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Dihydroartemisinin attenuates inflammation and fibrosis in rats by suppressing JAK2/STAT3 signaling

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Abstract

Coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2, has induced a worldwide pandemic since early 2020. COVID-19 causes pulmonary inflammation secondary to pulmonary fibrosis (PF); however, there are still no effective treatments for PF. The present study aimed to explore the inhibitory effect of dihydroartemisinin (DHA) on pulmonary inflammation and PF, and its molecular mechanism.

Morphological changes and collagen deposition were analyzed using hematoxylin-eosin staining, Masson staining, and the hydroxyproline content. DHA attenuated early alveolar inflammation and later PF in a bleomycin-induced rat PF model, and inhibited the expression of interleukin (IL) - 1 β , IL -6, tumor necrosis factor α (TNF α), and chemokine (C-C Motif) Ligand 3 (CCL3) in model rat serum. Further molecular analysis revealed that both pulmonary inflammation and PF were associated with increased transforming growth factor- β 1 (TGF- β 1), Janus activated kinase 2 (JAK2), and signal transducer and activator 3 (STAT3) expression in the lung tissues of model rats. DHA reduces the inflammatory response and PF in the lungs by suppressing TGF- β 1, JAK2, phosphorylated (p)-JAK2, STAT3, and p-STAT3 degradation. Thus, DHA exerts therapeutic effects against bleomycin-induced pulmonary inflammation and PF by inhibiting JAK2-STAT3 activation. Thus, DHA inhibits alveolar inflammation, and attenuates lung injury and fibrosis, possibly representing a therapeutic candidate to treat COVID-19.

Introduction

On 11 March 2020 World Health Organization (WHO) declared Coronavirus Disease 2019 (COVID-19), a respiratory disease caused by the SARS-CoV-2 virus, as a

pandemic [1]. The latest figures from 31 December 2020 show that more than 80 million people have been infected with the virus, causing more than 1.79 million deaths worldwide. SARS-CoV-2 crosses borders and races, infecting patients of any age [2]. The disease causes a wide range of symptoms, from asymptomatic to respiratory failure [3, 4]. Pulmonary fibrosis (PF) is one possible complication of pulmonary involvement in COVID-19, which is a chronic, progressive, and fatal form of fibrosing interstitial pneumonia [5, 6].

The pathophysiological characteristics of PF are the inability of damaged alveolar epithelial cells to reconstruct normally, excessive deposition of collagen and other extracellular matrix components, and the persistent presence of fibroblasts, leading to the destruction of the normal lung structure [6]. The interstitial matrix widens during the progression of PF and the normal pulmonary parenchyma becomes compressed, leading to respiratory failure [5].

Radiological imaging is the fastest and most direct method to assess pulmonary parenchymal involvement [7-9]. Computed tomography(CT) examination is an important method to evaluate PF. Some studies analyzed the chest CT characteristics of patients with COVID-19, and patchy ground-glass opacities were the most common changes; 85% of patients who showed progressive were accompanied by enlarged nodules and stripes [7, 8]. 12.7% patients had irregular solid nodules and 17.5% patients had fibrous stripes [10]. The results of these imaging studies for COVID-19 further confirmed that PF might be one of the major complications in patients with COVID-19 [11].

PF comprises the formation of heterogeneous pulmonary lesions by the transformation of abnormal pulmonary epithelial cells and pulmonary interstitial cells into invasive pulmonary myofibroblasts, resulting in changes to pulmonary parenchymal fibrosis. The specific mechanism of the origin and activation of invasive pulmonary myofibroblasts is still unknown. It is likely to be a multi-factor and multi-step process, including the recruitment of circulating fibroblasts' blood mesenchymal precursors, activation of fibroblasts, and mesenchymal transformation of endothelial cells, alveolar type II epithelial cells (ATII), mesothelial cells, and pericytes [11]. The process of PF involves the abnormal expression of multiple cytokines and the activation of multiple signaling pathways. Previous studies found that the Janus kinase 2 (JAK2) / signal transducer and activator of transcription 3 (STAT3) signaling pathways are involved in idiopathic pulmonary fibrosis. The levels of activated JAK2 and STAT3 were elevated in ATII cells and lung fibroblasts from patients with idiopathic pulmonary fibrosis, in which JAK2 and STAT3 participate in lung fibrosis by dependent and independent mechanisms [12]. Another study confirmed that various kinases could be phosphorylated by STAT3, several of which have been implicated in aberrant fibroblast activation in fibrotic diseases, and fibroblasts deficient in STAT3 were less sensitive to the pro-fibrotic effects of transforming growth factor beta 1 (TGF- β 1) [13]. All of these studies suggested that

STAT3 and its signaling pathways play an important role in PF.

STAT comprises an oncoprotein family encoded by tumor genes, and includes seven members. STAT3 can transduce peptide hormone signals from the cell surface to the nucleus and can be activated by many peptide hormones [14]. Various extracellular ligands can bind to receptors on the surface of the cell membrane to form cytokine-receptor kinase complexes or growth factor-receptor complexes. These complexes can phosphorylate the intracellular JAK proteins (e.g., JAK1/2 or src), and the activated JAK2 or src can phosphorylate Tyr705 sites to activate the STAT3 monomers to form dimers with STAT1 or STAT5 through their SH2 domains, after which they enter the nucleus to bind to specific target genes to activate their transcription [14].

Targeted inhibition of the STAT3 signaling pathway is expected to inhibit the formation of PF, and developing drugs that can effectively block STAT3 phosphorylation and activation might represent one of the most promising strategies to treat PF. However, currently, there is no STAT3 inhibitor that can be used effectively and safely in the clinic. Dihydroartemisinin (DHA) is an artemisinin derivative that is used widely as an antimalarial. Accumulating evidence indicates that DHA can effectively inhibit STAT3 phosphorylation [15]. Experiments *in vitro* and *in vivo* confirmed that DHA is a potent STAT3 inhibitor, acting as an effective antimetastatic via the inhibition of STAT3 phosphorylation and activation [15]. DHA could effectively suppress the invasion and migration of laryngeal cancer stem cells by inhibiting STAT3 activation, and affected the epithelial to mesenchymal transition (EMT) of cancer stem cells *in vitro* [16]. DHA significantly suppressed the phosphorylation of JAK2 in colon cancer cells, which might increase apoptosis of colon cancer cells by targeting the JAK2/STAT3 signaling [17]. In addition, DHA exhibited specific inhibitory effects on Head and Neck Squamous Cell Carcinoma through selectively blocking JAK2/STAT3 signaling [18]. This study hypothesized that DHA could attenuate PF and evaluated the effects of DHA in a rat bleomycin-induced PF model to determine the mechanisms.

Results

DHA attenuates early alveolar inflammation in a rat model of bleomycin-induced PF

Alveolar inflammation was induced on day 7 after intratracheal administration of bleomycin. As visualized by H&E staining (Figure 1A), the lung tissues from the control group of rats had intact alveoli, while the lung tissues from the rats stimulated with bleomycin exhibited obvious inflammatory cell infiltration in the alveolar cavity, pulmonary edema accompanying septal thickness, damage to the lung architecture, and alveolar disarray. DHA attenuated alveolar inflammation, and reduced the septal edema and alveolar damage. Lung tissues from the DHA-3 group exhibited slight infiltration of inflammatory cells and slight pulmonary edema in comparison to those in the BLM group (Figure 1A).

The Szapiel scoring method was used to assess the extent of pulmonary inflammation; the scoring rules are shown in Table 1. The score of the control group was 0.33 ± 0.47 , that of the BLM group was 2.83 ± 0.37 , that of the DHA-1 group was 2.33 ± 0.47 , that of the DHA-2 group was 1.67 ± 0.47 , and that of the DHA-3 group was 0.83 ± 0.37 . Compared with that of the control group, the Szapiel scores of BLM groups increased significantly ($P < 0.001$). Compared with that of the BLM group, the Szapiel score of the DHA2 and DHA3 groups decreased significantly ($P < 0.05$ and $P < 0.001$), and the difference between the groups was statistically significant ($P < 0.001$; Fig. 1B). In the early stage of the PF model, bleomycin caused pneumonia, and DHA effectively inhibited pulmonary inflammatory. In addition, DHA inhibited pulmonary inflammation in a dose-dependent manner, such that intraperitoneal injection of high dose DHA (100 mg/kg/d) could significantly inhibit alveolar inflammation.

Table 1. Szapiel scoring rules for alveolar inflammation

Lever	Morphological analysis under the microscope	Scored
1	No alveolar inflammation	0
2	Mild alveolitis, with an area less than 20% of the lung	1
3	Moderate alveolar inflammation, involving 20–50% of the lung	2
4	Severe alveolar inflammation, involving more than 50% of the lung	3

Figure 1. DHA attenuates early alveolar inflammation and the inflammatory response in a bleomycin-induced rat pulmonary fibrosis model . At 7 days after bleomycin intratracheal administration, with or without DHA treatment, the rats were sacrificed, and their lungs and blood were removed. (A) H&E staining was used to detect the lung histopathological changes and (B) Lung inflammation was scored (n = 6). (C) *Il1b*, (D) *Il6*, (E) *Tnfa*, (F) *Ccl3* mRNA in lung tissues were determined to assess the lung inflammatory response (n = 6). Data are expressed as the means $\pm$ standard error . * $P < 0.05$, ** $P < 0.001$. BLM, bleomycin; DHA, dihydroartemisinin; NS, not

statistically significant.

DHA inhibits the inflammatory response in the lungs and serum of bleomycin - exposed rats.

To reveal the role of DHA in the early inflammation of the bleomycin-induced rat model, we used ELISA and qRT-PCR to detect inflammatory cytokines in the lungs and serum of rats at 7 days after modeling. The qRT-PCR result demonstrated that stimulation with bleomycin significantly increase the mRNA levels of *Il1b*, *Il6*, *Tnfa*, and *Ccl3* in the rat lungs, while DHA significantly attenuated these increases (Fig. 1C-F) in a dose-dependent manner: A high dose of DHA (100 mg/kg/d) effectively inhibited the transcription of all the tested inflammatory cytokines in lung tissue. Consistent with the qRT-PCR results, the protein levels of IL-1 β , IL-6, TNF α , and CCL3 in the rat serum increased after bleomycin stimulation but decreased after DHA treatment (Fig. 2A-D). These results indicate that DHA attenuated the inflammatory response in the lungs and serum of bleomycin-exposed rats.

Figure 2. DHA reduces the inflammatory response in the lungs via the JAK2/STAT3 signaling pathways. (A) IL-1 β , (B) IL-6, (C) TNF α , and (D) CCL3 protein levels in blood were determined to assess the lung inflammatory response (n = 6). (E) *Tgfb1*, (F) *Jak2*, and (G) *Stat3* mRNA levels in the lungs were determined using qRT-PCR (n = 6). Data are expressed as the means $\pm$ standard error. * P <0.05, ** P <0.001. BLM, bleomycin; DHA, dihydroartemisinin; NS, not statistically significant; qRT-PCR, quantitative real-time reverse transcription PCR.

DHA reduces the inflammatory response in the lungs via JAK2/STAT3 signaling.

To explore the mechanism by which DHA inhibits the early alveolar inflammatory response in the rat bleomycin-induced PF model, we evaluated the *Tgfb1*, *Jak2*, and *Stat3* mRNA expression and TGF- β 1, JAK2, p-JAK2, STAT3, and p-STAT3 protein levels in lung tissues at day 7 after intratracheal administration of bleomycin, with/without DHA treatment, using qRT-PCR and immunocytochemical staining. In homogenized lung tissue, the *Tgfb1*, *Jak2*, and *Stat3* mRNA levels in the BLM group were higher than those in the control groups. DHA (25 mg/kg/d) significantly inhibited the expression the of *Tgfb1* mRNA ($P < 0.001$, Fig. 2E), high dose DHA(100 mg/kg/d)significantly inhibited the expression the of *Jak2* mRNA ($P < 0.001$, Fig. 2F), DHA (50 mg/kg/d) significantly inhibited the expression the of *Stat3* mRNA ($P < 0.001$, Fig. 2G). The IHC results also showed that BLM increased the levels of TGF- β 1, JAK2, p-JAK2, STAT3, and p-STAT3 in lung tissues (Fig. 3A), and the mean IOD values showed that DHA reduced the levels of TGF- β 1, JAK2, p-JAK2, STAT3, and p-STAT3 proteins in a dose-dependent manner (all $P < 0.001$, Fig. 3B-F). DHA could inhibit the phosphorylation of JAK2, thereby inhibiting the phosphorylation of STAT3. Thus, DHA might reduce the inflammatory response of the lungs by inhibiting the activation of JAK2/STAT3 signaling.

Figure 3. DHA inhibited the JAK2/STAT3 pathway in pneumonia tissue. (A) Representative images of TGF- β 1, JAK2, p-JAK2, STAT3, and p-STAT3 protein expression in lung tissue detected by IHC. Mean IOD of (B) TGF β 1, (C) JAK2, (D) p-JAK2, (E) STAT3, and (F) p-STAT3 protein by IHC staining. Data are expressed as the means $\pm$ standard error. * $P < 0.05$, ** $P < 0.001$. BLM, bleomycin; DHA, dihydroartemisinin; IHC, immunohistochemistry; IOD, integrated optical density; NS, not statistically significant.

DHA reduces PF induced by bleomycin in rats.

H&E staining was performed to explore the pathological alterations and Masson

staining was used to evaluate the collagen fibrils in the lung tissues on day 28 after intratracheal administration of bleomycin, with/without DHA treatment. Compared with those of the control group, under the microscope, the lung tissues in the BLM group showed loss of alveolar architecture, pulmonary septal thickening, and increased cell number, ultimately resulting in severe lung tissue damage. However, DHA attenuated the pathological alterations in the lung tissues at a dose of 100 mg/kg/d (Fig. 4A). The Szapiel score also showed that DHA inhibited alveolar inflammation, with the Szapiel score decreasing in a DHA dose-dependent manner ($P < 0.001$, Fig. 4B). ELISA assays were used to explore the inflammatory cytokines in serum of rats in the PF model, which demonstrated significantly higher levels than in the control group (all $P < 0.001$, Fig. 4C-F). By contrast, the IL-1 β , IL-6, TNF α , and CCL3 levels in serum were reduced by DHA in a dose-dependent manner (all $P < 0.001$, Fig. 4C-F). High dose DHA (100 mg/kg/d) could significantly attenuate the increased IL-1 β , IL-6, and TNF α levels in the serum of the model rats (all $P < 0.001$). Low dose DHA (25 mg/kg/d) could reduce the CCL3 level in the serum of the model rats ($P < 0.001$).

Figure 4. DHA reduces pulmonary fibrosis induced by bleomycin in rats. Rats were sacrificed at 28 days after bleomycin injection, with or without DHA treatment, their blood and lungs were removed. (A) H&E staining of bleomycin-treated rat lungs, with and without DHA. (B) Lung inflammation was scored ($n = 6$). (C) IL-1 β , (D) IL-6, (E) TNF α , (F) CCL3 protein in serum were determined by ELISA ($n = 6$). Data are expressed as the means $\pm$ standard error. * $P < 0.05$, ** $P < 0.001$. BLM, bleomycin ; DHA, dihydroartemisinin; NS, not statistically significant; H&E, hematoxylin and eosin; ELISA, enzyme-linked immunosorbent assay.

Masson staining also shown that massive deposits of collagen in the pulmonary septum (blue) in BLM group lung tissues, and DHA treatment reduced the size of the

collagen deposits (Fig. 5A). The Ashcroft score was used to evaluate the pathological alterations of pulmonary fibrosis. The Ashcroft score rules are shown in Table 2. The score of the control group was 0.83 ± 0.69 , the score of the BLM group was 5.16 ± 1.07 , the score of the DHA-1 group was 3.83 ± 1.46 , the score of the DHA-2 group was 2.33 ± 0.94 , and the score of the DHA-3 group was 1.33 ± 0.94 . The Ashcroft score of the BLM group was increased significantly ($P < 0.001$), and DHA could decrease the Ashcroft score in dose dependent manner ($P < 0.001$, Fig. 5B). The hydroxyproline content was measured to evaluate PF. At 28 days after bleomycin treatment, the rats of the BLM group demonstrated a higher hydroxyproline content than that of the control group ($P < 0.001$). Compared with that of the BLM group, DHA significantly reduced hydroxyproline content in lung tissue of the bleomycin-induced rats at doses of 50 mg/kg/d and 100 mg/kg/d (all $P < 0.001$; Fig. 5C).

Table 2. Ashcroft scoring rules for PF

Lever	Morphological analysis under the microscope	Scored
0	Normal	0
1	Partially enlarged alveoli, alveolar septum thickened slightly (3 times $\leq$ normal)	1
2	Alveolar septum thickened moderately (3 times normal $>$), no damage to the lung structure	2
3	Alveolar septum thickened moderately (3 times normal $>$), and increased fibrotic tissue increased	3
4	The area of fibrous tissue mass is less than 10% of the lung, with mild lung structural damage	4
5	The area of fibrous tissue mass is 10–50% of the lung, and with pulmonary structural damage	5
6	The area of fibrous tissue mass is more than 50% of the lung, and with obvious pulmonary structural damage	6
7	Severe lung damage, large areas of fibrosis, honeycomb lung	7
8	Full field fibrous tissue	8

Figure 5. DHA reduces collagen deposition in pulmonary fibrosis tissues through the JAK2/STAT3 pathway. The rats were sacrificed at 28 days after bleomycin injection, with or without DHA treatment, and their lung were removed and blood collected. (A) Representative images of Masson staining of bleomycin-treated rat lungs, with and without DHA. (B) Evaluation of pulmonary fibrosis using the Ashcroft score (n = 6). (C) Determination of hydroxyproline in lung tissue (n = 6). (D) *Tgfb1*, *Jak2*, and (F) *Stat3* mRNA levels in the lung tissues were determined using qRT-PCR (n = 6). Data are expressed as the means $\pm$ standard error. * $P < 0.05$, ** $P < 0.001$. BLM,

bleomycin; DHA, dihydroartemisinin; NS, not statistically significant; qRT-PCR, quantitative real-time reverse transcription PCR.

DHA inhibits PF via JAK2/STAT3 signaling

TGF- β 1 is known to be critical for PF; therefore, we used qRT-PCR to detect *Tgfb1* mRNA expression in the lung tissue of all rat groups. The results confirmed that the expression of *Tgfb1* mRNA was higher than that in the control group on day 28 after bleomycin injection. However, DHA could significantly reduce this increase (Fig. 5D). We also detected the mRNA of *Jak2* and *Stat3* in the rat lung tissues. Similar to the results for *Tgfb1*, high dose DHA (100 mg/kg/d) could decrease the expression of *Jak2* and *Stat3* mRNA significantly (all $P < 0.001$, Fig. 5E and 5F).

To further confirm DHA inhibition of PF through JAK2/STAT3 signaling, we used IHC to detect the levels of TGF- β 1, JAK2, p-JAK2, STAT3, and p-STAT3 proteins in lung tissues (Fig. 6A). The mean IOD score showed that DHA (50 mg/kg/d) could reduce the levels of TGF- β 1, JAK2, and p-JAK2 significantly (all $P < 0.001$; Fig. 6B-D), and a high dose of DHA (100 mg/kg/d) could decrease the levels of STAT3 and p-STAT3 significantly (all $P < 0.001$; Fig. 6E-F).

Figure 6. DHA inhibited pulmonary fibrosis via the JAK2/STAT3 pathway (A) Representative images of TGF-β1, JAK2, p-JAK2, STAT3, and p-STAT3 protein in lung tissues, detected using IHC. Mean IOD of (B) TGF-β1, (C) JAK2, (D) p-JAK2, (E) STAT3, and (F) p-STAT3 protein IHC staining. Data are expressed as the means $\pm$ standard error. * $P < 0.05$, ** $P < 0.001$. BLM, bleomycin; DHA, dihydroartemisinin; IHC, immunohistochemistry; IOD, integrated optical density. NS, not statistically significant.

Discussion

PF is a common pathological process after lung tissue injury, chronic relapses of

which affect the quality of life of patients and can even cause respiratory failure. PF will occur after severe pulmonary infection caused by COVID-19 [19]. Currently, COVID-19 is prevalent worldwide; however, there is still no specific drug treatment for PF. Therefore, exploring the mechanism of PF to find effective drugs is expected to improve the quality of life of patients with COVID-19.

Pulmonary inflammation is the initial cause of PF [19]. During the inflammatory response, the alveolar epithelium and vascular endothelium are damaged at first, and then inflammatory cells and immune effector cells enter the lung and release inflammatory mediators, resulting in the formation of alveolar exudates, including a variety of inflammatory cytokines [5]. In the present study, in the early stage of the PF model induced by bleomycin, the mRNA and protein levels of inflammatory factors IL-1 β , IL-6, TNF α , and CCL3 in the rat lung and serum increased significantly.

CCL3 is expressed on macrophage surfaces and can directly interfere with TGF- β 1 secretion [20, 21], and can regulate the course of acute pulmonary inflammation [20, 22]. Normally, the expression of CCL3 is low; however, when the target cells are stimulated by endotoxins, such as lipopolysaccharide, viral proteins, proinflammatory factors (IL-1 β), the related cell signaling pathway is activated and CCL3 expression increases. The earliest reports of COVID-19 clarified that IL-1 β , IL-6, TNF α , and CCL3 in the peripheral blood of patients with severe COVID-19 pneumonia were significantly higher than those of the non-severe patients, and the cytokine storm is an important factor contributing to death from COVID-19 [16]. In addition, our study found that IL-1 β , IL-6, TNF α , and CCL3 levels in rat blood increased significantly during the acute inflammation of PF induced by bleomycin. However, DHA could reduce the inflammatory response in the lungs of model rats significantly and reduced the expression of cytokines, such as CCL3, in the blood.

Previous studies have shown that CCL3 and its receptors promote fibrosis [20], and analysis of alveolar lavage fluid in patients with congenital PF showed increased CCL3 levels. Our study also found that the CCL3 level in the blood of rats with PF was increased significantly. However, the CCL3 level in the blood of rats treated with DHA (50 mg/kg/d) decreased significantly, and PF was inhibited. These findings suggested that CCL3 plays an important role in pulmonary inflammation and PF, and DHA inhibits CCL3 expression to attenuate pulmonary inflammatory responses and PF.

Cytokines can either directly activate myofibroblasts or induce the transformation of epithelial cells into fibroblasts, leading to the formation of fibrosis [23]. Furthermore, many cytokines, particularly the IL-6 family, can activate STAT3 rapidly within cells. With persistent STAT3 activation, chronic inflammation and fibrosis ensue [14]. The IL-6 receptor α and IL-6 receptor β (gp130) can form a heterodimeric receptor. IL-6 binds to the heterodimeric receptor and then undergoes transphosphorylation and activation via gp130-associated JAKs, notably JAK2. Activated JAK2 proteins phosphorylate themselves and the receptors, which results in STAT3, via its Src-homology (SH) 2 domain, binding to these phosphorylated sites. Phosphorylated JAK2 can activate STAT3 through phosphorylation at Tyr705 sites

Phosphorylated STAT3 monomers form dimers with STAT1 or STAT5 through their SH2 domains and enter the nucleus to bind to specific target genes to activate their transcription [14, 24].

In the early stage of the PF model induced by bleomycin, we observed activation of JAK2/STAT3 signaling. JAK/STAT signaling induces macrophage activation, and the activated macrophages secrete cytokines, such as platelet-derived factor, TNF α , IL-28, and nitric oxide [23]. TNF α directly induces the expression of TGF- β [25]; therefore, in the early stage of the bleomycin-induced rat model, the expression of TGF- β 1 was also high in the lung tissue. TGF- β 1 is a key direct fibrosis factor that can promote the transformation of pulmonary fibroblasts into myofibroblasts, produce more matrix protein components, and release them into extracellular matrix, thus leading to the occurrence and development of PF [26, 27]. Furthermore, TGF- β 1 signaling can induce phosphorylation and activation of JAK2, thereby activating the JAK2/STAT3 signaling pathway, and this vicious circle amplifies the inflammatory response and pulmonary fibrosis [28]. Our results showed that in the later stage of the PF model induced by bleomycin, Masson staining confirmed the presence of PF, and IHC staining confirmed the activation of JAK2/STAT3 signaling. Our findings suggested that JAK2/STAT3 signaling pathways play a crucial role in the early stage of inflammatory response in the bleomycin-induced PF model, as well as in the later stage of pulmonary fiber formation. Previous studies have also detected STAT3 phosphorylation in fibrotic lung tissue from patients with idiopathic pulmonary fibrosis, and demonstrated that phosphorylated STAT3 participated in both the lung epithelial cell damage and the fibroblast to myofibroblast transition, making STAT3 an attractive therapeutic target in PF[12, 28].

Recently, although the number of developed STAT3 inhibitors has been increasing, and some STAT3 inhibitors have been tested in clinical trials, there are potential defects in the clinical development of these drugs, and as yet none are available for clinical application.

Artemisinin is an active component extracted from the leaves and buds of *Artemisia annua*, a traditional Chinese medicine plant, and is a sesquiterpene lactone compound containing internal peroxides. Used in the treatment of various types of malaria, with high efficiency, Artemisinin (or its derivative DHA) induces few side effects and has other beneficial characteristics[29]. DHA has many pharmacological effects on virus infection [30], inflammation [31], and tumor cell proliferation [32]. In addition, DHA also shows relatively safe toxicity characteristics. Previous studies have found that DHA can effectively block the phosphorylation of STAT3, thereby inhibiting its activation, with negligible toxicities [16]. In addition, DHA has been reported to exert protective effects against lung inflammation [33, 34]. The present study revealed the role of DHA in the early inflammatory response in a rat model of bleomycin-induced PF. DHA effectively alleviated lung tissue damage and inhibited the release of inflammatory mediators. Notably, DHA attenuated pulmonary inflammatory responses in a dose-dependent manner, such that lung tissues from the rats treated with 100 mg/d of DHA exhibited only slight infiltration of inflammatory cells and little pulmonary edema, suggesting a therapeutic effect on bleomycin-induced

acute lung injury. Our findings suggested that DHA is a potential therapeutic agent for patients with PF in the future. In further research, we found that DHA reduced lung inflammation and lung tissue damage by inhibiting the phosphorylation of JAK2 and STAT3. The levels of phosphorylated JAK2 and phosphorylated STAT3 protein were reduced significantly in lung tissue by daily injection of 100 mg DHA. In the late stage of the bleomycin-induced PF model, we also found that DHA could significantly inhibit pulmonary fiber deposition in the lung tissues of the rat model, as well as the content of hydroxyproline acid, indicating that DHA could inhibit the formation of PF. Further experiments showed that DHA could significantly inhibit TGF- β 1 expression and inhibit the phosphorylation of JAK2 and STAT3.

In contrast to STAT3, the effect of DHA on JAK2 has not been studied. The present study further revealed that DHA inhibited STAT3 phosphorylation via inhibiting JAK2 phosphorylation. Regarding the mechanism by which DHA inhibits JAK2 phosphorylation, our study found that TGF- β 1 expression and TNF α expression were significantly decreased in lung tissues after DHA treatment. We concluded that DHA might inhibit macrophage activation through the TGF- β /JAK2/STAT3 signaling pathway, in which reducing the expression of TNF- α and IL-6, reduces the expression of TGF- β 1, and inhibits the inflammatory response and PF.

The COVID-19 pandemic is mounting severe health, economic, and social challenges to the global population. Under these conditions, it is important to anticipate and prepare for this challenge. PF is the ultimate outcome of many viral infections, leading to acute respiratory distress syndrome. Currently, there is still a lack of drugs with antiviral, anti-inflammatory, and anti-fibrosis effects and that have negligible toxicities. DHA has the potential to treat COVID-19 infections and prevent the long-term consequences of fibrosis that the epidemic may cause. Finally, we hope that our findings will help to treat COVID-19 and prevent PF induced by COVID-19.

Materials and methods

Ethics statement.

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Yang Zhou University (YZU-EC-JS202022, Jiangsu, China). The experiments were performed in accordance with the guidelines of the National Institutes of Health, and all procedures met the requirements of the ARRIVE (Animal Research: Reporting In Vivo Experiments) guideline. Rats were anesthetized with isoflurane, and all necessary efforts were taken to minimize suffering prior to the experiments.

Reagents and antibodies

Masson's trichrome was purchased from Beijing Solarbio Science & Technology, Ltd., (GB1340, Solarbio, Beijing, China). Anti-phospho-JAK2 (Y1007) antibodies were purchased from Abcam (ab195055, Cambridge, UK); anti-JAK2 and anti-phospho-STAT3 (Tyr705) antibodies were purchased from Cell Signaling Technology (3230 and 9145, Danvers, MA, USA); anti-STAT3 (bsm-52235) and

anti-TGF- β 1 (bs0086R) antibodies were purchased from Bioss (Beijing, China); horse radish peroxidase (HRP)-conjugated Goat anti-mouse IgG (sc-516102) and goat anti-rabbit IgG (sc-2357) were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Rat interleukin (IL)-1 β enzyme-linked immunosorbent assay (ELISA) Kit (70-EK301B/3-96), the Rat IL-6 ELISA Kit (70-EK306HS-96), the Rat tumor necrosis factor alpha (TNF α) ELISA Kit (70-EK382/3-96), and the Rat chemokine (C-C Motif) Ligand 3 (CCL3)/ macrophage inflammatory protein 1-alpha (MIP-1 α) ELISA Kit (CCL3, 70-EK361-96) were purchased from Lianke Bio (Hangzhou, Zhejiang, China). Isoflurane was obtained from Abbott Pharmaceutical Co., Ltd. (Shanghai, China), and the hydroxyproline determination kit was from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animal experiments.

Five-week-old male Wistar rats weighing 110–130 g were used in all experiments. The rats were bought from the Comparative Medicine Centre of Yangzhou University (Yang Zhou, JiangSu, China). The rats were bred under pathogen-free conditions with a 12 hour dark/light cycle, in a laminar flow cabinet at 24 °C and a relative humidity of 40–60%. Rats were provided with food and purified water, and rat were bred for 1 week to adapt the environmental before the experiment.

The rats were randomly divided into five groups (n = 12 in each group) as follows: 1. The BLM group received bleomycin (BLM; 5 mg/kg) for intratracheal instillation at a concentration of 4 mg/ml; 2. The Control group, who received the same volume of 0.9% NaCl solution for intratracheal instillation only; 3. The DHA-1 group, who received intratracheal instillation of BLM and daily intraperitoneal injection of DHA (25 mg/kg); 4. The DHA2 group, who received intratracheal instillation of BLM and daily intraperitoneal injection of DHA (50 mg/kg); and 5. The DHA3 group, who received intratracheal instillation of BLM and daily intraperitoneal injection of DHA (100 mg/kg). BLM was injected directly into the trachea under direct vision after the trachea had been exposed through a midline anterior neck incision. At 7 or 28 days after intratracheal BLM administration, with or without DHA treatment, the rats were sacrificed by inhaling excess isoflurane. Lung tissue was quickly removed and blood was collected for further analysis.

Experiments involving animals have been reported according to ARRIVE guidelines 54 . All efforts were made to minimize animal suffering and to reduce the number of animals used.

Hydroxyproline and collagen measurement

The content of hydroxyproline was measurement to calculate the total collagen content in lung tissue, since 12.5% of collagen comprises hydroxyproline; therefore, the total content of tissue collagen was calculated by dividing the hydroxyproline value by 0.125. The hydroxyproline determination kit was used for the hydroxyproline assay, operated in strict compliance with the manufacturer's

instructions. All experiments were performed in triplicate. Data for the hydroxyproline content are expressed as micrograms of hydroxyproline per milligrams of lung tissue ($\mu\text{g}/\text{mg}$).

Cytokine measurements

The levels of IL-1 β , IL-6, TNF α , and CCL3 in rat sera were detected using ELISA kits. All experiments were operated according to the manufacturer's instructions and the assays were performed in triplicate.

RNA extraction and quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA was extracted from lung tissue using an RNeasy Mini Kit (Invitrogen, Waltham, MA, USA). A reverse transcription kit (Takara, Shiga, Japan) was used to perform first strand cDNA synthesis, and an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used to conduct the quantitative real-time PCR, using SYBR Green dye (Roche Diagnostics, Mannheim, Germany). The *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene was used as an internal control to calculate the relative fold expression levels. The experiment was performed in triplicate. Primers for *Il1b* (IL-1 β), *Il6*, *Tnfa* (TNF α), *Ccl3*, *Tgfb1* (TGF- β 1), *Jak2*, *Stat3*, and *Gapdh* are shown in Table 3.

Table 3. Primers used for qRT-PCR in the present study

Gene	Primer sequence (5' to 3')
<i>Il1b</i>	F:GGGCCTCAAAGGAAAGAATC
	R:TACCAGTTGGGGAAGTCTGC
<i>Il6</i>	F:CTG GGGATGTCTGTAGCTCA
	R:CTGTGAAGTCTCCTCTCCGG
<i>Tnfa</i>	F:TCTCAAACTCGAGTGACAAG
	R:AGTTGGTTGTCTTTGAGATCC
<i>Ccl3</i>	F:ACCACAGTCCATGCCATCAC
	R:TCCACCACCCTGTTGCTGTA
<i>Tgfb1</i>	F: GTCATAGATTGCATTGTTGC
	R:AAGGAGACGGAATACAGGG
<i>Jak2</i>	F:TCTGGGGAGTATGTTGCAGAA
	R:AGACATGGTTGGGTGGATACC
<i>Stat3</i>	F: GTGTGACACCGTAAGTGGCT
	R: GACATCGGCAGGTCAATGGT
<i>Gapdh</i>	F:TGACTTCAACAGCGACACCCA
	R: CACCCTGTTGCTGTAGCCAAA

Immunohistochemistry (IHC)

IHC was performed using lung tissues. Primary antibodies recognizing TGF- β 1(dilution, 1:200), JAK2 (dilution, 1:200) , p-JAK2 (dilution, 1:100), STAT3 (dilution, 1:200), or p-STAT3 (dilution, 1:200) were incubated with tissue slides overnight at 4 °C, and then processed according to our previous report [35]. All staining was imaged digitally using the same light exposure and evaluated using Image Pro Plus (IPP), a digitalized IHC scoring program (Media Cybernetics, San Diego, CA, USA). All experiments were performed in triplicate. The immunostaining results are expressed as the mean integrated optical density (IOD).

Histological analysis

Hematoxylin-Eosin (H&E) staining and Masson staining were used to analyze the grades of pulmonary inflammation and fibrosis. Lung tissues were fixed using 4% paraformaldehyde for 24 h at room temperature, and then embedded in paraffin. Sections for pathological analysis were cut at 4- μ m thickness and were stained with H&E, Masson staining, and using immunohistochemistry. An optical microscope was used to observe the H&E and Masson stained sections and lung injury was scored by two pathologists who were blinded to the treatments. The severity of pulmonary inflammation was scored using the Szapiel scoring method [36] and PF was scored using the Ashcroft scoring method [37]; the scoring rules are shown in tables 1 and 2

Statistical analysis

SPSS 20.0 (IBM Corp., Armonk, NY, USA) was used to conduct the statistical analysis. The means $\pm$ standard error were used to express the continuous variables, one-way analysis of variance (ANOVA) and Dunnett's t test was used for comparisons between groups based on the normal distribution of the data. Values of $P < 0.05$ were regarded as statistically significant.

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Author Contributions

Conceptualization, You,X.L.; guo,T.;and Jiang,X.Y.; Data curation, Zhang,C.M.; Jiang,X.Y.; Formal Analysis, You,X.L.; and Jiang,X.Y; Funding Acquisition, You,X.L.; Investigation, Zhao,X.J. and Bao,J.J.;Methodology, You,X.L.; Jiang,X.Y.;and Jiang,K.J .; Guo,T.; Bao,J.J.and Zhu,X.W.; Project Administration,

You,X.L.;and Zhang,C.M.; Resources, Guo,T.;and Zhu,X.W.; Software, Zhang,C.M.; Supervision, You,X.L.; Zhao,X.J.; and Zhang,C.M.; Validation, Zhao,X.J. and Bao,J.J.; Visualization, Bao,J.J.; Writing-Original Draft Preparation, You,X.L.;and Jiang,X.Y.; Writing-Review & Editing, You,X.L.; and Jiang,X.Y.; All authors read and approved the final manuscript.

Informed Consent Statement

Not applicable

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Figures

Figure 1

DHA attenuates early alveolar inflammation and the inflammatory response in a bleomycin induced rat pulmonary fibrosis model . At 7 days after bleomycin intratracheal administration, with or without DHA treatment, the rats were sacrificed, and their lungs and blood were removed. (A) H&E staining was used to

detect the lung histopathological changes and (B) Lung inflammation was scored (n = 6). (C) IL1 β , (D) IL6, (E) Tnfa, (F) Ccl3 mRNA in lung tissues were determined to assess the lung inflammatory response (n = 6). Data are expressed as the means $\pm$ standard error. *P < 0.05, **P < 0.001. BLM, bleomycin; DHA, dihydroartemisinin; NS, not statistically significant.

Figure 2

DHA reduces the inflammatory response in the lungs via the JAK2/STAT3 signaling pathways. (A) IL-1 β , (B) IL-6, (C) TNF α , and (D) CCL3 protein levels in blood were determined to assess the lung inflammatory response (n = 6). (E) Tgfb1, (F) Jak2, and (G) Stat3 mRNA levels in the lungs were determined using qRT-PCR (n = 6). Data are expressed as the means $\pm$ standard error. *P < 0.05, **P < 0.001. BLM, bleomycin; DHA, dihydroartemisinin; NS, not statistically significant; qRT-PCR, quantitative real-time reverse transcription PCR.

Figure 3

DHA inhibited the JAK2/STAT3 pathway in pneumonia tissue. (A) Representative images of TGF β 1, JAK2, p JAK2, STAT3, and p STAT3 protein expression in lung tissue detected by IHC. Mean IOD of (B) TGF β 1, (C) JAK2, (D)p-JAK2,(E) STAT3, and (F) p STAT3 protein by IHC staining. Data are expressed as the means $\pm$ standard error. *P < 0.05, **P < 0.001. BLM, bleomycin; DHA, dihydroartemisinin; IHC, immunohistochemistry; IOD, integrated optical density; NS, not statistically significant.

Figure 4

DHA reduces pulmonary fibrosis induced by bleomycin in rats. Rats were sacrificed at 28 days after bleomycin injection, with or without DHA treatment, their blood and lungs were removed. (A) H&E staining of bleomycin treated rat lungs, with and without DHA. (B) Lung inflammation was scored (n = 6). (C) IL-1 β , (D) IL-6, (E) TNF α , (F) CCL3 protein in serum were determined by ELISA (n = 6). Data are expressed as

the means $\pm$ standard error. * $P < 0.05$, ** $P < 0.001$. BLM, bleomycin ; DHA, dihydroartemisinin; NS, not statistically significant; H&E, hematoxylin and eosin; ELISA, enzyme linked immunosorbent assay assay.

Figure 5

DHA reduces collagen deposition in pulmonary fibrosis tissues through the JAK2/STAT3 pathway. The rats were sacrificed at 28 days after bleomycin injection, with or without DHA treatment, and their lung were removed and blood collected. (A) Representative images of Masson staining of bleomycin treated

rat lungs, with and without DHA. (B) Evaluation of pulmonary fibrosis using the Ashcroft score (n = 6). (C) Determination of hydroxyproline in lung tissue (n = 6). (D) Tgfb1, Jak2, and (F) Stat3 mRNA levels in the lung tissues were determined using qRT-PCR (n = 6). Data are expressed as the means $\pm$ standard error. *P < 0.05, **P < 0.001. BLM, bleomycin; DHA, dihydroartemisinin; NS, not statistically significant; qRT-PCR, quantitative real-time reverse transcription PCR.

Figure 6

DHA inhibited pulmonary fibrosis via the JAK2/STAT3 pathway (A) Representative images of TGF β 1, JAK2, p JAK2, STAT3, and p STAT3 protein in lung tissues, detected using IHC. Mean IOD of (B) TGF β 1, (C) JAK2, (D) p JAK2, (E) STAT3, and (F) p STAT3 protein IHC staining. Data are expressed as the means $\pm$ standard error . *P < 0.05, **P < 0.001. BLM, bleomycin; DHA, dihydroartemisinin; IHC, immunohistochemistry; IOD, integrated optical density. NS, not statistically significant.