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Suppression of NLRP3 inflammasome by ivermectin ameliorates bleomycin-induced pulmonary fibrosis

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Abstract: Ivermectin is a US Food and Drug Administration (FDA)-approved antiparasitic agent with antiviral and anti-inflammatory properties. Although recent studies reported the possible anti-inflammatory activity of ivermectin in respiratory injuries, its potential therapeutic effect on pulmonary fibrosis (PF) has not been investigated. This study aimed to explore the ability of ivermectin (0.6 mg/kg) to alleviate bleomycin-induced biochemical derangements and histological changes in an experimental PF rat model. This can provide the means to validate the clinical utility of ivermectin as a treatment option for idiopathic PF. The results showed that ivermectin mitigated the bleomycin-evoked pulmonary injury, as manifested by the reduced infiltration of inflammatory cells, as well as decreased the inflammation and fibrosis scores. Intriguingly, ivermectin decreased collagen fiber deposition and suppressed transforming growth factor-β1 (TGF-β1) and fibronectin protein expression, highlighting its anti-fibrotic activity. This study revealed for the first time that ivermectin can suppress the nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome, as manifested by the reduced gene expression of *NLRP3* and the apoptosis-associated speck-like protein containing a caspase recruitment domain (*ASC*), with a subsequent decline in the interleukin-1β (IL-1β) level. In addition, ivermectin inhibited the expression of intracellular nuclear factor-κB (NF-κB) and hypoxia-inducible factor-1α (HIF-1α) proteins along with lowering the oxidative stress and apoptotic markers. Altogether, this study revealed that ivermectin could ameliorate pulmonary inflammation and fibrosis induced by bleomycin. These beneficial effects were mediated, at least partly, via the downregulation of TGF-β1 and fibronectin, as well as the suppression of NLRP3 inflammasome through modulating the expression of HIF-1α and NF-κB.

Key words: Intra-tracheal instillation; Immunohistochemistry; Transforming growth factor-β1 (TGF-β1); Nuclear factor-κB (NF-κB); Lung fibrosis

1 Introduction

Pulmonary fibrosis (PF), as an interstitial fibrotic pathology affecting the lower respiratory tract, is characterized by a progressive deterioration of lung function, eventually leading to respiratory failure and death (Raghu et al., 2017; Gad et al., 2020). With few effective drugs available for the treatment of PF, there is an urgent need for new therapeutic agents capable of reversing the pulmonary fibrotic cascades (Han et al., 2021).

Upon exposure to destructive agents, the lungs respond to tissue damage through a cascade of synchronized biological processes (Kan et al., 2021). After repeated exposure to injurious events, a pro-fibrotic reprogramming pathway with extensive pro-inflammatory mediators develops in the lung (Ruiz-Riol et al., 2017). Such a vicious cycle starts with the activation of alveolar epithelial cells to promote the expression of several pro-fibrotic growth factors. Importantly, transforming growth factor-β (TGF-β) is the master regulatory factor promoting the activation of fibroblasts and myofibroblasts. The abnormal activation of fibroblasts boosts

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the production of extracellular matrix (ECM) components with the deposition of collagens, fibronectin, and α-smooth muscle actin (α-SMA) (Blobe et al., 2000).

Over the past decade, many reports discussed the role of the nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome in the pathogenesis and regulation of various pulmonary insults (Tian et al., 2017; Huang et al., 2019). An inflammasome complex is composed of a sensor, an adaptor, and a zymogen. In response to a specific pathogenic stimulus, the inflammasome sensor is activated, and the adaptor subsequently forms discrete foci in the activated cell. This causes the activation of zymogen via complex intermediate signaling mediators involved in the stimulation of nuclear factor-κB (NF-κB), which is considered a key regulator in the stimulation of the inflammasome components (Latz et al., 2013). The activation of inflammasome induces the production of various inflammatory cytokines including interleukin-1β (IL-1β) and IL-18, followed by triggering a programmed form of inflammatory lytic cell death called pyroptosis, which releases additional inflammatory mediators (Sagoo et al., 2016).

Bleomycin is a glycopeptide antibiotic with an anti-neoplastic effect. Interstitial lung fibrosis is a common side effect of bleomycin, which can be used to establish an experimental model for inducing PF in rodents. Besides, many molecular signatures, as well as histopathological hallmarks of bleomycin-induced lung fibrosis, were found to resemble human fibrotic lung diseases (Liu et al., 2017).

Hypoxia-inducible factor-1α (HIF-1α) is a ubiquitous transcription factor that is normally degraded via the ubiquitin-proteasome pathway. Under hypoxic conditions, however, HIF-1α degradation is arrested, leading to accumulation and subsequent translocation of HIF-1α into the nucleus where it can control the expression of its target genes (Vriend and Reiter, 2016). Additionally, HIF-1α was proven to regulate NLRP3 inflammasome activation and the secretion of IL-1β through NF-κB signaling pathway in animal models of bleomycin-induced acute lung damage (Huang et al., 2019).

Ivermectin is a broad-spectrum antiparasitic agent with prominent antiviral and anti-inflammatory properties (Steinhoff et al., 2016; Caly et al., 2020; Mansour et al., 2021). Previous experimental studies reported that ivermectin exerts anti-inflammatory effects through inhibiting the activation of T cells (Steinhoff et al., 2016) and suppressing the production of pro-inflammatory cytokines (Zhang et al., 2008). However, the effect of ivermectin on PF has neither been explored nor fully discussed.

In the above context, the present study aimed to investigate the potential of ivermectin to alleviate bleomycin-induced biochemical derangements and histological changes in an experimental model of PF. Additionally, we explored the molecular and biochemical mechanisms implicated in NLRP3-inflammasome activation in bleomycin-induced PF.

2 Materials and methods

2.1 Animals

Forty 8-week-old male Wistar rats weighing 200–220 g were purchased from Theodor Bilharz Research Institute (Giza, Egypt). The animals were kept under standard controlled conditions of temperature ((23±2) °C) and relative humidity ((55±2)%) with a 12-h light/dark cycle at the animal house facility of the Faculty of Pharmacy, Cairo University (Cairo, Egypt). Rats were fed a standard chow diet and had access to water ad libitum throughout the experiment.

2.2 Drugs and chemicals

Bleomycin (Bleocip 15IU, Cipla Pharmaceutical Company, Mumbai, India) was dissolved in saline to a final concentration of 5 mg/mL. Ivermectin (Iverzine, Unipharma Company, Cairo, Egypt) was dissolved in distilled water to a final concentration of 0.6 mg/mL. Thiopental sodium was obtained from Egyptian Int. Pharmaceutical Industries Co. (E.I.P.I.CO), Egypt. The other chemicals and reagents used were of high analytical grade.

2.3 Induction of pulmonary fibrosis

PF was induced by the intra-tracheal instillation of bleomycin at a dose of 5 mg/kg with an adjusted volume of 1 mL/kg (Chen et al., 2009). Rats were anesthetized using thiopental sodium (50 mg/kg, intraperitoneal) (Gazdhar et al., 2013).

2.4 Experimental design

As shown in Fig. 1, 40 male Wistar albino rats were weighed and randomly allocated into four groups

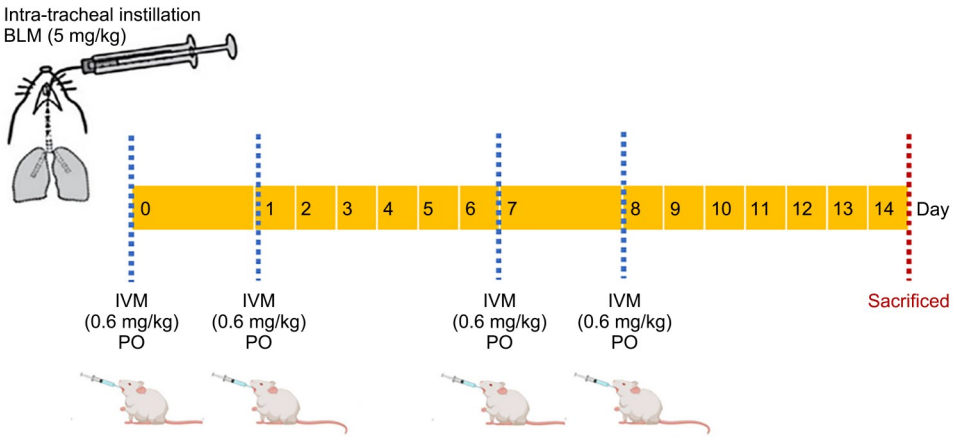

Fig. 1 Schematic representation of the experimental timeline. BLM: bleomycin; IVM: ivermectin; PO: per oral.

(10 rats each). The animals of Group 1 were injected with saline (1 mL/kg) by intra-tracheal instillation and served as the normal control (CTRL) group. In Group 2, rats were given 0.6 mg/kg ivermectin (per oral (PO), once daily) on Days 0, 1, 7, and 8 to serve as the ivermectin control (IVM) group. The ivermectin dose used herein was extrapolated from that reported to be given to coronavirus disease 2019 (COVID-19) patients (Shin et al., 2010; Buonfrate and Bisoffi, 2021; Krolewiecki et al., 2021). Animals in Group 3 were given a single dose of 5 mg/kg bleomycin by intra-tracheal instillation (Chen et al., 2009; Wang JP et al., 2021) to serve as the PF model (BLM) group. Furthermore, rats of Group 4 were injected with a single intra-tracheal dose of bleomycin (5 mg/kg) in addition to ivermectin (0.6 mg/kg, PO, once daily) on Days 0, 1, 7, and 8 to serve as the BLM+IVM group.

Fourteen days after bleomycin injection, retro-orbital blood samples were withdrawn from the orbital plexus under anesthesia (thiopental sodium 50 mg/kg, intraperitoneal) (Gazdhar et al., 2013) using heparinized micro-capillaries (Optilab, Berlin, Germany). Serum was separated by centrifugation at 4000 r/min for 10 min at −4 °C (Heraeus Biofuge, Berlin, Germany). Next, the animals were euthanized by cervical dislocation; the lungs were dissected, washed with cold saline, and dried on filter paper. Thereafter, the right lungs were used for histological and immunohistochemical examinations, and the left lungs were used for biochemical investigations.

The histopathological and biochemical measurements are fully described in the supplementary materials and methods.

3 Results

3.1 Effect of ivermectin on bleomycin-induced histopathological changes

The lung tissues of bleomycin-exposed rats revealed severe lung lesions, with a marked thickening of the interstitial alveolar septa (interstitial pneumonia), as well as bronchopneumonia shown by the hyperplasia of goblet cells, excessive mucous secretion in the bronchial lumen, and extensive inflammatory cell infiltration. Fibroblast proliferation and collagen deposition in interstitial space, in addition to the marked infiltration of perivascular inflammatory cells, were also evident. However, these lesions were significantly ameliorated and regressed by ivermectin treatment. As illustrated in Fig. 2, The light microscopic examination of both CTRL and IVM rats displayed the normal histoarchitecture of lung tissues consisting of normal thin-walled alveoli, normal bronchi, and regular bronchioles (Figs. 2a and 2b). Meanwhile, the lung tissues of rats treated with combined bleomycin and ivermectin exhibited slight focal thickening of the alveolar wall (focal interstitial pneumonia) and mild perivascular inflammatory cell infiltration (Fig. 2f).

It was further revealed that bleomycin administration increased the inflammation, fibrosis score, as well as Ashcroft's score compared to the CTRL and IVM groups. Meanwhile, ivermectin significantly ameliorated the inflammation and reversed the fibrosis score and Ashcroft's score compared to the BLM group (Figs. 3a–3c). In the same context, the weights were markedly decreased among rats that received bleomycin compared to the CTRL and IVM groups,

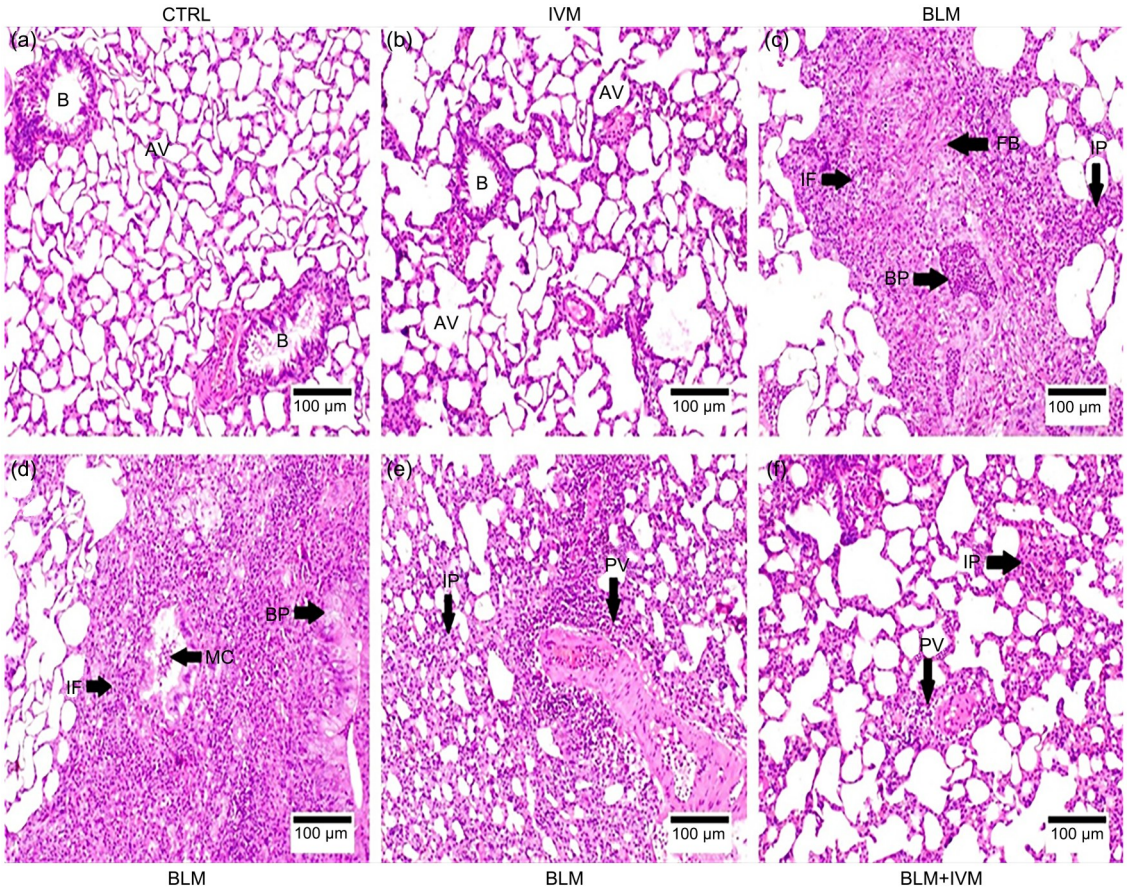

Fig.2 Representative photomicrographs of H&E-stained lung sections of different groups. (a, b) CTRL (a) and IVM (b) groups showing normal histological architecture consisting of normal thin-walled alveoli (AV), normal bronchi, and regular bronchioles (B). (c–e) BLM groups showing interstitial pneumonia (IP), fibroblast proliferation (FB), bronchopneumonia (BP), mucous exudate in the bronchial lumen (MC), extensive inflammatory cell infiltration (IF), and marked perivasculitis (PV). (f) BLM+IVM group showing slight focal IP and mild perivascular inflammatory cell infiltration (PV). BLM: bleomycin; CTRL: normal control; H&E: hematoxylin and eosin; IVM: ivermectin.

whereas remarkable weight gain was achieved upon treatment with ivermectin after bleomycin exposure (Figs. 3d and 3e).

3.2 Effect of ivermectin on bleomycin-induced changes by Masson's trichrome (MTC) staining

As demonstrated in Figs. 4a, 4b, 4e, and 4f, the microscopic examination of lungs of CTRL and IVM rats showed normal weak MTC-stained collagen fibers. In contrast, rats exposed to bleomycin exhibited a marked increase in collagen fiber deposition and showed apparent fibrosis (Figs. 4c and 4g). Furthermore, treatment with ivermectin after bleomycin exposure ameliorated collagen fiber deposition, as confirmed by weak MTC-positive reaction (Figs. 4d and 4h). Histomorphometric analyses of MTC-stained sections revealed a significant increase in the percentage area of collagen deposition in the bleomycin-exposed group in comparison to the CTRL and IVM groups. However, ivermectin treatment after bleomycin administration induced a statistically significant decline in the percentage area of collagen deposition in comparison to the BLM group (Fig. 4i).

The immunohistochemical expression of IL-1β, TGF-β1, NF-κB p65, and caspase-3 in the lung tissues was demonstrated in Fig. 5. Briefly, the CTRL rats revealed no immune expression (Figs. 5a, 5e, 5i, and 5m). The lungs of rats treated with IVM alone exhibited normal immune reactivity (Figs. 5b, 5f, 5j, and 5n). Furthermore, significant strong positive immunostaining reaction was found in the BLM group compared to the other three groups (Figs. 5c, 5g, 5k, and 5o). Meanwhile, treating bleomycin-induced PF with ivermectin markedly downregulated the immune expression of

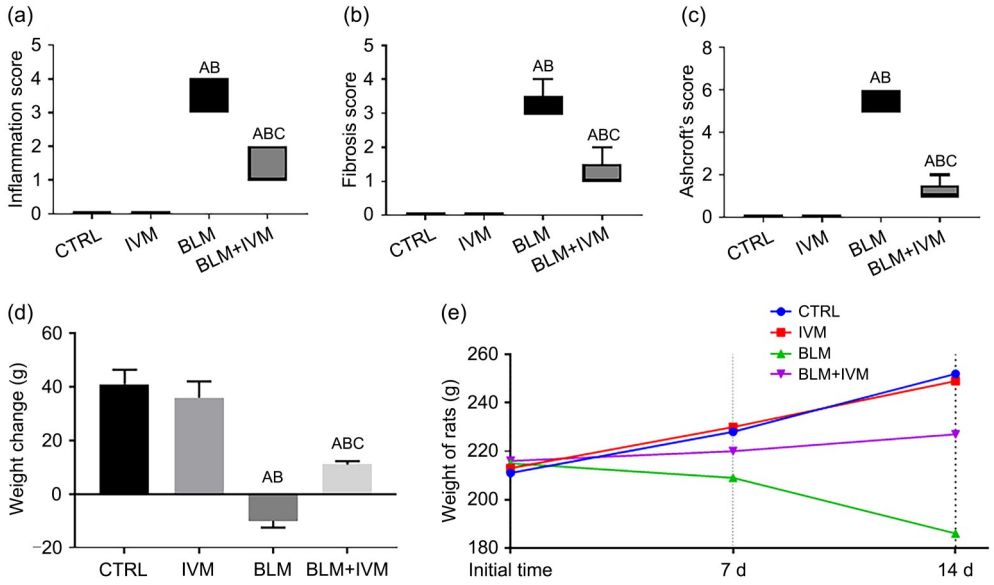

Fig. 3 Box plots representing the inflammation score (a), fibrosis score (b), and Ashcroft's score (c) of the different groups, and the recorded weight change (d) and weight of rats (e) at different time intervals. Data are expressed as mean±standard deviation (SD), *n*=6. A: significantly different from CTRL group; B: significantly different from IVM group; C: significantly different from BLM group; and the same below. BLM: bleomycin; CTRL: normal control; IVM: ivermectin.

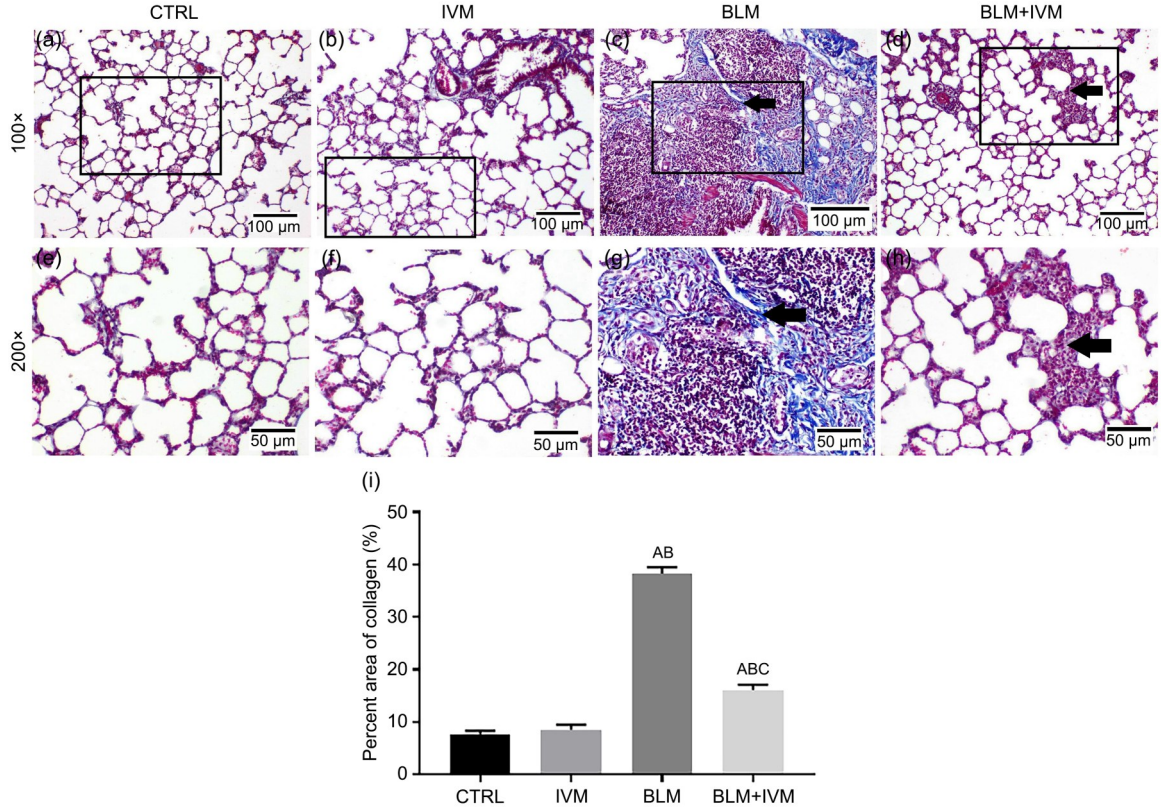

Fig. 4 Representative photomicrographs of MTC-stained lung tissues from different groups. (a, e) CTRL group showing normal weak MTC-stained collagen fibers. (b, f) IVM group showing normal weak MTC-stained collagen fibers. (c, g) BLM group showing marked increase in collagen fiber deposition (arrows). (d, h) BLM+IVM group showing weak collagen fiber deposition (arrows). (i) Area percentage of positive MTC staining in different groups. BLM: bleomycin; CTRL: normal control; IVM: ivermectin; MTC: Masson's trichrome.

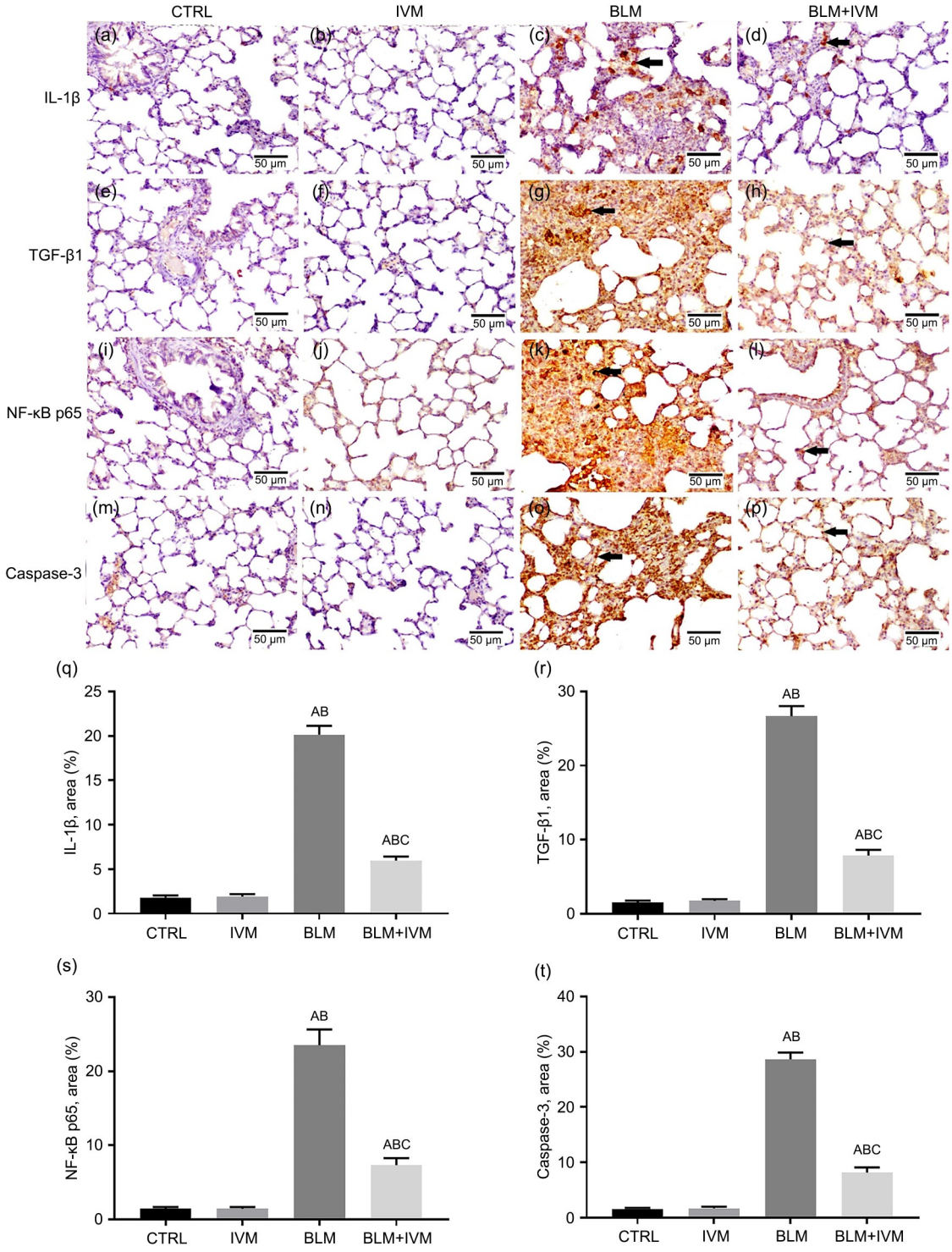

Fig. 5 Effects of ivermectin on IL-1β, TGF-β1, NF-κB p65, and caspase-3 on the lung tissues of rats with bleomycin-induced PF. (a–p) Representative photomicrographs of the immunohistochemical evaluation of IL-1β, TGF-β1, NF-κB p65, and caspase-3 in the lung tissues of rats. The brown color in the figure indicates the immunostaining of IL-1β (a–d), TGF-β1 (e–h), NF-κB p65 (i–l), and caspase-3 (m–p), as follows: negative immune expression in rats from the CTRL (a, e, i, and m) and IVM (b, f, j, and n) groups; strong immune expression in BLM (c, g, k, and o) rats; weak immune expression in BLM+IVM (d, h, l, and p) rats. (q–t) The summarized data of IL-1β, TGF-β1, NF-κβ p65, and caspase-3 (as area percentages). BLM: bleomycin; CTRL: normal control; IL-1β: interleukin-1β; IVM: ivermectin; NF-κB: nuclear factor-κB; TGF-β1: transforming growth factor-β1; PF: pulmonary fibrosis.

IL-1β, TGF-β1, NF-κB p65, and caspase-3 in the lung tissues compared to the BLM group (Figs. 5d, 5h, 5l, and 5p, respectively).

3.3 Effect of ivermectin on bleomycin-induced alterations in NLRP3-inflammasome activation

As shown in Figs. 6a and 6c, the intra-tracheal administration of bleomycin significantly upregulated the gene expression of *NLRP3* and apoptosis-associated speck-like protein containing a caspase recruitment domain (*ASC*), reaching seven and six times the expression of CTRL and IVM groups, respectively. Meanwhile, treating rats with ivermectin significantly downregulated the gene expression of *NLRP3* (Fig. 6a) and *ASC* (Fig. 6c) to reach only 51% and 60% of the value for BLM group, respectively. For further validation, the protein concentration of NLRP3 was determined, which revealed that bleomycin administration significantly increased the protein level of NLRP3 compared to both the CTRL and IVM groups, whereas treating bleomycin-induced PF rats with ivermectin significantly lowered the level of NLRP3 compared to the BLM group (Fig. 6b).

3.4 Effects of ivermectin on bleomycin-induced changes in fibronectin and HIF-1α protein levels

Both HIF-1α and fibronectin levels were significantly elevated in bleomycin-treated rats reaching 2.6-fold and 3.0-fold, respectively, compared to the CTRL group, and 3.6-fold and 3.7-fold, respectively, compared to the IVM group (Figs. 6d and 6e). On the other hand, the levels of HIF-1α and fibronectin were significantly decreased in the BLM+IVM group, reaching 51% and 42%, respectively, of the value for the BLM group (Fig 6d and 6e).

3.5 Effect of ivermectin on bleomycin-induced alterations in the oxidative stress status

Additionally, intra-tracheal administration of bleomycin to rats caused a significant increase in lipid peroxidation, as represented by lung MDA content reaching about 300% compared to the control animals (Fig. 6f). The reduction in GSH concentration (Fig. 6g) was more intense in IVM animals (115%) in comparison with the BLM group; the latter showed a significant decrease in GSH content to 51% of the CTRL value. Importantly, ivermectin administration in PF rats alleviated bleomycin-induced oxidative stress, where the level of GSH was 86% higher and the level of MDA was 48% lower in BLM+IVM rats compared to the BLM group.

4 Discussion

PF is one of the most common interstitial lung diseases worldwide with high morbidity and mortality, particularly amidst the COVID-19 pandemic (Lechowicz

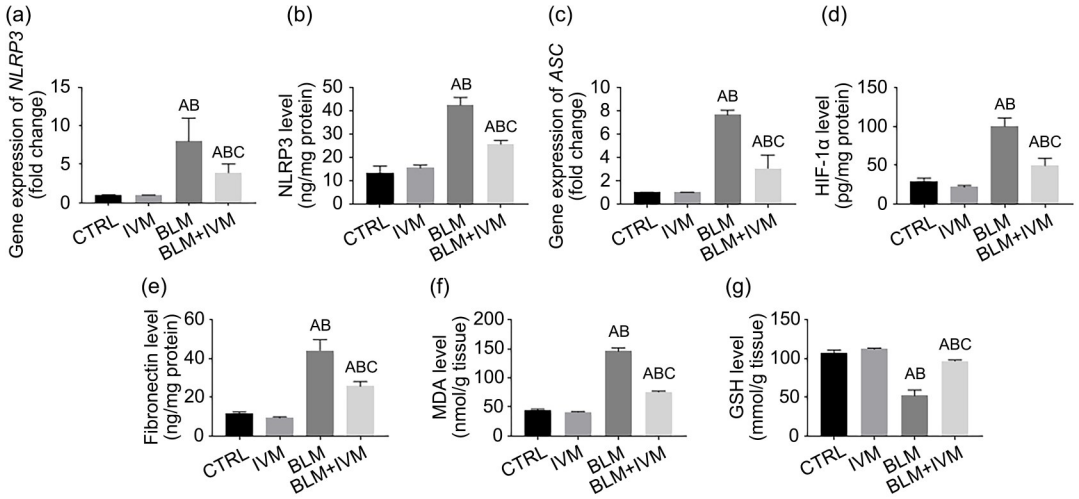

Fig. 6 Effects of ivermectin on the gene expression of *NLRP3* (a) and *ASC* (c), concentration of NLRP3 protein (b), and levels of HIF-1α (d), fibronectin (e), MDA (f), and GSH (g) in bleomycin-induced PF rats. BLM: bleomycin; CTRL: normal control; GSH: glutathione; HIF-1α: hypoxia-inducible factor-1α; IVM: ivermectin; MDA: malondialdehyde; NLRP3: nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain-containing protein 3; PF: pulmonary fibrosis.

et al., 2020; Ambardar et al., 2021). Currently, only few effective drugs are available for the treatment of PF (Han et al., 2021). Recently, the clinical utility of ivermectin as a treatment route for COVID-19-associated respiratory diseases was conjectured (Mittal and Mittal, 2021). However, to our knowledge, this is the first study reporting the anti-fibrotic effect of ivermectin in rats with experimentally induced PF.

In our report, the histopathological alterations observed in the BLM group were ameliorated and reversed by ivermectin treatment. These findings were evident by the decreased inflammation and fibrosis scores along with reduced collagen deposition in the lung tissues of BLM+IVM rats, highlighting the possible anti-fibrotic role of ivermectin. Moreover, ivermectin succeeded in suppressing the upregulation of TGF-β1 and fibronectin expression by bleomycin-induced rats, which further confirms the anti-fibrotic activity of ivermectin.

Notably, it has been recently reported that ivermectin promotes wound healing via modulating the levels of TGF-β1 and inflammatory cytokines (Sia et al., 2020). TGF-β1 is one of the key signals in the pathogenesis of PF as TGF-β1 signaling contributes to myofibroblast activation and subsequent collagen production (Han et al., 2021). Moreover, TGF-β1 is known to induce apoptosis in the alveolar epithelial cells (Han et al., 2021). As previously reported, apoptosis is a pathological hallmark of fibrotic lungs (Han et al., 2021). This was demonstrated in the current study by the enhanced protein expression of the pro-apoptotic caspase-3 in a rat model of bleomycin-induced PF, which was significantly downregulated by ivermectin treatment.

The role of inflammation in the pathogenesis of PF has been well established (Lin et al., 2019). The NLRP3 inflammasome was reported to potentially regulate the expression of TGF-β1 via modifying the level of IL-1β, which can elevate TGF-β1 in PF (Tian et al., 2017; Chen et al., 2021). In the current study, bleomycin administration triggered NLRP3 inflammasome activation, as evidenced by the enhanced gene expression of *NLRP3* and *ASC* and the subsequent increase in IL-1β level. The increase in the expression of NLRP3 inflammasome along with the development of inflammation and lung fibrosis suggests its involvement in the pathogenesis of PF, consistent with recent reports highlighting the role of NLRP3 inflammasome in mediating cell response to bleomycin-induced pulmonary injury (Tian et al., 2017; Lin et al., 2019).

Our study provides an explanation for the anti-inflammatory effects observed in ivermectin-treated COVID-19 patients; it was revealed for the first time that ivermectin treatment can significantly suppress the inflammasome activation via downregulating both *NLRP3* and *ASC* expression in addition to the reduction in IL-1β level. In fact, the observed reduction in IL-1β level in the BLM+IVM group agrees well with a previous study by Ci et al. (2009), which reported that ivermectin could inhibit lipopolysaccharide (LPS)-induced pro-inflammatory cytokines including IL-1β. Importantly, NLRP3 is thought to play a crucial role in COVID-19-induced lung inflammation and the production of pro-inflammatory cytokines (Rodrigues et al., 2021).

Accumulating proof highlights the importance of NF-κB signaling for NLRP3 inflammasome priming and assembly. Moreover, the activated NLRP3 can regulate the NF-κB signaling pathway to establish the detrimental inflammation (Chen et al., 2021). Our study found that NF-κB was upregulated in the BLM group compared to the CTRL group. In the fibrosis state, the NLRP3 inflammasome promotes the production of a considerable amount of IL-1β through NF-κB signaling, and then further activates NF-κB to enhance the expression of many inflammation-related proteins along with upregulating the collagen content (Ding et al., 2020). It is worth mentioning that the BLM+IVM group showed significantly reduced expression of NF-κB compared to the BLM group. Based on these findings, we suggest that ivermectin could suppress NLRP3 inflammasome activated in bleomycin-evoked PF through modulating the NF-κB levels. This suggestion is corroborated by the results of Ci et al. (2009), who demonstrated that ivermectin exerts an anti-inflammatory effect via downregulating NF-κB.

In the present work, the elevated expression of HIF-1α in rats with bleomycin-induced PF suggests its involvement in hypoxia and inflammatory damage of the lung tissue, which is consistent with Wang ZY et al. (2021). Notably, treatment with ivermectin could reduce HIF-1α level in the lung tissues of rats with bleomycin-induced PF compared to untreated rats, supporting the results of Kosyna et al. (2015). Furthermore, a recent study by Gonçalves et al. (2020) showed that ivermectin inhibits the viral replication of COVID-19

via the modulation of HIF-1α level. In this context, the regulation of the NLRP3 activation was reported by HIF-1α, which modulates NF-κB level and promotes the expression of IL-1β in bleomycin-induced PF (Huang et al., 2019). In addition, it was revealed that HIF-1α mediates NLRP3 inflammasome regulation, thus influencing apoptotic cell death, which further corroborates our results (Jiang et al., 2020).

The role of reactive oxygen species (ROS) production in the activation of NLRP3 inflammasome is well-documented (Chen et al., 2021). In our study, rats with PF showed increased MDA level and reduced GSH level, indicating enhanced ROS formation that was curbed by ivermectin administration. This is in line with the study by Wang JP et al. (2021), who showed that the accumulation of oxygen free radicals by bleomycin resulted in alveolar epithelial and vascular endothelial cell damage. According to a study by Chen et al. (2021), ROS production could promote the expression of NLRP3 and pro-inflammatory cytokines through the activation of intracellular NF-κB signaling pathway, which induced the transcription of genes involved in inflammation.

Finally, the current study is primarily descriptive, prompting future functional and mechanistic studies to validate our results. We recommend examining the role of NLRP3 in IVM-induced effects through gain-of-function and lose-of-function assays. Moreover, to further demonstrate the possible impact of gender on ivermectin-induced improvements in PF, our experiment should be replicated using female animals. Additional studies should also be directed to investigating the effect of ivermectin on established lung fibrosis, where PF is induced by intra-tracheal instillation of bleomycin (5 mg/kg), followed by ivermectin treatment (0.6 mg/kg) after 10 d, to ensure that fibrosis is already established in the lung and continues for the following two weeks.

5 Conclusions

Taken together, the current work has revealed that ivermectin attenuates the pulmonary inflammation and fibrosis induced by bleomycin in rats. These beneficial effects were mediated, at least partly, via the suppression of NLRP3 inflammasome through the regulation of NF-κB and HIF-1α expression. This may add to the clinical usefulness of ivermectin in treating patients with idiopathic PF and patients with COVID-19 complications. However, further investigations are required to validate these effects in clinical settings.

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Author contributions

All authors contributed to the study conception and design. Mai A. ABD-ELMAWLA, Heba R. GHAIAD, and Maha ABDELMONEM: study conception, material preparation, data collection & analysis, and writing; Enas S. GAD and Kawkab A. AHMED: material preparation, data collection & analysis, and writing. All authors wrote the first draft of the manuscript, and they all commented on previous versions of the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data. The authors declare that all data were generated in-house and that no paper mill was used.

Compliance with ethics guidelines

Mai A. ABD-ELMAWLA, Heba R. GHAIAD, Enas S. GAD, Kawkab A. AHMED, and Maha ABDELMONEM declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed. All animals' procedures were performed in accordance with the Research Ethics Committee of the Faculty of Pharmacy, Cairo University (REC-FOPCU), Egypt (No. BC3203) and with the Helsinki Declaration of 1975, as revised in 2013.

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Supplementary information

Materials and methods