Histol Histopathol (2021) 36: 317-324

http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE **Open Access**

Shufeng Jiedu capsules protect rats against LPS-induced acute lung injury via activating NRF2-associated antioxidant pathway

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Summary. Shufeng Jiedu capsule (SFJDC) is a traditional Chinese medicine, which has been used for the treatment of respiratory infections for more than thirty years in Hunan (China). SFJDC protected rats against LPS-induced acute lung injury (ALI); however, the molecular mechanisms underlying the therapeutic effects of SFJDC remain unclear. Therefore, this study aimed at analyzing the major anti-inflammatory compounds of SFJDC and exploring the underlying molecular mechanisms. SFJDC dissolved in water was fingerprinted by UPLC/Q-TOF. Inflammation response was assessed by histopathological examination and ELISA assay. Arterial blood gases were also analyzed to evaluate the function of rat lungs. The expression levels of Kelch-like ECH-associating protein 1 (Keap1), Nrf2, heme oxygenase-1 (HO1), Cullin 3 (CUL3) and NQO1 were analyzed by Western blotting. Results indicated that SFJDC alleviated inflammation response by reducing the level of inflammatory cytokines, and upregulation of glutathione-S-transferase (GST) and superoxide dismutase (SOD) in lung tissues. Furthermore, SFJDC suppressed LPS-induced upregulation of Keap 1 and CUL3 in rat lungs. The expression of NRF2 HO1 and NQO1 were further upregulated by SFJDC in the presence of LPS, indicating that SFJDC might activate the NRF2-associated antioxidant pathway. In conclusion, SFJDC treatment may protect the rat lungs from LPS by alleviating the inflammation response via NRF2-associated antioxidant pathway.

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DOI: 10.14670/HH-18-293

Key words: Shufeng Jiedu capsule (SFJDC), Acute lung injury, Antioxidant activity, NRF2

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are characterized by an acute hypoxemic respiratory insufficiency or respiratory failure; the mortality rate of ALI/ARDS is 35-45% (Hu et al., 2013; Tao et al., 2013). As we know, neutrophil-mediated inflammatory response imbalance and reactive oxygen species (ROS)-mediated injury are associated with the pathogenesis of ALI/ARDS (Athale et al., 2012; Reddy et al., 2012; Davidson et al., 2013; El Ali et al., 2013; Lin et al., 2013). Inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 can trigger the inflammation process that leads to a systemic inflammatory response syndrome (Luh and Chiang, 2007). These inflammatory cytokines and ROS contribute to lung injury and lead to ALI or ARDS (Ji et al., 2010; Jin et al., 2011; Choi et al., 2012; Artaud-Macari et al., 2013). Therefore, inhibiting the secretion of inflammatory cytokines and ROS generation may play a major role in the treatment of ALI/ARDS (Chaves de Souza et al., 2013; Potteti et al., 2013; Sahin et al., 2013; Tao et al., 2014).

Lipopolyssacharides (LPS) are components of the cell wall of Gram-negative bacteria, which are the main reagents causing infections in patients with ALI (Luh and Chiang, 2007). LPS could induce ALI in mice via promoting inflammation through modulating TLR4/MyD88/NF-ϰB pathway (Ju et al., 2018). Macrophages (main immune cells) play multiple important roles in protecting lungs from infection by

responding to LPS. In addition, macrophage-induced ROS play a critical role in fighting against pathogens, but at the cost of lung damage (Yan et al., 2008). In order to deal with the transiently increased ROS-induced damage, lung cells activate an anti-oxidative stress response system (Jin et al., 2009). This coordinated response is regulated by upstream gene antioxidant response element (ARE) (Jin et al., 2009). In addition, ARE was activated by nuclear factor erythroid 2 (NF-E2)-related factor 2 (NRF2) (Cho et al., 2015); NRF2 plays an important role in defending against oxidative stress (Kim et al., 2013; Colín-González et al., 2014; Tsai et al., 2014). Moreover, human BTB-Kelch protein KEAP1 negatively regulates NRF2 activity by binding to Cullin 3 (Cul3) and NRF2 (Kobayashi et al., 2006).

Presently, the main treatment strategies against ALI/ARDS still rely on antibiotics and corticosteroids (Reddy et al., 2009). Shufeng Jiedu capsule (SFJDC) is a traditional Chinese medicine used for treating many respiratory illnesses in Hunan (China) (Tao et al., 2013, 2014). SFJDC is composed of *Polygonum cuspidatum* Sieb et Zucc., *Forsythia suspensa* (Thunb) Vahl., *Isatis indigotica* Fortune ex Lindl., *Bupleurum chinense* DC., *Thlaspi arvense* L., *Verbena officinalis* L., *Phragmites communis* Trin, and *Glycyrrhiza uralensis* Fisch. SFJDC contains active compounds such as resveratrol and flavonoids, which demonstrate significant antioxidant effects by regulating NRF2/ARE pathways (Cheng et al., 2015a). In our previous studies, SFJDC protect against ALI by suppressing the MAPK/NF-κB pathway (Tao et al., 2014). Nevertheless, the mechanisms by which SFJDC regulated ALI remain unclear. In the present study, we aimed at analyzing the anti-inflammatory components of SFJDC and exploring the underlying mechanisms.

Materials and methods

Ultra high performance liquid chromatography/ quadrupole-time of fly (UPLC/Q-TOF) sample preparation

SFJDC was purchased from Anhui Jiren Pharmaceutical Co., Ltd. (Anhui, China, batch #Z20090047). The SFJDC solution was obtained by dissolving 0.2 g of SFJDC in 12 mL of 70% methanol. Then, SFJDC solution was sonicated for 1 h and centrifuged for 15 min at 12000 rpm; the supernatant was collected for UPLC/Q-TOF analysis.

Ultra performance liquid chromatography (UPLC)

Fingerprinting analysis was conducted at the Shanghai School of Medicine, Fudan University (Shanghai, China). UPLC was performed using an Acquity UPLC system (Wasters, Milford, MA, USA). The chromatographic column of Waters ACQUITY UPLC BEH C18 (1.7 μm, 2.1×100 mm) was equipped and eluted with a linear gradient of A (acetonitrile) and (B) ultrapure water with 0.1%(v/v) formic acid) at flow rate of 0.4 mL/min at 35°C. The injection volume was 4 μL in this study. Gradient elution program is provided as indicated in Table 1.

Mass spectrum (MS) condition

Waters Q-TOF Premier mass spectrometer (Waters) with an electrospray ion source was used for acquiring MS data. Q-TOF mass spectrometer was operated in V mode with a scanning range of m/z 100 to 1500 Da in both positive-ion and negative-ion mode. The conditions of ESI source were set as follows: source temperature 110°C; desolvation gas temperature, 350°C; desolvation gas flow rate, 600L/h; capillary voltage, 3.0 kV (positive ion mode) or 2.5 kV (negative ion mode); cone gas, 50L/h; detector voltage, 1.9 kV (positive ion mode), or 2.0 kV (negative ion mode). The mass data were processed by MassLynx 4.1 software. Leucine enkephalin acetate salt was used for calibration ([M+H]+ = 555.2931, [M-H]- = 553.2775).

Animal experiment

Specific pathogen-free (SPF) Sprague-Dawley rats (weighing 250±25 g) were purchased from the Medical School Clinical Animal Research Center of Fudan University (Shanghai, China). All rats were housed in a SPF facility at Fudan University. All experimental procedures were performed according to the Chinese Guidelines for the Care and Use of Laboratory Animals. All experiments were approved by the Institutional Animal Care Committee of Fudan University. Rats were randomized to four groups (20 rats per group): PBS group, SFJDC treatment, LPS treatment, and LPS plus SFJDC. Rats were intraperitoneally injection with LPS (10 mg/kg) (L4005-100MG; Sigma, St Louis, MO, USA) for establishment of ALI model. Half an hour later, the rats received intragastric feeding of SFJDC (100 mg/kg; SFJDC is soluble in water). Three days after SFJDC treatment, rats were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg). The trachea was exposed and cannulated with a 20-gauge intravascular catheter. The abdominal cavity was opened at the ventral midline to obtain 2-5 ml of aorta blood for

Table 1. Gradient elution program.

Time /min	Flow rate mL/min	A (Acetonitrile/%)	B (ultrapure water with 0.1% (v/v) formic acid)
0.0	0.4	2.0	98.0
2.0	0.4	2.0	98.0
12.0	0.4	15.0	85.0
15.0	0.4	15.0	85.0
18.0	0.4	20.0	80.0
35.0	0.4	50.0	50.0
40.0	0.4	100.0	0.0

arterial blood gas analysis.

Enzyme-linked immunosorbent assay (ELISA)

The bronchoalveolar lavage fluid (BALF) was collected after treatment. The levels of IL-1β, IL-6, TNF-α, GST, SOD and andmonocyte chemotactic protein 1 (MCP-1) in BALF were detected using ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols.

HE staining

To prepare paraffin embedding, the posterior lobe of the right lung was fixed in 4% paraformaldehyde firstly. Then, the tissue sample was cut at a thickness of 5 μm and the samples were deparaffinized, rehydrated and stained with hematoxylin-eosin (HE) for 5 min. After HE staining, the sections were observed with a light microscope at 200× magnification.

Blood gas analysis

Rat arterial blood was taken for blood gas analysis under anesthetic. Samples were analyzed immediately for detection of partial pressure of oxygen (PO_2) using a blood gas analyzer I-STST1 (300) mode (Abbott, Lake Bluff, IL, USA).

TUNEL staining

TUNEL staining was conducted using an ApopTag Plus In Situ Apoptosis Detection Kit (Millipore, Danvers, MA, USA) in accordance with the manufacturer's instructions. Positive-stained cells were counted under a fluorescence microscope (Olympus, Japan).

Western blot analysis

RIPA buffer (Beyotime, Shanghai, China) was used to acquire total proteins. The concentrations of proteins were detected with bicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for protein separation. Separated proteins were transferred into a PVDF membrane (Thermo Fisher) and blocked with 5% nonfat milk for 2 h. After that, membranes were incubated with the following primary antibodies overnight at 4°C: anti-Keap 1 (1:1000, Abcam, Cambridge, MA, USA) (1:1000), anti-Nrf2 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HO1 (1: 1000, Abcam), anti-CUL3 (1:2000, Abcam), anti-NQO1 (1:1000, Abcam), anti-GAPDH (1:1000, Abcam). After washing with TBST three times, the PVDF membrane was incubated with secondary antibody anti-rabbit (Abcam; 1:5000) for 1 h at room temperature. The protein samples were determined by chemiluminescence using ECL reagent (Pierce, Rockford, IL, USA).

Statistical analysis

Data were analyzed using GraphPad Prism version 7 (GraphPad Software, version 7.0). All data are presented as mean ± standard deviation (SD). The homogeneity of variance and the normal distribution were verified. One-way ANOVA was then used for analysis. $P<0.05$ was considered statistically significant.

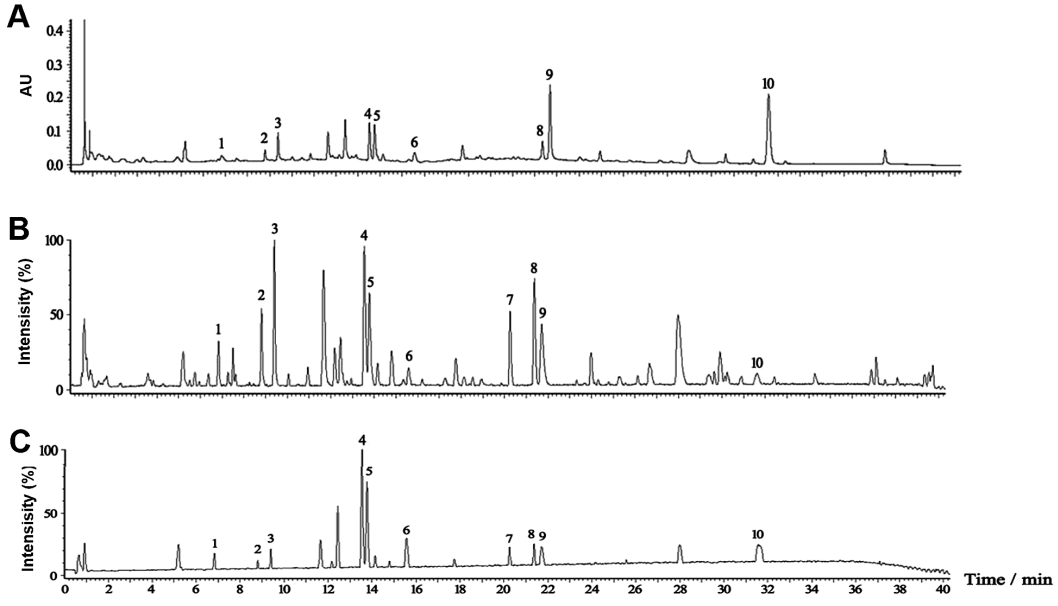

Fig. 1. UPLC/Q-TOF chromatograms to identify the active ingredients of SFJDC. **A.** Chromatogram at 254 nm **B.** 4321` in positive ion mode **C.** Mass spectrum in negative ion mode. Anti-inflammatory ingredients of SFJDC (1-10).

Results

Identification of anti-inflammatory components of SFJDC

Since the anti-inflammatory effects of SFJDC remain unclear (Cheng et al., 2015a), UPLC/Q-TOF was used to identify the anti-inflammatory components of SFJDC firstly. UPLC/Q-TOF of SFJDC was performed to acquire precise molecular mass and the retention time for indentification of unknown ingredients. The molecular mass was precisely determined within a reasonable degree of measurement error using Q-TOF. The fingerprint of SFJDC is presented in Fig. 1. Unknown compounds were characterized by analyzing the fragmentation behaviors in MS spectrum as well as referring to the available literature. The fragmentations were determined by accurate high resolution mass measurements. The total ion chromatogram of SFJDC is shown in Fig. 1A. The total ion current chromatogram at positive ESI mode is shown in Fig. 1B. The total ion current chromatogram at negative ESI mode is shown in Fig. 1C. Eventually, 10 anti-inflammatory components of SFJDC were identified (Table 2). These 10 anti-inflammatory components were classified as follows based on their structures: Phenylethanolic glycosides (forsythoside E, forsythoside A, isoforsythoside A, verbascoside); Iridoid glycosides (hastatoside, verbenalin); Lignans (phillyrin); Flavonoids (3-Hydroxyglabrol, vitexin); Anthraquinones (emodin).

SFJDC protects rat against LPS-induced ALI

Next, H&E staining was performed to evaluate the protective effect of SFJDC on LPS-stimulated rat lungs. In the control group (Fig. 2A), normal lung histology was observed, including clear alveolar space, normal alveolar septum, and complete structural integrity (Liu et al., 2019). In LPS treated group, infiltration of inflammatory cells in the alveoli, necrotic epithelial cells, the congestive edema and increased interstitial thickness were observed (Fig. 2A). However, LPS-induced pathomorphologic changes of rat lungs were significantly alleviated by SFDJC treatment; solely SFJDC administration had no effect on lung histology (Fig. 2A). In addition, arterial blood gas analysis was applied to evaluate the role of SFJDC in LPS-induced rat ALI. As demonstrated in Fig. 2B, LPS notably decreased PO2 level in rat lungs, while this phenomenon was reversed by SFJDC. Moreover, TUNEL staining indicated LPS-induced cell apoptosis in rat lungs was significantly alleviated by SFJDC (Fig. 2C,D). Taken together, LPS-induced pathomorphologic changes, pulmonary dysfunction and cell apoptosis in rat lungs were all alleviated by SFJDC.

SFDJC significantly reversed LPS-induced inflammatory response in lung of rats

We next used ELISA assays to estimate the level of inflammatory cytokines, as well as the activities of GST and SOD in rat lungs. After injection of LPS, a significant increase in the levels of inflammatory cytokines, including IL-1β, IL-6, TNF-α and MCP-1 was observed in rat lungs (Fig. 3A-D). In addition, LPS-induced secretion of IL-1β, IL-6 TNF-α and MCP-1 in rat lungs was obviously inhibited by the administration of SFJDC, while single SFJDC treatment had no effect on the secretion of iinflammatory cytokines (Fig. 3A-D). Meanwhile, GST and SOD activities were increased in the presence of LPS. The treatment of SFJDC further

Table 2. Anti-inflammatory components of SFJD.

Peak No.	tR (min)	MS (m/z)	MSMS	UV(nm)	Molecular formula	Molecular weight (Da)	Identification	Herbal source
1	6.83	461.1628	315[M-H-Rha]-,135[M-H-Rha-Glu]-	196, 221, 284	$C_{20}H_{30}O_{12}$	462.4510	Forsythoside E	*Forsythia suspensa* (Thunb) Vahl
2	8.81	405.1388	243[M+H-Glu]+, 225[M+H-Glu-H_2O]+, 207[M+H-Glu-2H_2O]+, 193[M+H-Glu-H_2O-CH_4O]+	232, 192	$C_{17}H_{24}O_{11}$	404.3710	Hastatoside	*Verbena officinalis* L.
3	9.40	389.1407	227[M+H-Glu]+, 195[M+H-Glu-CH_4O]+, 177[M+H-Glu-CH4O-H_2O]+	238	$C_{17}H_{24}O_{10}$	388.3716	Verbenalin	*Verbena officinalis* L.
4	13.52	623.2007					Forsythoside A	*Forsythia suspensa* (Thunb) Vahl
5	13.77	623.1971	623[M-H]-, 461[M-H-Rha]-, 161[M-2H-461]-	198, 220, 326	$C_{29}H_{36}O_{15}$	624.5958	Verbascoside	*Verbena officinalis* L.
6	15.58	623.1978					Isoforsythoside A	*Forsythia suspensa* (Thunb) Vahl
7	20.25	535.2187	557[M+Na]+, 355[M+H-Glu]+, 249[M+H-Glu-anisole]- 189[M+H-Glu-anisole-2CH_2O]-	200, 230, 277	$C_{27}H_{34}O_{11}$	534.5604	Phillyrin	*Forsythia suspensa* (Thunb) Vahl
8	21.35	407.1294	407[M-H]-, 245[M-H-2C_5H_7-CO]-	237	$C_{25}H_{28}O_5$	408.4943	3-Hydroxyglabrol	*Glycyrrhiza uralensis* Fisch
9	21.77	431.0843	269[M-H-Glu]-, 225[M-H-Glu-CO_2]-	222, 271, 194	$C_{21}H_{20}O_{10}$	432.3838	Vitexin	*Isatis indigotica* Fortune ex Lindl
10	31.61	269.0423	269[M+H]+, 241[M+H-CO]+, 225[M+H-CO_2]+	287, 266, 224	$C_{15}H_{10}O_5$	270.2414	Emodin	*Polygonum cuspidatum* Sieb et Zucc, *Isatis indigotica* Fortune ex Lindl

promoted the activities of GST and SOD in rat lungs (Fig. 3E,F). These results indicated that SFJDC significantly decreased the LPS-induced inflammation response by promoting the activities of GST and SOD in rat lungs.

SFJDC activated NRF2-associated antioxidant activity in rat lungs

Since SFJDC promoted activities of GST and SOD in rat lungs, this indicated that signaling pathways associated with antioxidant responses might be involved. The Keap1-Nrf2-ARE signaling pathway has been regarded as one of the major regulators of cytoprotective responses against oxidative stress (Yoshizaki et al., 2017). Therefore, Western blot assay was performed to determine the expression of Keap1-Nrf2-ARE associated effectors. As indicated in Fig. 4A, LPS significantly stimulated the expressions of Keap1, Nrf2, HO1 and CUL3 in rat lungs. Given that NRF2 is negatively regulated by CUL3 and KEAP1 (Furukawa and Xiong, 2005), SFDJC alleviated the LPS-induced responses by upregulation of Keap 1 and CUL3 in rat lungs (Fig. 4A-C). Since Nrf2 level is associated with HO-1 and NQO1 expressions (Yoshizaki et al., 2017), SFJDC significantly increased the expression of Nrf2, HO1 and NQO1 compared with the LPS-treated group (Fig. 4A,D-F). All these data demonstrated that SFJDC protected rat lungs against LPS-induced ALI through regulating NRF2-associated antioxidant pathway.

Discussion

SFJDC is a traditional Chinese medicine that has been used for years for the treatment of many respiratory symptoms in China. In our previous studies, we found that SFJDC inhibited the p38MARK/NK-κB pathway, thereby alleviating the inflammatory response (Tao et al., 2014). In this study, we aimed to further explore the role of SFJDC in a rat model of ALI. Our findings indicated that SFJDC alleviated inflammation in the rat lungs by promoting the activation of GST and SOD expression. Through activating the NRF2-associated antioxidant pathway, SFJDC protected rat lungs against LPS-induced ALI by enhancing antioxidant activities. In summary, SFJDC alleviated LPS-induced ALI in rat model via suppression of inflammation and activation of

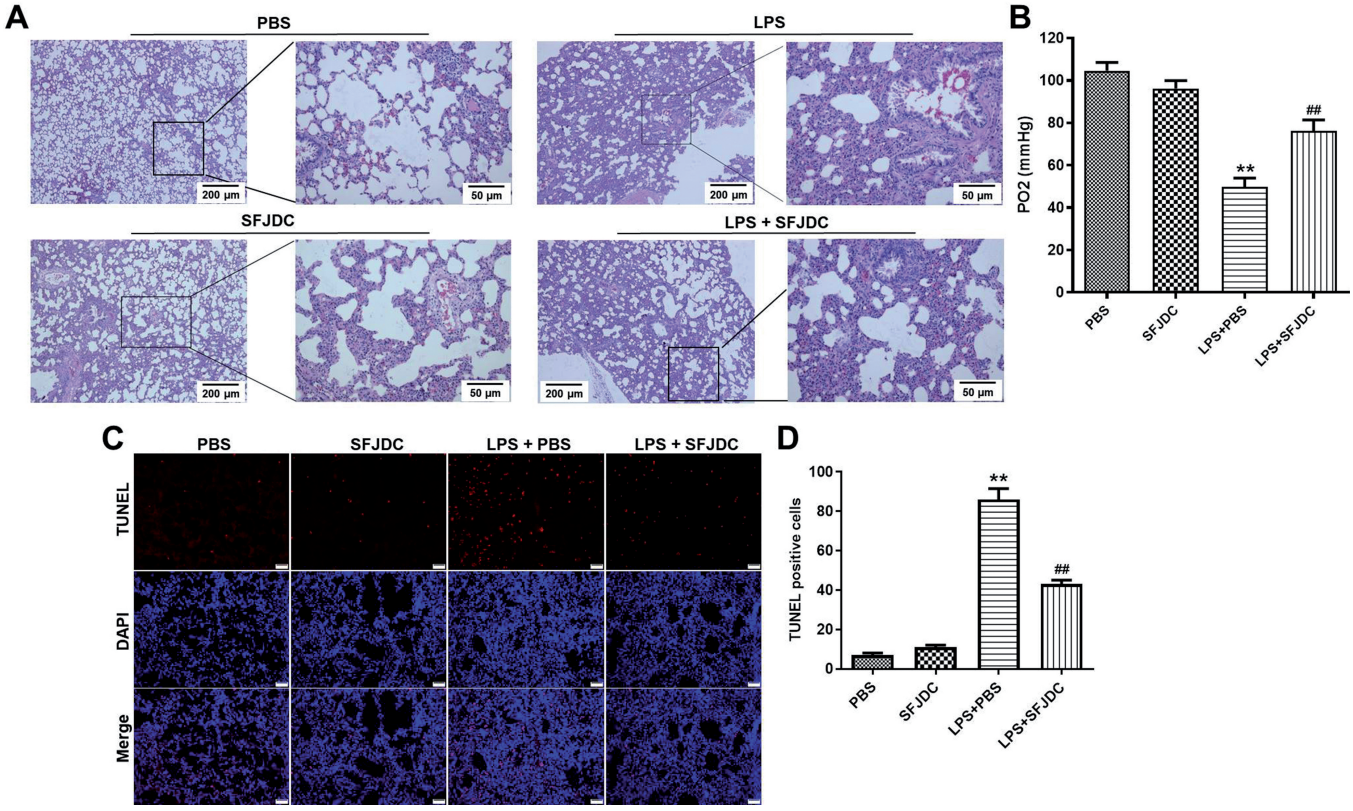

Fig. 2. SFJDC protected rats against LPS-induced acute lung injury. **A.** Lung sections of the rat were stained with HE for histological test after LPS administration for 72 h. Representative photos of lung histology from each group were captured. Normal lung tissues were used as control. **B.** PO2 was detected with arterial blood gas analysis. **P<0.01, compared with PBS group. ##P<0.01, compared with LPS+PBS group. **C.** Lung sections of the rat were stained with TUNEL staining for histological test after LPS administration for 72 h. **D.** TUNEL positive cells were counted. **P<0.01, compared with the PBS group. ##P<0.01, compared with LPS+PBS group. A, x 200.

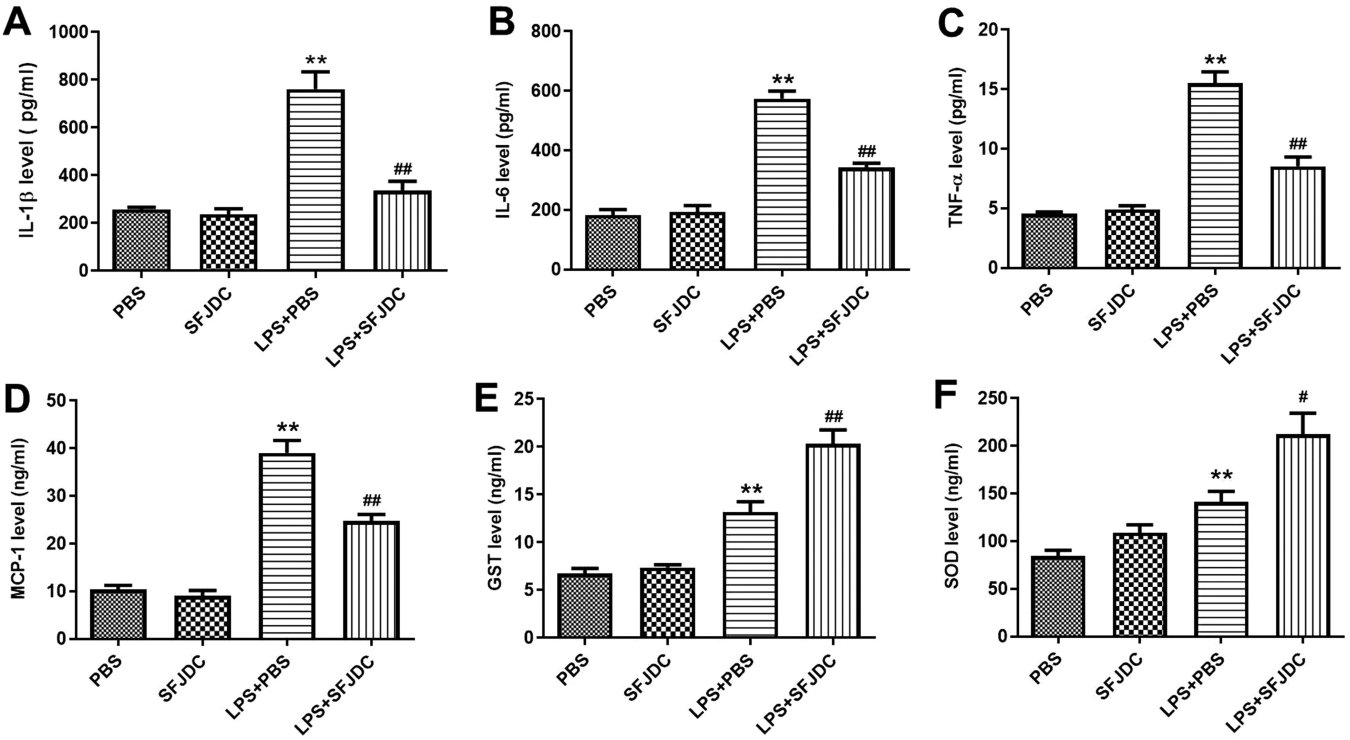

Fig. 3. SFJDC significantly reversed LPS-induced inflammatory response in rat lungs. **A-F.** ELISA assay was conducted to detect the levels of IL-1β, IL-6, TNF-α, MCP-1, GST and SOD in each group. **P<0.01, compared with the PBS group. #P<0.05, ##P<0.01 compared with the LPS+PBS group.

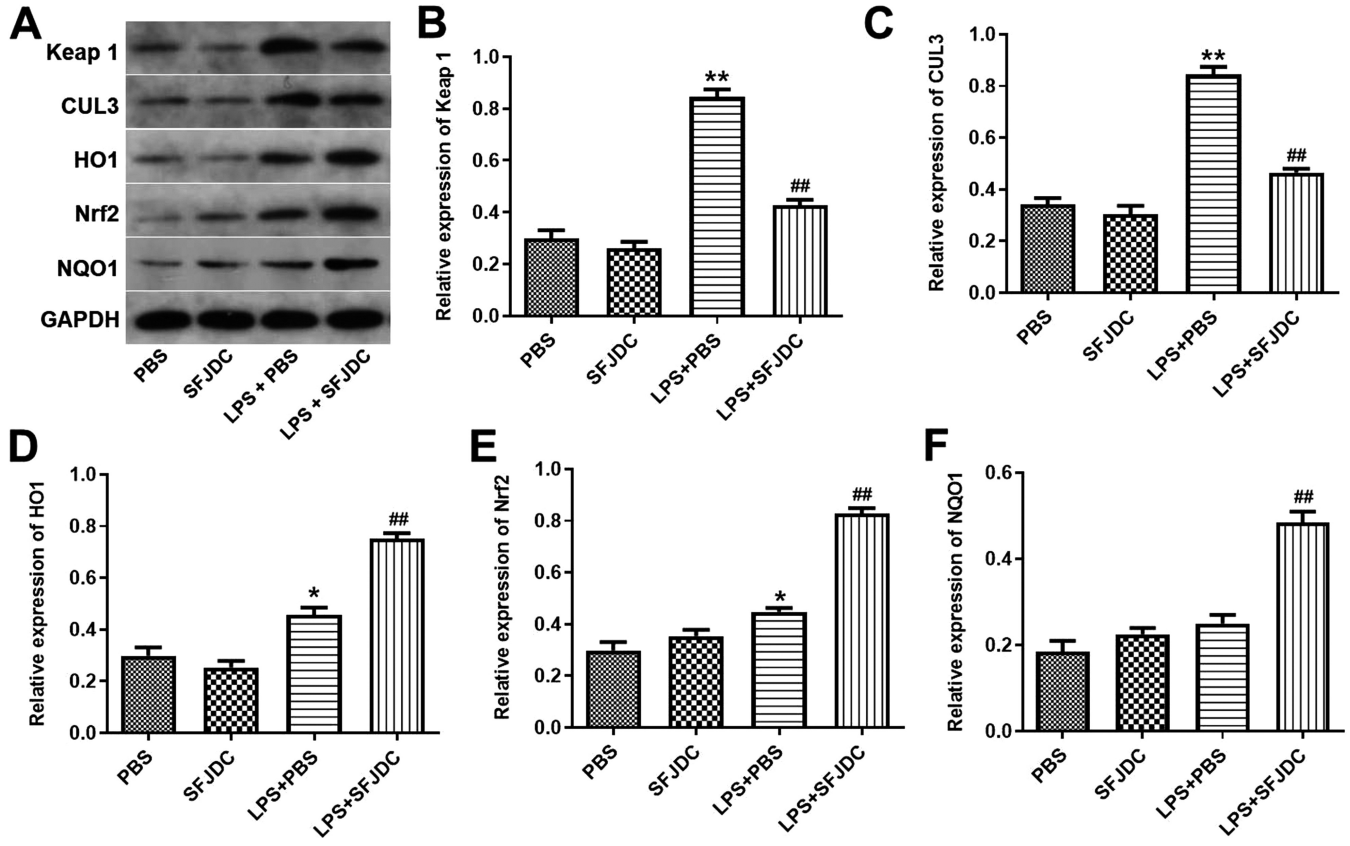

Fig. 4. SFJDC activated Keap1-Nrf2-ARE signaling pathway in the lung of rats. **A.** Western blotting analysis of major effectors involved in Keap1-Nrf2-ARE signaling pathway. **B-E.** The expressions of Keap 1, Nrf2, HO1, CUL3 and NQO1 were quantified, respectively. GAPDH was used as a loading control. *P<0.05, **P<0.01, compared with the PBS group. ##P<0.01, compared with LPS+PBS group.

NRF2-associated antioxidant pathway.

Regarding the anti-inflammatory components of SFJDC, flavonoids were previously proved to be a group of main active components which play key roles in the treatment of lung injury (Xie et al., 2009; Wang et al., 2012; Chen et al., 2013; Chu et al., 2014; Lee et al., 2015). Moreover, Li Cheng et al reported that forsythiaside inhibited cigarette smoke-induced lung inflammation in mice (Cheng et al., 2015b). In line with these previous studies, we proved that SFJDC notably alleviated lung injury via suppression of inflammation. In addition, the identification of anti-inflammatory components of SFJDC inspired more research on each single anti-inflammatory component, such as isoforsythoside, hastatoside, phillyrin and so on. However, a more profound analysis is necessary to explore extensive active components of SFJDC and to determine the most effective compound in SFJDC. Moreover, comprehensive studies in the future will allow us to determine synergistic or antagonist effects between the active compounds.

To investigate the mechanisms by which SFJDC exhibits anti-inflammation activities, the NRF2-associated antioxidant pathway were identified. The present study indicated that SFJDC stimulated the NRF2-associated antioxidant expression of proteins in rat lungs. Interestingly, one of our previous studies demonstrated that SFJDC protected rat lung tissue against ALI by suppressing the MAPK/NF-κB pathway. Moreover, Weiting Zhong proved that phillyrin (one of the main chemical constituents of Forsythia suspensa) attenuated LPS-induced pulmonary inflammation via suppression of MAPK/NF-κB activation in acute lung injury mice (Zhong et al., 2013). Another study of Yanmei Li et al demonstrated that SFJDC exhibited anti-inflammatory effects for upper respiratory infection via regulating the ERK pathway (Li et al., 2017). Considering SFJDC exhibited anti-inflammatory effects via regulation of these different pathways in different situations, more research is required to explore the interaction.

Conclusion

In summary, this study strongly suggests that SFJDC treatment could protect rats against LPS-induced ALI through alleviating the inflammation response and activation of NRF2-associated antioxidant pathway. Therefore, SFJDC might serve as a therapeutic drug against ALI.

Acknowledgements. The authors acknowledge the invaluable help of the staff at the SPF facility at Fudan University.

Funding. This research was supported by the National Center for Protein Science, Shanghai Institutes for Biological Sciences Chinese Academy of Sciences, and the National Natural Science Foundation of China (NO.81171837, and NO.81773967).

Conflict of interests. The authors declare that they have no conflict of interests.

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Accepted December 21, 2020