar septa. In contrast, the non-prophylactic pulmonary fibrosis group showed excessive collagen deposition around bronchioles and within interalveolar septa. The pirfenidone and azilsartan groups each demonstrated focal areas of increased collagen deposition, with the remaining lung tissue showing minimal to moderate

interstitial collagen. Notably, the combination group exhibited a marked reduction in collagen deposition within the interalveolar septa, approaching near-normal architecture.

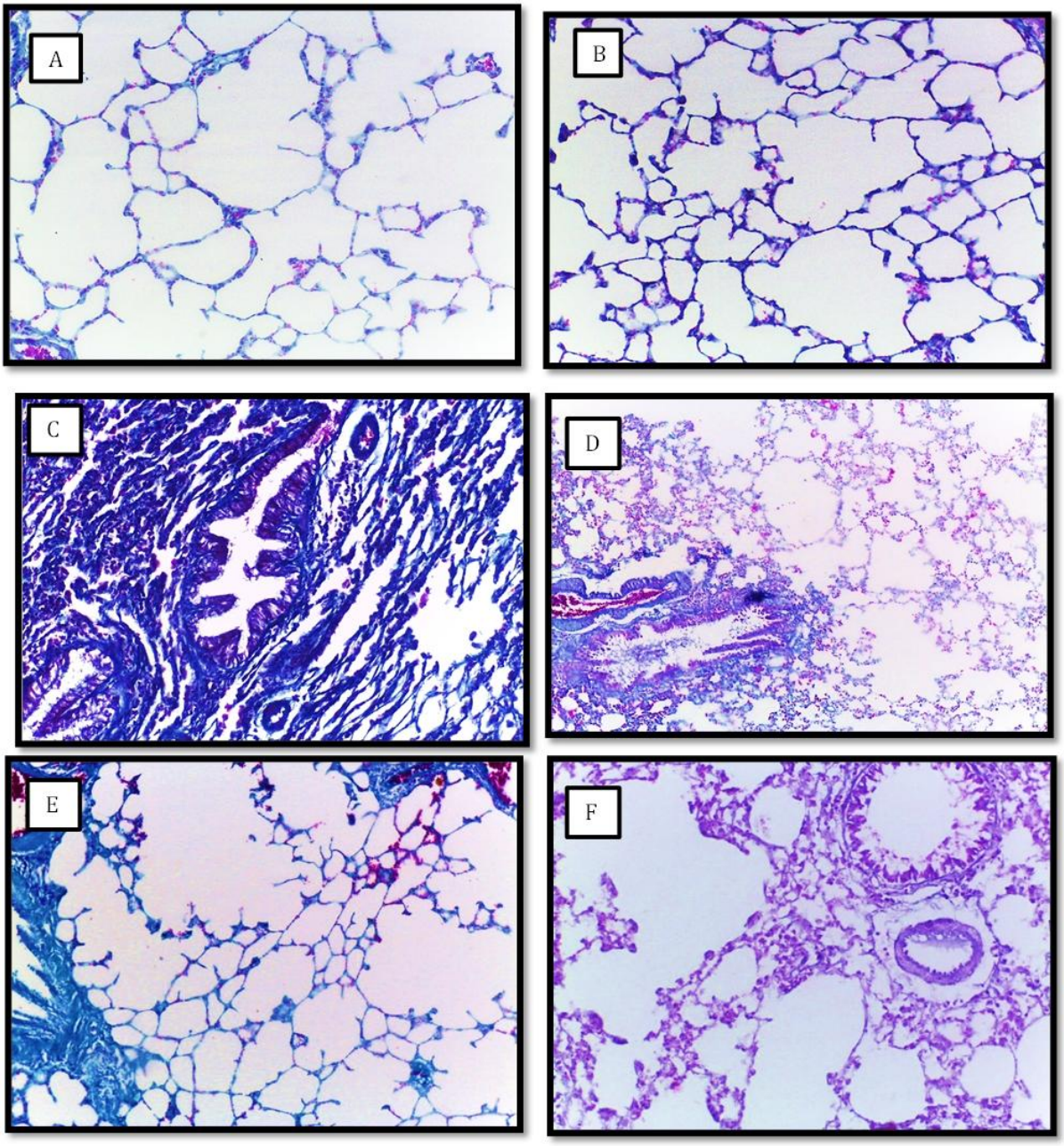

Figure (3): Photomicrographs of lung sections (Masson trichome, magnification x200): (A)normal control group, (B)DMSO control group, (C) non prophylactic pulmonary fibrosis, (D)pirfenidone prophylactic pulmonary fibrosis group, (E) azilsartan prophylactic pulmonary fibrosis group, (F) Pirfenidone and azilsartan prophylactic pulmonary fibrosis group.

4) Discussion

Pulmonary fibrosis (PF) is a progressive interstitial lung disease characterized by impaired gas exchange, respiratory failure, and reduced quality of life, arising from dysregulated repair processes, inflammation, and excessive extracellular matrix deposition [21, 22].It represents a major complication in several respiratory disorders, particularly idiopathic pulmonary fibrosis (IPF) and systemic sclerosis, both of which are associated with significant morbidity and mortality [23, 24]. Despite advances in understanding molecular mechanisms, including the roles of DAMPs, TLR4 signaling, and profibrotic cytokines, current therapies such as pirfenidone

and nintedanib slow but do not reverse disease progression, leaving lung transplantation as the only curative option [25-27].

Given this therapeutic gap, the present study evaluated the prophylactic effects of pirfenidone and azilsartan, individually and in combination, in an amiodarone-induced pulmonary fibrosis rat model. Amiodarone administration successfully produced key pathological and functional features of fibrosis, including inflammatory activation, profibrotic signaling, collagen accumulation, epithelial injury, histological remodeling, and impaired gas exchange, consistent with previous reports [17, 28].

In the present work, animals were randomly allocated into six groups: a normal control group, DMSO control group, non-prophylactic pulmonary fibrosis group, and three prophylactic groups treated during amiodarone exposure with pirfenidone, azilsartan, or their combination. This experimental design enabled direct comparison between untreated fibrosis and single versus combined prophylaxis, allowing assessment of relative and potentially synergistic protective effects.

The present results demonstrated that amiodarone administration (40 mg/kg/day) for four weeks induced marked pathological and biochemical hallmarks of pulmonary fibrosis, consistent with previous literature [28]. The amiodarone non-prophylactic group exhibited significantly elevated myeloperoxidase, transforming growth factor beta 1, hydroxyproline, and p53 levels, together with severe fibrotic changes in histopathological sections, consistent with Sharaf El-Din & Abd Allah [17]. These findings support the fidelity of the model in producing a reproducible fibrotic phenotype suitable for evaluating pharmacologic prophylaxis.

The present results demonstrated that myeloperoxidase activity was highest in the amiodarone non-prophylactic group, indicating a strong neutrophil-dominant inflammatory response with increased oxidative stress, consistent with Punithavathi [29]. This pattern supports that amiodarone-induced lung injury involves a substantial inflammatory component that contributes to downstream remodeling. Mechanistically, amiodarone disrupts cellular membranes and lysosomal function, injures alveolar epithelial cells, activates innate immunity, and promotes chemokine-driven neutrophil infiltration; activated neutrophils release myeloperoxidase and generate reactive oxidants such as hypochlorous acid, thereby amplifying tissue injury and promoting fibrosis [30].

Among the treated groups, the present findings showed that pirfenidone prophylaxis significantly decreased myeloperoxidase compared with the amiodarone non-prophylactic group, supporting reduced neutrophil-associated inflammatory activity and oxidative burden. This finding agrees with previous studies [31, 32] showing that pirfenidone suppresses inflammatory and oxidative pathways and reduces inflammatory cell infiltration, which plausibly explains lower myeloperoxidase activity. Similarly, azilsartan prophylaxis significantly reduced myeloperoxidase relative to the amiodarone non-prophylactic group, consistent with evidence that angiotensin receptor blockade can attenuate neutrophil-driven inflammation and oxidative stress [33]. This effect is mechanistically supported by azilsartan-mediated reduction of angiotensin II–driven oxidative stress, including suppression of NADPH oxidase–derived reactive oxygen species, which limits neutrophil recruitment and activation [33]. Notably, the combined pirfenidone and azilsartan group produced the most pronounced reduction in myeloperoxidase, showing no significant difference from controls, suggesting additive or synergistic suppression of inflammation and oxidative stress. This can be explained by complementary pathway coverage, whereby azilsartan reduces upstream oxidant

amplification and pirfenidone suppresses inflammatory mediator signaling, jointly limiting neutrophil recruitment and activation [32, 34].

Transforming growth factor beta 1 is a central mediator of pulmonary fibrosis that promotes fibroblast proliferation, extracellular matrix deposition, and epithelial-to-mesenchymal transition and is widely used as a fibrosis marker due to its key role in fibrogenesis [35]. The present results demonstrated that transforming growth factor beta 1 was markedly elevated in the amiodarone non-prophylactic group, indicating strong profibrotic activation. This is consistent with the report that drug-induced pulmonary fibrosis is initiated by oxidative stress and epithelial damage, which activates macrophages and epithelial cells to release transforming growth factor beta 1 and drive fibrotic remodeling [5]. Mechanistically, transforming growth factor beta 1 promotes profibrotic chemokine and cytokine signaling, increases extracellular matrix synthesis, suppresses collagen degradation, and drives fibroblast-to-myofibroblast transition [5, 35].

The present findings showed that pirfenidone prophylaxis significantly reduced transforming growth factor beta 1 compared with the amiodarone non-prophylactic group, reflecting suppression of profibrotic signaling. This agrees with mechanistic evidence that pirfenidone interferes with transforming growth factor beta 1–driven fibrotic pathways, including inhibition of epithelial-to-mesenchymal transition and fibroblast-to-myofibroblast differentiation through disruption of key transcriptional signaling complexes [36]. The azilsartan prophylactic group also showed a significant decrease in transforming growth factor beta 1, supported by previous studies demonstrating that angiotensin receptor blockers attenuate transforming growth factor beta 1 signaling and collagen synthesis [37-39]. Importantly, the combination group achieved a significantly greater reduction than either monotherapy, suggesting additive or synergistic antifibrotic activity through simultaneous inhibition of downstream transforming growth factor beta 1 signaling by pirfenidone and reduction of upstream profibrotic activation via angiotensin II blockade [36-39]. However, transforming growth factor beta 1 remained higher than control values, which can be attributed to persistent epithelial injury and macrophage activation and to local activation of latent transforming growth factor beta 1 stored in the extracellular matrix that is not fully blocked by either drug [5, 35].

Hydroxyproline is a gold-standard biochemical marker for fibrosis quantification because it reflects tissue collagen content and correlates with histological collagen staining [5, 8]. The present results demonstrated that hydroxyproline was significantly elevated in the amiodarone non-prophylactic group compared with controls, confirming excessive collagen deposition and fibrotic remodeling, consistent with Ibrahim Fouad and Mousa [5]. Prophylactic pirfenidone significantly reduced hydroxyproline compared with the amiodarone non-prophylactic group, indicating attenuation of collagen accumulation, consistent with Huang [40]. This observation is mechanistically supported by evidence that pirfenidone limits collagen synthesis by suppressing profibrotic mediators, particularly transforming growth factor beta 1–driven pathways, thereby reducing fibroblast activation and extracellular matrix overproduction [41]. Azilsartan prophylaxis also produced a significant reduction in hydroxyproline, supporting a class-related antifibrotic effect of angiotensin receptor blockers and aligning with evidence that valsartan reduced lung hydroxyproline in experimental pulmonary fibrosis [42]. The apparent discrepancy with the findings of Hama Amin & Aziz [43] can be explained by fundamental differences in model pathophysiology and in what hydroxyproline represents in each setting. In non-fibrotic injury models, reduced hydroxyproline may reflect collagen depletion and impaired tissue repair; therefore, restoration of hydroxyproline is interpreted as improved

healing. In contrast, in the amiodarone-induced pulmonary fibrosis model, elevated hydroxyproline reflects pathological collagen overproduction and progressive extracellular matrix accumulation; accordingly, the reduction observed with azilsartan indicates an antifibrotic effect rather than impaired repair. Notably, the combined pirfenidone and azilsartan group reduced hydroxyproline to values not significantly different from controls, suggesting near normalization of collagen burden and supporting enhanced antifibrotic efficacy with combination prophylaxis.

p53 is upregulated in alveolar epithelial cells in fibrotic lung disease and reflects cellular stress and apoptosis-related signaling; it has also been proposed as a biomarker for fibrosis severity and progression [44, 45]. While restoration of intracellular p53 function in fibroblasts may reduce fibrosis, tissue-level p53 increases in lung homogenates more commonly reflect epithelial injury and apoptosis severity [46, 47]. The present results demonstrated that p53 was significantly elevated in the amiodarone non-prophylactic group compared with control groups, consistent with epithelial injury–driven stress responses linked to fibrogenesis [47]. The apparent difference from the findings reported by Nagaraja [46], which described a protective role of intracellular p53 in fibroblasts, can be explained by differences in the site of measurement and cellular context. In that study, p53 was assessed within fibroblasts, where its activity may regulate cell proliferation and limit fibrotic progression. In contrast, the present study measured p53 in whole-lung homogenates, which reflect overall tissue levels, including p53 released from injured or apoptotic cells. Therefore, the elevated p53 observed here primarily indicates epithelial injury and cellular stress rather than a protective intracellular regulatory effect within fibroblasts.

The present findings showed that pirfenidone prophylaxis significantly reduced p53 compared with the amiodarone non-prophylactic group, indicating attenuation of epithelial stress and injury. This effect is mechanistically plausible because pirfenidone suppresses transforming growth factor beta signaling and related profibrotic cascades that intersect with stress-response pathways [49]. Azilsartan prophylaxis also significantly reduced p53, consistent with evidence that azilsartan downregulates p53 expression and reduces cellular stress and apoptosis in injury models [50]. The present work also showed that the difference in p53 reduction between monotherapies was nonsignificant. Importantly, combination prophylaxis produced a significantly greater reduction in p53 than either monotherapy, supporting broader protection through complementary mechanisms, with pirfenidone limiting downstream profibrotic signaling and azilsartan reducing upstream oxidative injury that drives p53 activation [49, 50]. Residual elevation of p53 compared with controls is plausibly explained by persistent epithelial damage and ongoing remodeling signals that are not fully reversible within the experimental period [47, 51].

The present results demonstrated severe and diffuse pulmonary injury in the amiodarone non-prophylactic group, characterized by extensive inflammatory infiltration, lymphoid follicle formation, disrupted alveolar architecture, and lesions spanning early fibrosis to honeycombing and marked architectural distortion, confirming progressive pulmonary fibrosis. These findings are consistent with reports describing amiodarone lung toxicity as a combination of inflammatory injury and fibrotic remodeling resembling interstitial lung disease pathology [5, 52] and are mechanistically attributable to epithelial injury, oxidative stress, and sustained inflammatory activation that promote fibroblast proliferation and extracellular matrix deposition [30].

Among the treated groups, pirfenidone prophylaxis showed clear improvement of lung architecture with reduced inflammatory infiltrates and less extensive fibrotic remodeling,

consistent with evidence that pirfenidone attenuates lung injury and inflammatory infiltration in fibrosis models [53]. Azilsartan prophylaxis also improved histopathological architecture, supporting its protective effects through suppression of oxidative stress, inflammation, and apoptosis; evidence of histological preservation in injury models further supports this interpretation [50]. Notably, the combined prophylactic group exhibited the most pronounced histological preservation, with minimal fibrotic remodeling compared with the amiodarone non-prophylactic group. This pattern aligns with evidence that combining an antifibrotic agent with angiotensin receptor blockade can enhance antifibrotic and antioxidant effects and reduce collagen accumulation more effectively than monotherapy [54].

Consistent with the histopathological findings, Masson's trichrome staining showed preserved architecture and negligible collagen deposition in the normal and DMSO control groups, supporting absence of solvent-related fibrosis [55]. In the amiodarone non-prophylactic group, Masson's trichrome staining revealed extensive interstitial collagen deposition with marked distortion and elevated Ashcroft scoring, consistent with established reports using Masson's trichrome and Ashcroft scoring in amiodarone-induced fibrosis [16, 55]. Pirfenidone prophylaxis reduced collagen burden and architectural distortion, indicating attenuation from severe toward moderate fibrosis, consistent with recent evidence linking pirfenidone to reduced collagen deposition and fibrosis scoring [53]. Azilsartan prophylaxis also produced moderate improvement with reduced collagen deposition and partial preservation of lung architecture, as reflected by the reported Ashcroft score, and this is supported by evidence that renin–angiotensin system blockade reduces fibrosis severity in experimental pulmonary fibrosis [54]. The combined pirfenidone and azilsartan group demonstrated the greatest reduction in collagen deposition and near-normal architecture, concordant with combination-therapy evidence demonstrating superior reductions in collagen and fibrosis severity compared with monotherapy [54].

Based on the cumulative evidence, the present findings support that amiodarone-induced pulmonary fibrosis is driven by an interrelated cycle of neutrophil-dominant inflammation and oxidative stress (myeloperoxidase), activation of profibrotic pathways (transforming growth factor beta 1), collagen accumulation (hydroxyproline), and epithelial stress/apoptosis signaling (p53), resulting in progressive remodeling and architectural distortion [8, 35 ,51]. The present results demonstrated that pirfenidone and azilsartan each significantly attenuated these pathways, while their combination produced the most pronounced protection across inflammatory, fibrogenic, collagen, apoptotic, and histological endpoints, supporting additive or synergistic benefit through complementary mechanisms [36, 54, 57].

Up till now, this study provides the first evidence that azilsartan exerts a protective effect in lung fibrosis, reducing transforming growth factor beta 1, hydroxyproline, p53, myeloperoxidase, and Ashcroft scores, consistent with the concept that angiotensin receptor blockers have antifibrotic potential across organ systems [39,58,59]. Collectively, these findings provide a rationale for repurposing angiotensin receptor blockers as adjunct antifibrotic therapies for high-risk settings such as drug-induced pulmonary fibrosis, while emphasizing that confirmation in clinical studies remains necessary.

5) Conclusion

The present study demonstrates that amiodarone induces profound pulmonary injury characterized by oxidative stress, inflammation, epithelial apoptosis, collagen accumulation, and severe histological distortion. Prophylactic administration of pirfenidone or azilsartan significantly ameliorated these pathological alterations; however, their combination produced a markedly superior protective effect. Dual therapy suppressed MPO, TGF-β1 activity,

hydroxyproline, and p53 expression, and achieved the lowest Ashcroft fibrosis score with near-complete preservation of lung architecture.These findings suggest that simultaneous targeting of inflammatory, oxidative, apoptotic, and profibrotic pathways offers synergistic protection against the early events driving Amiodarone-induced pulmonary fibrosis. The combined regimen of pirfenidone and azilsartan represents a promising prophylactic strategy that warrants further mechanistic and translational investigation.

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