

β -Amyrin ameliorates pulmonary fibrosis by inhibiting inflammatory response and oxidative stress in mice

Zhiyong Cai, Jindi Liu, Hongliang Bian* and Jinlan Cai

Newborn Department, Yancheng Maternity and Child Health Care Hospital, Yancheng, Jiangsu Province, China

Abstract: The study aimed to determine efficacy and mechanisms of β -Amyrin on pulmonary fibrosis. Use bleomycin (BLM) to induce the marine model of pulmonary fibrosis. β -Amyrin (20, 40, 80 mg/kg) was once treated via intragastrical administration for five consecutive days when after BLM stimulation. HE/Masson staining, hydroxyproline (HYP) content, Arterial blood gas analysis (BGA), inflammatory cytokines and oxidative stress factors were performed in this study. The lung gas-exchange function was significantly improved after being treated β -Amyrin with different concentrations, while IL-6, IL-1 β , TNF- α and MCP-1 levels were decreased. And the increased fibrotic lesion in lung was also determined after treatment of β -Amyrin. Additionally, reduced MDA level and increase levels of GPX, SOD and GSH were also demonstrated using β -Amyrin in BLM-induced mice in a dose-dependent manner. In conclusions, our study determined that β -Amyrin has a potent efficacy in protecting against BLM-induced pulmonary fibrosis via suppressing inflammatory response and oxidative stress.

Keywords: β -Amyrin, pulmonary fibrosis, inflammation, oxidative stress.

INTRODUCTION

Pulmonary fibrosis, characterized by diffuse alveolitis, pulmonary interstitial inflammation and interstitial fibrosis, is a chronic inflammatory disease (Kropski JA *et al.*, 2019). The prevalence of this disease is increasing year by year. 5-year survival rate is less than 50% (Kinoshita and Goto, 2019). Bleomycin (BLM) is often used as a chemotherapeutic drug to treat cancer, but it has obvious lung toxicity and easily leads to lung injury and fibrosis (Kolb P *et al.*, 2020). Under normal circumstances, BLM is metabolized by hydrolase, but the lack of hydrolase in the lungs makes the lungs very sensitive to the toxicity of BLM (Egea-Zorrilla A *et al.*, 2022). High concentrations of BLM can cause DNA strand breaks, generate free radicals, induce oxidative stress and ultimately lead to cell necrosis and/or apoptosis (Ravanetti and Ragonieri, 2020). A one-time injection of BLM into the trachea is often used to establish an animal model of pulmonary fibrosis.

Recently, clinically used drugs to treat pulmonary fibrosis mainly include immunosuppressants, corticosteroids and new drugs such as nintedanib and pirfenidone (Liu *et al.*, 2017). However, due to their complex etiology, the pathogenesis is still unclear. The curative effect of these existing treatment methods is still not ideal, so finding new drugs that effectively treat pulmonary fibrosis has become a hot and difficult point (Bellaye *et al.*, 2018). Natural products are always been a research hotspot for pulmonary fibrosis treatment (Qian *et al.*, 2018). β -Amyrin, pentacyclic triterpenoid compound (Park *et al.*, 2020), has exhibit anti-fibrotic, anti-inflammatory and

anti-apoptotic effects (Thirupathi *et al.*, 2017). But its protective effect on pulmonary fibrosis is not clear. So, we determine efficacy and mechanisms of β -Amyrin on pulmonary fibrosis.

MATERIALS AND METHODS

Establishment of pulmonary fibrosis mice model

Totally 50 male C57 mice (weighting 20-25g), after habituation for 1 week, mice were initially separated into 5 groups: control group (control group treated with saline), BLM group (model group) and β -Amyrin groups (20, 40 and 80mg/kg). Mice in BLM groups received a one-time intratracheally injection of 5mg/kg BLM in 50 μ L saline per mouse to induce pulmonary fibrosis. From 5 days before BLM to 14 days after BLM, mice in β -Amyrin groups treated with β -Amyrin 20, 40 and 80 mg/kg, respectively. The operation was performed under anesthesia by pentobarbitone intraperitoneal injection (20 mg/kg). All animal experiments were approved by Institutional Animal Care.

SOD, GPX, GSH and MDA assay

Assay kits of GPX, SOD, GSH and MDA were used to detect these levels in lung tissues as per the manufacturer's instructions. All assay kits were provided by R&D Systems Inc., USA

Arterial blood gas analysis

Abdominal aortic arterial blood of mice was collected at the end of experiments. Oxygen saturation (SO₂), partial pressure of oxygen (PO₂), partial pressure of carbon dioxide (PCO₂) and blood power of hydrogen (pH) were assessed according to the BGA.

*Corresponding author: e-mail: hlbian05@126.com

Cytokine analysis

Cytokines in the sample from serum were detected by ELISA as per the manufacturer's instructions (IL-6, IL-1β, TNF-α and MCP-1, Puxin, Shanghai, China).

Assay of hydroxyproline (HYP) content

Hydroxyproline (HYP) content of lung tissues were detected by kit as per the manufacturer's instructions.

Pathological examination

Used Hematoxylin and eosin to detect the pathological change and Masson's trichrome staining to detect fibrotic extent of lung tissue. After fixed in 4% formaldehyde for 48h, lung tissue then was cut into 4 μm thick sections for HE and Masson.

STATISTICAL ANALYSIS

All values are presented as the mean ± SD. Student's t test and one-way or two-way ANOVA evaluate significant differences. GraphPad Prism 8.0 statistic software (LaJolla, CA, USA) was used.

RESULTS

Effect of β-amyrin on HYP and lung index

Collagen level of fibrosis in tissue samples was determined using the HYP level. Compared to control group, the HYP content in BLM group was significantly increased (fig. 1A). After being treated with different doses of β-Amyrin, the HYP gradually recovered to normal. Moreover, we also determined the lung index, which indicated that β-Amyrin reduced lung index, especially at the dose of 80mg/kg (fig.1B).

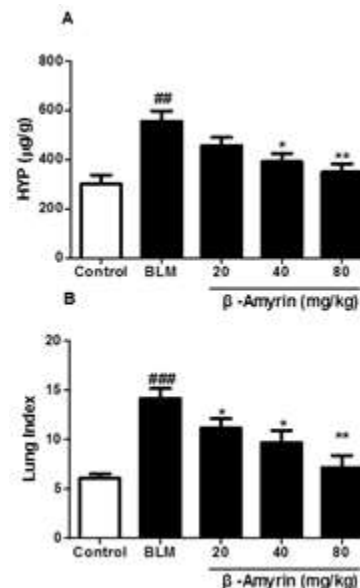
Effects of β-Amyrin on blood gas analysis (BGA) in BLM-induced pulmonary fibrosis

In this study, we detected the arterial blood gas to assess the pulmonary fibrosis. The results demonstrated that blood pH in BLM group was significantly lower (fig. 2A) than control group ($p < 0.01$), while the value of PCO_2 in BLM group was significantly higher than controls (fig. 2B). In addition, both PO_2 value (fig. 2C) and SO_2 value (fig. 2D) were markedly reduced in BLM group compared with control group. β-Amyrin dynamically elevated value of blood pH. Moreover, β-Amyrin also markedly promoted the values of SO_2 and PO_2 and inhibited the value of PCO_2 .

Effect of β-amyrin on BLM-induced pulmonary fibrosis

Morphological observation of the lung tissue of mice stained with HE indicated that compared the normal lung tissue in control group, the alveolar cavity was intact, the alveolar wall was thin and there was no thickening, alveolar cavity and lung interstitium had almost no inflammatory cell exudation and no fiber. In BLM group, the lung tissue has numbers of inflammatory cell

infiltration, the diffuse alveolar septum thickens and part of the alveoli in the lesion collapses and forms flaky lung tissue. Compared with BLM group, mice the β-Amyrin groups had a significant improvement in the lung injury (fig. 3).



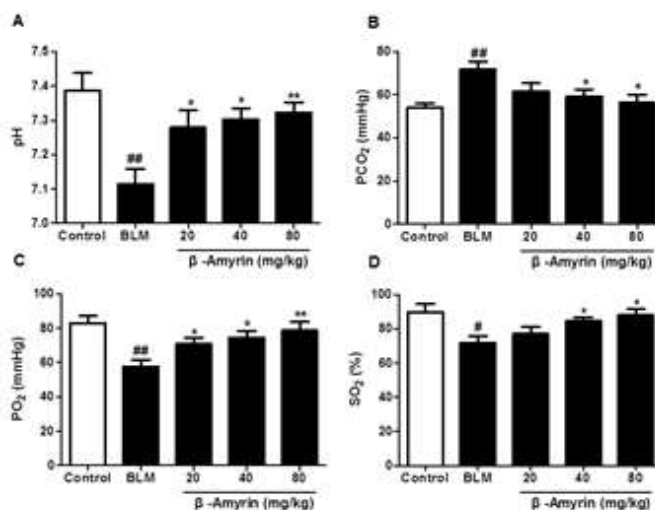
Pulmonary fibrosis was induced with intraperitoneal injection of a single intratracheal administration of BLM. Mice were administered saline or β-Amyrin orally 4 days prior to BLM and continued until to the end of experiment. (A) HYP levels 8 weeks after BLM treatment. (B) Pulmonary index 8 weeks after BLM treatment. ^{##} $P < 0.01$, ^{###} $P < 0.001$, BLM group compared to control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$, β-Amyrin-treated groups compared to BLM group.

Fig. 1: Effects of β-Amyrin on HYP content and pulmonary index.

Morphological observation of lung tissue of mice stained with Masson also found that the distribution of collagen fibers in lung tissue was basically normal in the control group, with only sporadic collagen fibers. However, numbers of reticular fibers were formed in the lung tissue of BLM group and the alveolar structure was destroyed. A lot of collagen was deposited and pulmonary fibrosis lesions formed. Compared with BLM group, β-Amyrin treatment could improve pulmonary fibrosis in a dose-dependent manner, especially at the dose of 80mg/kg ($P < 0.01$). Collectively, our data showed that that β-Amyrin could effectively ameliorate pulmonary fibrosis induced by BLM.

Effects of β-Amyrin on serum levels of IL-6, IL-1β, TNF-α and MCP-1

In the end of experiments, we determined the levels of IL-6, IL-1β, TNF-α and MCP-1 in serum using ELISA. The results suggested the significantly raised level of TNF-α (fig. 5A), IL-1β (fig. 5B), IL-6 (fig. 5C) and MCP-1 (fig. 5D) in BLM group.



Mice were administered saline or β -Amyrin orally 5 days prior to BLM and continued until to the end of experiment. Arterial blood was collected in the syringe and sealed. Key indicators of blood gas testing including power of hydrogen (pH, A), partial pressure of carbon dioxide (PCO₂, B), partial pressure of oxygen (PO₂, C), oxygen saturation (SO₂, D) were measured. #P<0.05, ##P<0.01, BLM group compared to control group; *P<0.05, **P<0.01, β -Amyrin-treated groups compared to BLM group.

Fig. 2: Arterial blood gas analysis in BLM-induced pulmonary fibrosis.

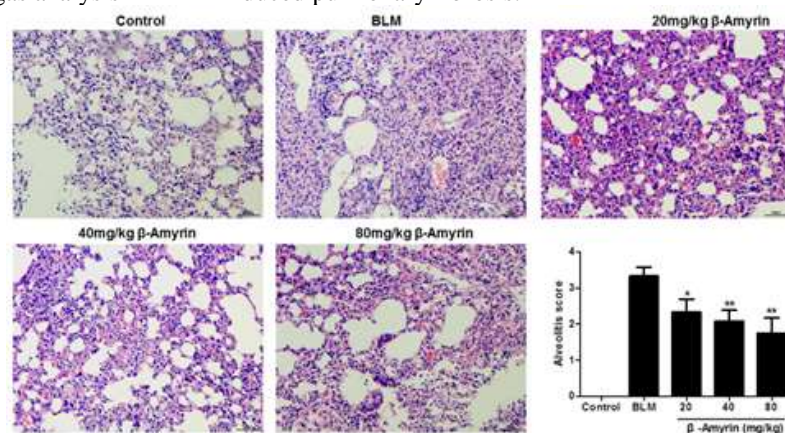
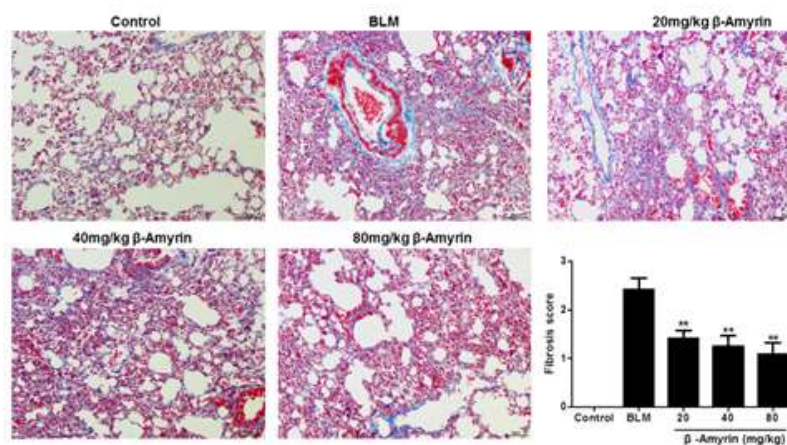
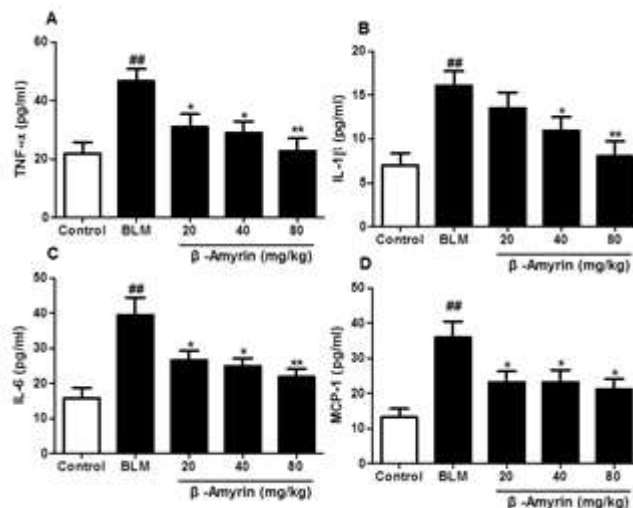


Fig. 3: Effect of β -Amyrin on pathomorphology (H&E staining) of BLM-induced pulmonary fibrosis in mice ($\times 200$).



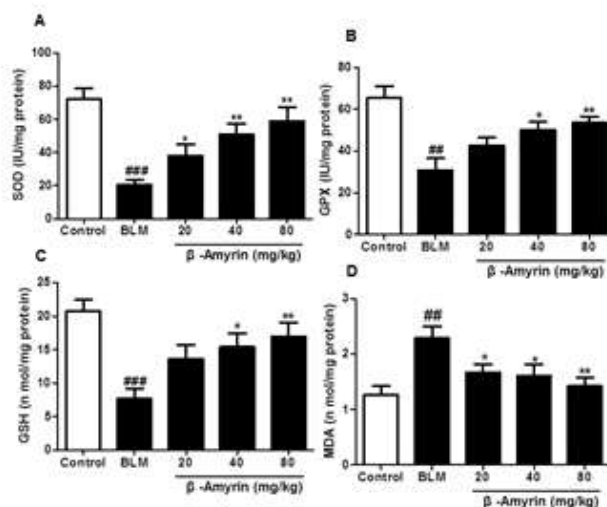
#P<0.05, ##P<0.01, BLM group compared to control group; *P<0.05, **P<0.01, β -Amyrin-treated groups compared to BLM group.

Fig. 4: Effect of β -Amyrin on pathomorphology (Masson's trichrome staining) of BLM-induced pulmonary fibrosis in mice ($\times 200$).



The blood was collected from abdomen artery and serum TNF- α (A), IL-1 β (B), IL-6 (C) and MCP-1 (D) were measured with ELISA. ##P<0.01, BLM group compared to control group; *P<0.05, **P<0.01, β -Amyrin-treated groups compared to BLM group.

Fig. 5: Effects of β -Amyrin on serum levels of inflammatory factor.



The lung tissue was collected and the levels of SOD (A), GPX (B), GSH (C) and MDA (D) were measured with ELISA. ##P<0.01, ###P<0.001, BLM group compared to control group; *P<0.05, **P<0.01, β -Amyrin-treated groups compared to BLM group.

Fig. 6: Effects of β -Amyrin on tissue levels of oxidative stress.

As the increase of β -Amyrin concentrations, the level of IL-6, IL-1 β , TNF- α and MCP-1 in treatment groups returned to normal as control group and the effect was dose-dependent. These findings determined that β -Amyrin could significantly inhibit the inflammatory response to attenuate the pulmonary fibrosis.

Effects of β -amyrin on oxidative stress

Our data suggested that BLM decreased the activities of SOD, GPX and GSH compared with control group (p<0.001). Interestingly, β -Amyrin treatment could up regulate the activities of GPX, SOD and GSH (fig. 6A-C). In addition, BLM increased MDA levels (p<0.01) and β -Amyrin decreased lung tissue MDA levels (fig. 6D).

DISCUSSION

Pulmonary inflammation is the main manifestation in pulmonary fibrosis, inflammation persists and eventually forms fibrosis (Kamio *et al.*, 2018). 3-year survival rate of pulmonary fibrosis is only 50%. For a long time, there has been a lack of effective treatments for pulmonary fibrosis. Until 2014, the US FDA simultaneously approved pirfenidone and nintedanib to treat idiopathic pulmonary fibrosis (Chan *et al.*, 2017). However, there are no anti-pulmonary fibrosis drugs with independent intellectual property rights in my country at present and the above two new drugs are expensive, long-term medication is unbearable for ordinary patients and there are also adverse

reactions such as liver toxicity. Thus, it is necessary to develop anti-pulmonary fibrosis drugs that are economical, efficient and low-toxic.

Mice are usually used to induce the model animal. Commonly used methods of administration include intratracheal drip, intraperitoneal injection, subcutaneous injection, tail vein injection, etc (Shieh *et al.*, 2019). Intratracheal instillation of BLM is currently commonly used method to model the pulmonary fibrosis. BLM induces DNA strand breaks, generates free radicals and induces oxidative stress, which leads to cell damage. Cell necrosis and apoptosis are accompanied by the development of inflammation and fibrosis (Pan *et al.*, 2014). Pathologically, it mostly presents the histopathological characteristics of ordinary interstitial pneumonia, mainly the damage and destruction of alveolar structure, massive deposition of extra cellular matrix, inflammatory cell infiltration and fibrosis with the formation of cellular lung. The pathological and physiological changes of the BLM-induced pulmonary fibrosis model are similar to those of PF and are used to simulate the pathological process of human pulmonary fibrosis (Shieh *et al.*, 2019). In our present study, we found that BLM could induce the pulmonary fibrosis and reduced the BGA function, oxidative stress and inflammation response. According to the results of this study, we determined the protective effects of β -Amyrin on pulmonary fibrosis.

Study has found that the anti-fibrotic effect of liver depends on the reduction of TNF- α level (Krithika *et al.*, 2016). Activated expression of MCP-1 is usually found in inflammatory exudative tissue. In our research, IL-6, IL-1 β , TNF- α and MCP-1 levels were higher in BLM-induced pulmonary fibrosis mice group but their levels were reduced significantly in β -Amyrin-treated group, suggesting that β -Amyrin alleviates pulmonary fibrosis by reducing inflammatory markers of liver injury. MDA level is an indicator of oxidative damage. β -Amyrin treatment significantly reduced MDA level and increased antioxidant enzymes SOD, GPX and nonenzyme GSH, suggesting that β -Amyrin alleviates pulmonary fibrosis by reducing oxidative stress.

In our study, we found that β -Amyrin not only markedly decreased HYP content, lung index but also improved the gas-exchange function of lung, as well as alleviated the lung tissue fibrosis.

CONCLUSION

Our study demonstrates the mechanism of β -Amyrin inhibited BLM-induced pulmonary fibrosis, which may be regulated by inhibiting inflammatory response and oxidative stress.

REFERENCES

- Bellaye PS, Yanagihara T, Granton E, Sato S, Shimbori C, Upagupta C, Imani J, Hambly N, Ask K, Gauldie J, Iglarz M and Kolb M (2018). Macitentan reduces progression of TGF- β 1-induced pulmonary fibrosis and pulmonary hypertension. *Eur. Respir. J.*, **52**(2): 1701857.
- Chan P, Bax L, Chen C, Zhang N, Huang SP, Soares H, Rosen G and Abutarif M (2017). Model-based meta-analysis on the efficacy of pharmacological treatments for idiopathic pulmonary fibrosis. *CPT: Pharmacomet. Syst. Pharmacol.*, **6**(10): 695-704.
- Duecker R, Baer P, Eickmeier O, Strecker M, Kurz J, Schaible A, Henrich D, Zielen S and Schubert R (2018). Oxidative stress-driven pulmonary inflammation and fibrosis in a mouse model of human ataxia-telangiectasia. *Redox Biology*, **14**: 645-655.
- Egea-Zorrilla A, Vera L, Saez B and Pardo-Saganta A (2022). Promises and challenges of cell-based therapies to promote lung regeneration in idiopathic pulmonary fibrosis. *Cells*, **11**(16): 2595.
- Kamio K, Azuma A, Matsuda K, Usuki J, Inomata M, Morinaga A, Kashiwada T, Nishijima N, Itakura S, Kokuho N, Atsumi K, Hayashi H, Yamaguchi T, Fujita K, Saito Y, Abe S, Kubota K and Gemma A (2018). Resolution of BLM-induced murine pulmonary fibrosis via a splenic lymphocyte subpopulation. *Respir Res.*, **19**: 71.
- Kinoshita T and Goto T (2019). Molecular mechanisms of pulmonary fibrogenesis and its progression to lung cancer: A Review. *Int J Mol Sci.*, **20**(6):1461
- Kolb P, Upagupta C, Vierhout M, Ayaub E, Bellaye PS, Gauldie J, Shimbori C, Inman M, Ask K and Kolb MRJ (2020). The importance of interventional timing in the bleomycin model of pulmonary fibrosis. *Eur. Respir J.*, **55**(6): 1901105.
- Krithika R, Jyothislakshmi V and Verma RJ (2016). Phyllanthin inhibits CCl4-mediated oxidative stress and hepatic fibrosis by down-regulating TNF- α /NF- κ B, and pro-fibrotic factor TGF- β 1 mediating inflammatory signaling. *Toxicol. Ind. Health*, **32**(5): 953-60.
- Kropski JA and Blackwell TS (2019). Progress in understanding and treating idiopathic pulmonary fibrosis. *Annu. Rev. Med.*, **70**: 211-224.
- Pan Y, Wang Y, Zhao Y, Peng K, Li W, Wang Y, Zhang J, Zhou S, Liu Q, Li X, Cai L and Liang G (2014). Inhibition of JNK phosphorylation by a novel curcumin analog prevents high glucose-induced inflammation and apoptosis in cardiomyocytes and the development of diabetic cardiomyopathy. *Diabetes*, **63**(10): 3497-511.
- Park HJ, Kwon H, Lee JH, Cho E, Lee YC, Moon M, Jun M, Kim DH and Jung JW (2020). β -Amyrin ameliorates alzheimer's disease-like aberrant synaptic plasticity in the mouse hippocampus. *Biomolecules & therapeutics*, **28**(1): 74-82.

- Qian W, Cai X, Qian Q, Zhang W and Wang D (2018). Astragaloside IV modulates TGF-β1-dependent epithelial-mesenchymal transition in BLM-induced pulmonary fibrosis. *J Cell Mol Med.*, **22**(9): 4354-4365.
- Ravanetti F and Ragonieri L (2020). Modeling pulmonary fibrosis through BLM delivered by osmotic minipump: a new histomorphometric method of evaluation. **318**(2): L376-1385.
- Shieh JM, Tseng HY, Jung F, Yang SH and Lin JC (2019). Elevation of IL-6 and IL-33 levels in serum associated with lung fibrosis and skeletal muscle wasting in a bleomycin-induced lung injury mouse model, 7947596.
- Thirupathi A, Silveira PC, Nesi RT and Pinho RA (2017). β-Amyrin, a pentacyclic triterpene, exhibits anti-fibrotic, anti-inflammatory and anti-apoptotic effects on dimethyl nitrosamine-induced hepatic fibrosis in male rats. *Hum Exp Toxicol.*, **36**(2): 113-122.