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Electroacupuncture ameliorates cardiopulmonary bypass induced apoptosis in lung via ROS/Nrf2/NLRP3 inflammasome pathway

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ABSTRACT

Aims: Electroacupuncture (EAc) has a pulmonary protective effect during cardiopulmonary bypass (CPB), but its molecular mechanisms including inflammasome activation signaling pathways remains unclear.
Materials and methods: Male Sprague Dawley rats were divided into control, CPB + EAc and CPB groups. Lung injury model was developed by CPB treatment and EAc (2/100 Hz) was carried out before CPB in the CPB + EAc group. Lung tissues were collected at two time points (0.5 h; 2 h) to determine cytokines release by ELISA kits, and protein expressions by Western blot. Serum collected at two time points (0.5 h; 2 h) from CPB and CPB + EAc treated groups were used in NR8383 cells to confirm the effect of EAc.
Key findings: CPB significantly increased the inflammatory mediators, histological damage and expression of inflammasome related protein and apoptosis, when compared with control group. The level of tumor necrosis factor-α(TNF-α), interleukin (IL)-18 and IL-1β in the CPB + EAc treated group was significantly decreased along with histological changes compared to CPB. Moreover, EAc inhibited the activation of Nod like receptor protein-3 (NLRP3) inflammasome complex, caspase-8 and activated NF-E2-related factor 2 (p-Nrf2). In addition, serum from the CPB + EAc group prevented CPB induced activation of inflammasome and related mediators, reducing ROS generation and apoptosis in NR8383 macrophages.
Significance: These findings indicate that EAc had a critical anti-apoptotic role by suppression of ROS/Nrf2/NLRP3 inflammasome pathway. EAc might be a possible therapeutic treatment for CPB-induced acute lung injury.

1. Introduction

Cardiopulmonary bypass (CPB) is a complex set of non-physiological condition where specific parts of our body are subjected to functional modifications. Following the CPB operation, generation of cytokines, proteins and imbalanced redox level are recorded [1]. It has been reported that acute lung injury or pulmonary disease is a post-operative difficulty of CPB [2,3]. After the CPB operation, inflammatory responses are activated by a myriad of events, including surgical trauma, ischaemic reperfusion and hypothermia [4]. Immune cells such as neutrophils, monocytes and macrophages are activated, after induction of foreign particles in the infected area of lung where they attribute to respiratory dysfunction as observed in perfusion lung syndrome due to high levels of cytokine secretion [5–7]. Moreover, CPB induces reactive oxygen species (ROS) generation and inflammatory response that are involved in the severity of lung injury. ROS act as chemical messengers which serve also to balance of redox system. Once there is hindrance in redox system, cellular macromolecules such as lipids and proteins, finally leads the cell to the apoptotic cell death [1]. Additionally, ROS scavenging or knockdown of NADPH oxidase subunits revealed that in absence of ROS production, NOD-like receptor protein 3 (NLRP3) induced IL-1β generation is impaired, indicates the pivotal roles of ROS in inflammasome activation [8]. Moreover, increased secretion of ROS is considered an important event that regulates several intracellular signalling pathways such as Nrf2 (NF-E2-related factor 2) [9], nuclear factor kappa B (NF-κB) pathways [10] etc.

Inflammasome is a cytosolic protein complex and has versatile function in numbers of inflammatory disorders [11]. NLRP3 consists of

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List of abbreviations

EAc	Electroacupuncture
CPB	Cardiopulmonary Bypass
ROS	reactive oxygenises species
TNF-α	tumor nacrosis factor-alpha
IL	interleukin
Nrf2	NF-E2-related factor 2
NF-κB	nuclear factor kappa B
MAPK	mitogen-activated protein kinese
JNK-1	c-Jun N-terminal kinase 1
NLRP3	Nod like receptor protein-3
HO	hemeoxygenese
DAMP	dangerous associated molecular pattern
PAMP	pathogen associated molecular pattern
LPS	lipopolysaccharide
ATP	adenosine triphosphate
MSU	monosodium urate
COPD	chronic obstructive pulmonary diseases
DCF	2′, 7-dichlorofluorescein
SEM	Scanning electron microscope
H_2O_2	hydrogen peroxide
NAC	N-acetyl-L-cysteine
PI	propidium iodide

three major domains including an apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) interacting domain known as the pyrin domain, a leucine rich domain with modulator function and a nucleotide tri-phosphatase domain, responsible for oligomerization, which is an elementary event to activate the NLRP3 protein [12]. The inflammasome meditates a rapid immune response against pathogen associated molecular pattern (PAMP) and dangerous associated molecular pattern (DAMP), which are involved in tissue damage by stimulation with lipopolysaceride (LPS), adenosine triphosphate (ATP), Nigericin and monosodium urate (MSU) crystals etc [13]. After the activation of inflammasome, caspase-1 proteolytically activates the biologically active form of IL-1β from its inactive state [14,15]. Activation of inflammasome also turns on the programmed cell death, such as apoptosis and pyroptosis. Apoptosis as programmed cell death process, activated by cascades of caspases such as caspase-8 [16], while pyroptosis is activated by gasdermin D (GSDMD) [17]. Nrf2 is a basic leucine zipper type transcription factors which bind with the antioxidant response element (ARE) to synthesize numbers of cytoprotective gene. A recent observation showed that inhibition of p-Nrf2 leads to the prevention of inflammation in a time dependent manner [9]. In addition, inflammasome such as NLRP3 complex is activated by Nrf2 signalling pathway [18], though involvement of this signalling pathway in CPB-induce lung injury is yet to uncover.

Acupuncture is well known traditional Chinese treatment process for the inflammatory diseases and has attracted increasing attention from the ancient era due to its least side effects [19]. In Europe and the USA, this integral part of traditional Chinese medicine has become a visible element of treatment strategy and has steadily claimed its usefulness in complementary medicine [20]. It is reported that in 2007 around 14 million Americans had received acupuncture treatment while in 2002 it was 8 million [21]. Interestingly, 3.1% of respondents had received acupuncture treatment for respiratory diseases alone in the USA, but reports on patients with respiratory disease who have received acupuncture therapy are hard to find [22]. In the previous decades, clinical and experimental studies have claimed that it has therapeutic effect to relieve chronic obstructive pulmonary diseases (COPD) [23]. Huang et al., 2019 have found that EAc attenuates NLRP3 inflammasome activation in CPB induced lung injury [24], but the upstream and downstream mechanisms are unclear. Our previous study had demonstrated that EAc could reduce apoptosis via mitogen-activated protein kinase (MAPK) pathway in CPB-induced acute lung injury [4]. But there is uncertainty regarding the involvement of inflammasome in CPB induced apoptosis.

Therefore, in this study, we investigated the effect of EAc on inflammasome and apoptosis in CPB-induced lung injury model both in *in vivo* and *in vitro*. And our results suggested that EAc could suppress the activation of inflammasome, and inhibition of caspase 8-dependent apoptosis through intervening ROS/Nrf2 pathway.

2. Materials and methods

2.1. Animals and group

Healthy Male Sprague Dawley rats (400–450 g) were bought from the Sino-British SIPPR/BK Lab (Shanghai, China). All studies were conducted in accordance with the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine. To establish the CPB model we followed our previous study [4]. Briefly, rats were randomly divided into three groups, with six rats per group: Control group (sham), CPB group: rats were anesthetized with 5% pentobarbital sodium (100 mg/kg,ip) for 1 h and then subjected to CPB for 1 h; CPB + EAc group: Before CPB treatments rats were anesthetized with 5% pentobarbital sodium (100 mg/kg) for 1 h and followed by EAc at two acupuncture points [pericardium 6 (PC6) and large intestine 4 (LI4)] for 30 min before CPB, and then were subjected to CPB. Thereafter, the CPB treatment at different time points 0.5 h and 2 h accordingly, followed by euthanasia, then lung tissues were collected for histopathological study, enzyme-linked immunosorbent assay (ELISA), and Western blot etc. Moreover, rat serum was harvested to treat the rat macrophage to investigate the anti-inflammatory effects of EAc.

2.2. Cell culture and treatment

NR8383 macrophages (ATCC, Rockville, MD) is rat alveolar macrophages, was supplemented with 15% fetal bovine serum (FBS) (Sigma, USA) and 1% penicillin/streptomycin (Sigma, USA). Cells with 4×10^6 cells/ml density were seeded on 6 well plates for Western blot and ELISA experiments, whereas cells with 1×10^5 cells/ml density were grown on 96 well plates for ROS experiment and 24 well plates for scanning electron microscope (SEM), respectively. After 24 h incubation, the cells were stimulated with the 10% serum of CPB + EAc (0.5 h and 2 h) and CPB (0.5 h and CPB 2 h) for 2 h. Later on, cells were centrifuged to collect supernatant and lysate separately for ELISA and Western blot analysis. In addition, cells were fixed onto slides by glutaraldehyde, 1% Osmium tetroxide (OsO_4) for scanning electron microscope.

2.3. Histopathological analysis of lung

For histopathological analysis, part of the left lower lobe was fixed in 10% neutral formalin for 3 days and dehydrated in a series of graded ethanol, embedded in paraffin wax, and cut into 4 μm thick sections. The tissue sections were stained by hematoxylin and eosin (H & E) dyeing solution, and evaluated with an Olympus CH 30 microscope (Olympus Corporation, Tokyo, Japan). The histopathological conditions of lung injury was scored following the Official American Thoracic Society Workshop Report [25] (a) neutrophils in the alveolar space, (b) neutrophils in the interstitial space, (c) hyaline membranes, (d) proteinaceous debris filling the airspaces, and (e) alveolar septal thickening. To find out the total score was calculated using this bellow

formula:
Score = [(20 × a) + (14 × b) + (7 × c) + (7 × d) + (2 × e)]/(number of fields × 100).

2.4. ELISA assay to detect the cytokines level

The levels of cytokines in lung tissues and supernatant of NR8383 cells were detected following the instructions of ELISA kits [TNF-α (DY410), IL-1β (DY401), R&D system, Minneapolis city, USA; IL-18 (50073-R167), Sino biological, Beijing, China].

2.5. Western blot assay

Protein (Lung tissues and NR8383 cells) was extracted by cold RIPA (Radio Immunoprecipitation Assay) lysis buffer (including 1% protease inhibitor cocktail (Roche), 2% PMSF (Sigma) and 1x PhosSTOP (Roche) and centrifuged at 13000 × *g* at 4 °C for 15 min. Protein concentration was evaluated by the Bio-rad reagent (Bio-Rad Inc., California, USA). 5 × loading buffer (Beyotime Inc., Nantong, China) was added to protein sample and then denatured at 100 °C for 5 min, stored at −80 °C. 50 μg of protein sample was separated by 10% and 15% SDS-PAGE at 70 V for 45 min and 130 V for 1 h respectively, transferred to nitrocellulose membranes (NC) membranes (Schleicher and Schuell; 300 mA for 90 min) by a Mini-protein II system (Bio-Rad). After transfer, the membrane was blocked with 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.05% Tween-20 (T-TBS) (v/v) containing 5% bovine serum albumin (BSA) for 1 h at room temperature and incubated with the primary antibodies: anti p-Nrf2 (1:500) (db523, Digbio, Hangzhou, China); anti-NLRP3 (1:1000) (AG-20B-0014, Adipogen Life Sciences, San diego, USA); anti-procaspase-1/cleaved cas-1 (1:1000) (ab179515, Abcam, Massachusetts, USA); anti-caspase-8 (1:1000) (ab227430, Abcam, Massachusetts, USA); Anti-Asc (1:1000) (sc-514559, Santa cruz biotechnology, Dallas, TX, USA) and anti-GAPDH (1:5000) (db106, Digbio, Hanzhou, China). After washing with Tris Buffer Saline and Tween-20 (TBST) for three times, secondary antibodies (IRDye 800CW goat anti-rabbit; IRDye 680CW goat anti-mouse (LI-COR Biosciences, Cambridge, UK) were added at a final dilution of 1:5000 and incubated for 1.5 h. Then the membrane was washed with 1 × TBST for three times and odyssey CLx infrared laser dual colors imagine analysis system (LI-COR, Inc., Lincoln, Nebraska, USA) was used to visualize the protein bands and relative expression of protein was calculated as the ratio of protein target and GAPDH.

2.6. Measurement of reactive oxygen species production

NR8383 cells (1 × 10^5 cells/ml) were seeded in 96 well plates. After the 24 h incubation, cells were treated with the 10% serum of CPB + EAc (0.5 h or 2 h) and CPB (0.5 h or 2 h) for 2 h, respectively. ROS scavenger N-acetyl-L-cysteine (NAC) (3 mM, HY-B0215, MedChemExpress, Shanghai, China) was used as a negative control, and

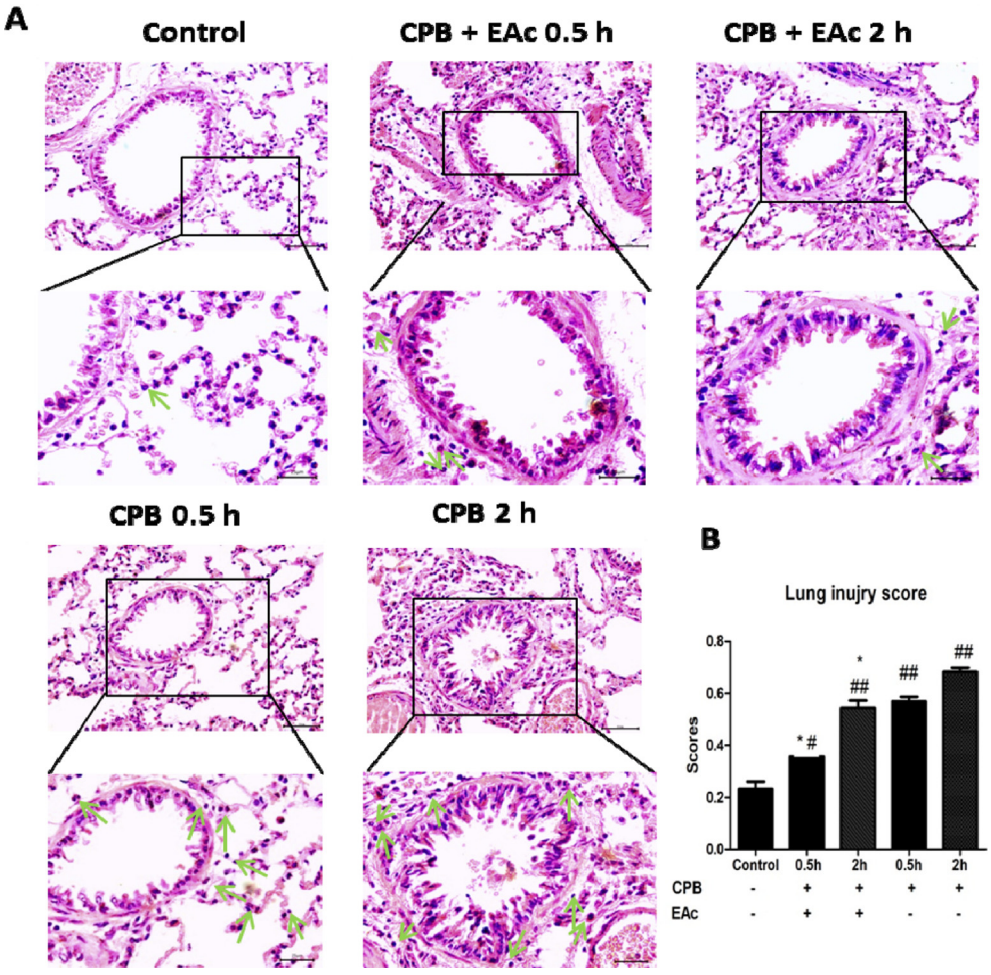

Fig. 1. Representative images (A) and histopathological score (B) of lung tissue. Lung tissues were stained with hematoxyline and eosin (original magnification, × 40). Neutrophils were marked with the green arrow in control, CPB + EAc and CPB treated lung injury in rats at different time points (Mean ± SEM, n = 6). $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ versus control group; $^{*}p < 0.05$, and $^{**}p < 0.01$ versus CPB same time point. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

hydrogen peroxide H_2O_2 (100 μM, E004-1-1, Reactive oxygen species Assay Kit, Nanjing Jiancheng Biotechnology, Nanjing, China) was used as positive control. Intracellular ROS production was determined by using 2′, 7′-dichlorofluorescein diacetate (DCFH-DA, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) which oxidized to the 2′, 7′-dichlorofluorescein (DCF) in the presence of peroxides. The cells were incubated at 37 °C with 10 μM DCFH-DA for 45 min in a 5% CO_2 incubator and washed twice with Phosphate Buffer Saline. The fluorescence intensity of 2′, 7′-DCF was detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm by Varioskan Flash microplate reader (Thermo Scientific, Vantaa, Finland).

2.7. Measurement of apoptosis by scanning electron microscope

Cells slides were fixed in glutaraldehyde, 1% OsO_4. Afterward, the samples were dehydrated by graded ethanol series (50%, 70%, 95% and 100%) and dried by automatic critical point dryer (Leica EM CPD 300, Leica Mikrosysteme GambH, Hernalser Hauprstrasse, Austria) using CO_2 as the transitional fluid. Specimens were sputter-coated with a thin layer of platinum by a sputtering device (Leica EM ACE600 High Vacuum Sputter Coater) and observed by scanning electron microscope (Novo nano SEM 450, Thermo Fisher Scientific, Waltham, USA).

2.8. Measurement of apoptosis by flow cytometry analysis

Cells were seeded in 6-well plates and treated with 10% serum of CPB + EAc (0.5 h and 2 h) and CPB (0.5 h and 2 h) for 10 h. Cell apoptosis was measured by an Annexin V-FITC and propidium iodide (PI) Apoptosis Detection Kit (8072965, BD Biosciences, San Jose, California, USA). Cells were treated as descripted above and washed twice with 1x cold PBS. Then, the cells were stained with Annexin V-FITC and PI, and incubated in the dark place for 20 min. The apoptosis was evaluated by flow cytometry (Beckman Coulter Dxflex, Suzhou, China). Experiments were performed in duplicate.

2.9. Statistical analysis

We performed duplicated experiment independently. All data are expressed as the mean ± SEM (n = 2–6). Data sets are analysed by using one-way ANOVA and Student-Newman-Keuls multiple-comparison test in Graph prism 5 software (Graphpad Software, SanDiego, CA, USA). A value of $p < 0.05$ is considered as the criterion for statistical significance.

3. Results

3.1. EAc attenuats pathological changes in CPB-induced lung injury

The effect of EAc on CPB-treated acute lung injury was revealed by H&E staining to detect the histological alteration of lung. Lung tissues in the CPB group at 0.5 h and 2 h showed the prominent number of neutrophils around the alveolar space ($p < 0.05$ vs control), whereas CPB + EAc group exhibited lower cellular infiltration which shown significant improvement ($p < 0.05$ vs CPB)(Fig. 1). These findings indicated that neutrophils recruitment is responsible to enhance lung injury, which was attenuated by EAc therapy.

3.2. EAc suppresses CPB-induced TNF-α, IL-18, and IL-1β production in vivo and in vitro

To explore the effect of EAc on inflammasome related response, we determined the level of TNF-α, IL-18 and IL-1β in CPB-treated lung tissues and NR8383 cells. In lung tissues, the contents of TNF-α, IL-18 and IL-1β were higher in CPB group as compared to control group ($p < 0.05$). In contrast, EAc treatment reduced the levels of TNF-α, IL-18 and IL-1β at 2 h by 1.74, 1.74 and 3.15 folds, respectively ($p < 0.05$

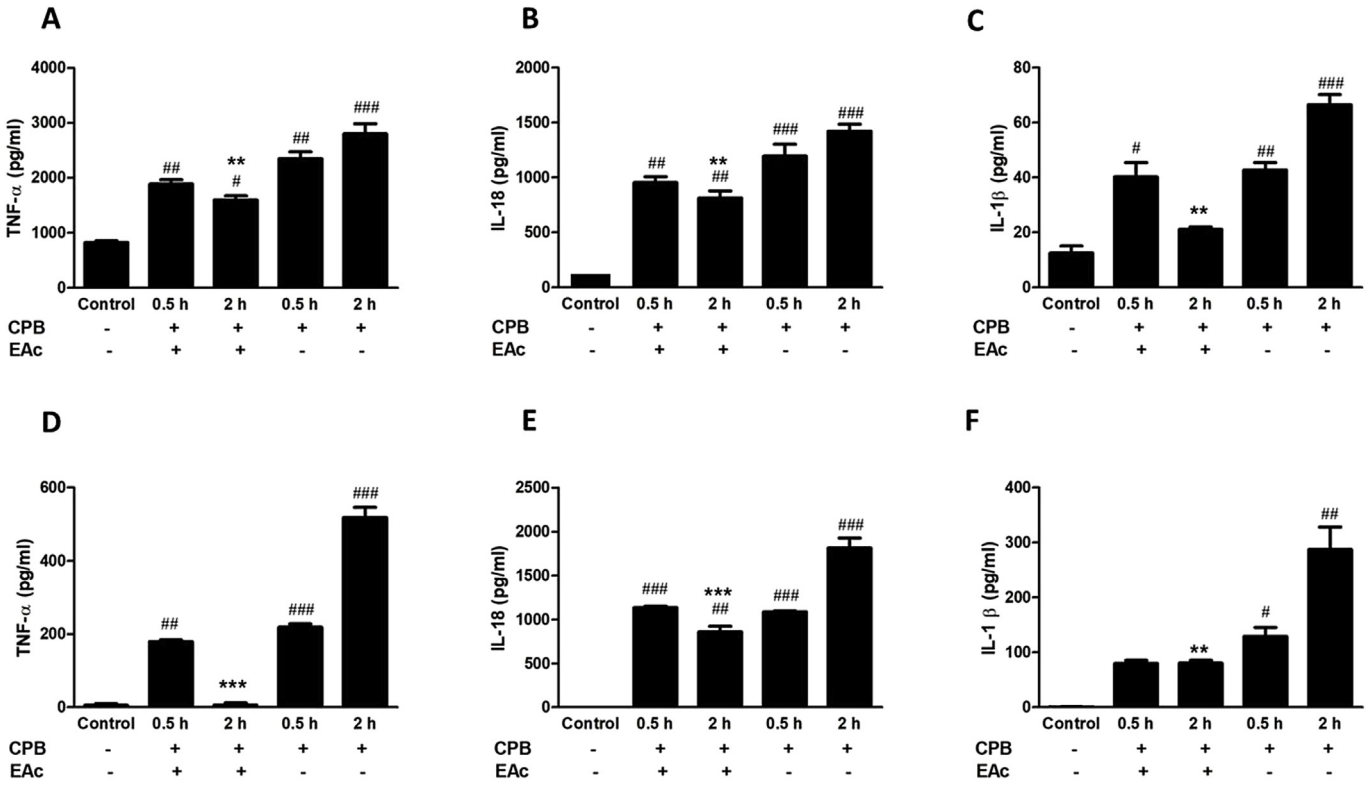

Fig. 2. The effect of on the level of inflammatory cytokines TNF-α, IL-18 and IL-1β in CPB injured lung tissues in rats (A–C) and supernatant from NR8383 cells treated by CPB + EAc or CPB serum (D–F) were determined by ELISA kits. The results are expressed as the mean ± SEM (n = 2). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ versus control group; $^{**}p < 0.01$, and $^{***}p < 0.001$versus CPB same time point.

vs CPB) (Fig. 2A–C). Additionally, EAc reduced the production of pro-inflammatory cytokines in *in vitro* model (NR8383 cells). As shown in Fig. 2 D-F, Serum from CPB group triggered the secretion of TNF-α, IL-18 and IL-1β into the culture medium ($p < 0.05$ vs Control), while CPB + EAc serum significantly repressed the cytokines TNF-α, IL-18 and IL-1β release by 8.23, 2.10 and 3.57 folds, respectively ($p < 0.05$ vs CPB). These results illustrated that EAc significantly alleviates the production of inflammatory cytokines then attenuates CPB-treated lung injury.

3.3. EAc inhibits the activation of NLRP3 inflammasome via Nrf2 pathway in vivo and in vitro

To evaluate the mechanism behind the anti-inflammatory effect on EAc, we measured the expression NLRP3 protein complex, p-Nrf2 in lung tissue. CPB treatment increased the expression of NLRP3, caspase-1 and p-Nrf2 proteins at 0.5 h and 2 h ($p < 0.05$ vs Control). In comparison to the CPB group at 2 h, NLRP3, Caspase-1 and p-Nrf2 in CPB + EAc group at the same time point decreased 4.34, 3.22 and 3.12 folds, respectively ($p < 0.05$) (Fig. 3A). To confirm this effect of EAc, we determined NLRP3 protein complex and p-Nrf2 in NR8383 cells. Consistent with the *in vivo* results, serum from the CPB group enhanced the expression of NLRP3, cleaved caspase-1 and p-Nrf2 proteins ($p < 0.05$ vs control), but the expressions of ASC and procaspase-1 were unaffected (Fig. 3B), whereas CPB + EAc serum at 2 h markedly decreased the expression of NLRP3, Caspase-1 and p-Nrf2 by 4.96, 7.55, and 3.20 folds, respectively ($p < 0.05$ vs CPB). These results indicated that EAc can suppress the activation of NLRP3, Caspase-1 and p-Nrf2 proteins as well as ameliorate the lung injury.

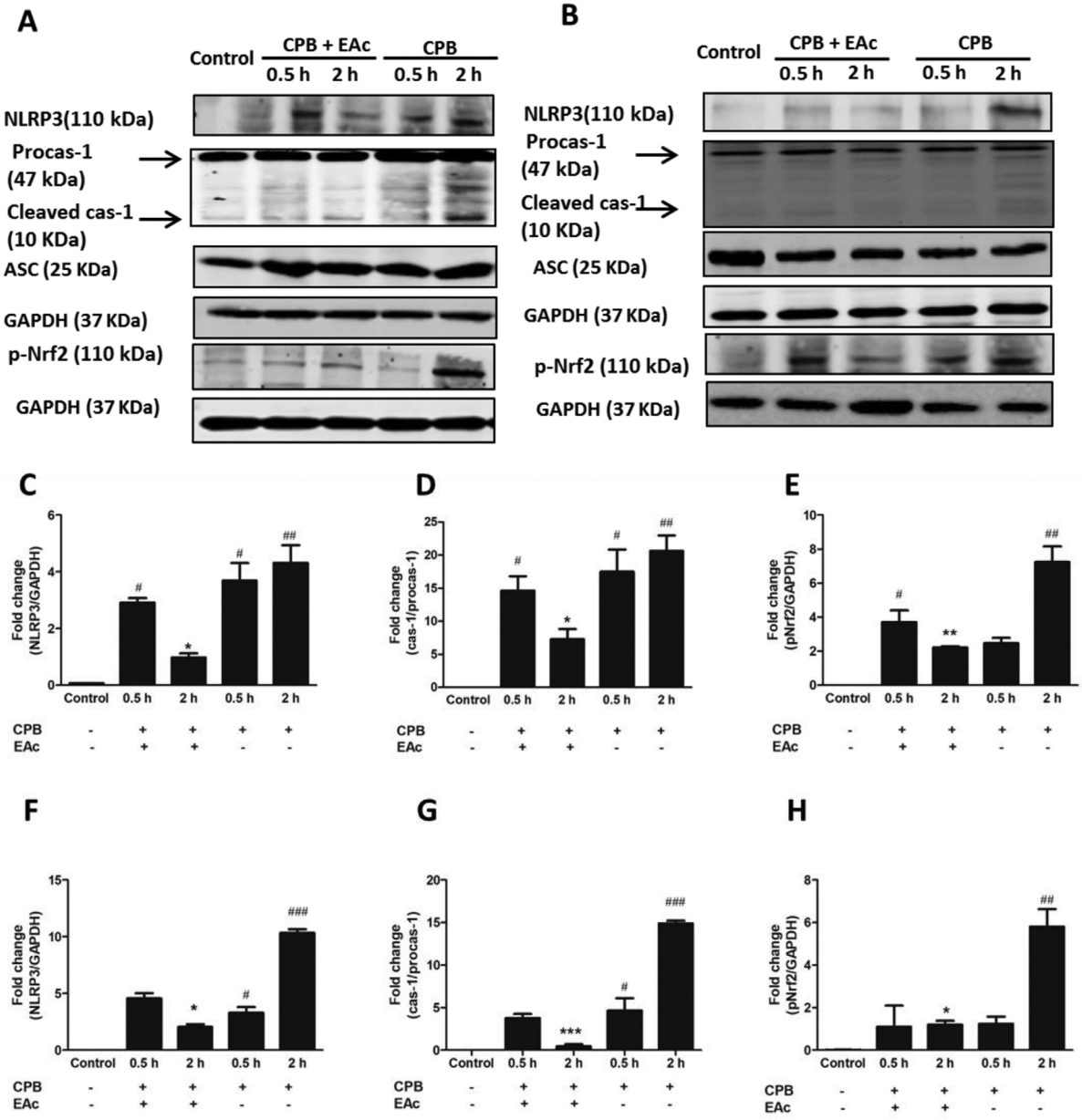

Fig. 3. Effect of EAc on NLRP3, procaspase-1, cleaved cas-1 and p-Nrf2 in CPB-injured lung tissues (A, C-E) and CPB and CPB + EAc serum-treated NR8383 cells (B, F-G). (A, B) Representative pictures of Western blots. NLRP3(C, F), Procaspase-1 and cleaved caspase-1 (D, G), and p-Nrf2 (E, H) were measured by the specific antibodies. Expression of GAPDH was shown as a loading control. Relative expression levels of the proteins were determined by densitometric analysis. The results are expressed as the mean ± SEM (n = 2). $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ versus control group; $^{*}p < 0.05$, versus CPB same time point.

3.4. EAc attenuates apoptosis in vitro

To investigate the effect of EAc on programmed cell death, treatment with CPB + EAc (0.5 h and 2 h) and CPB (0.5 h and 2 h) serum for 2 h, we observed remarkable morphological changes of NR8383 cells by Scanning Electron Microscope. The results revealed that CPB treatment (0.5 h and 2 h) produced multiple bubbles as an indication of apoptosis on the surface of cells, whereas CPB + EAc treatment (0.5 h and 2 h) shown reduced number of bubbles in Fig. 4A. Therefore, these results suggest that CPB induced programmed cell death was indeed apoptosis, pyroptosis had not been observed. This is in agreement with our previous study [4]. To further investigate the role of CPB in apoptosis, we used 10% blood serum of CPB + EAc (0.5 h and 2 h) and CPB (0.5 h and 2 h) groups to treat NR8383 cells, and then detected the early and late apoptosis by flow cytometry. Phosphatidylserine (PS) externalization is one of the biomarkers of apoptosis. Based on PS externalization, the cells are distinguished into four groups, namely viable (annexin V– PI^-), early apoptosis (annexin V^+ PI^-), late apoptosis (annexin V^+ PI^+) and necrotic (annexin V– PI^+) cells. The results suggested that CPB 2 h serum alone induced robust increase of late apoptosis to 9.52%. Interestingly, EAc sharply reduced apoptotic population to 0.11% at 10 h, as shown in Fig. 4B.

3.5. EAc suppresses caspase 8 mediated apoptosis in vivo and in vitro

To find out the linker between inflammasome and apoptosis, we measured the caspase-8 proteins. Our results suggested that apoptosis was triggered after the CPB at 0.5 h and 2 h, which was proved by the increased cleaved caspase-8 protein ($p < 0.05$ vs Control). Treatment with the EAc suppressed the cleaved caspase-8 in lung tissues by 2.79 folds at 2 h (Fig. 5A). Thus, EAc prevented apoptosis in lung tissue though inhibiting cleaved caspase-8. Additionally we analysed same process in NR8383 cells. The result suggested that the serum from CPB group (0.5 h and 2 h) increased the level of caspase-8 in NR8383 cells while treatment with the serum from CPB + EAc group markedly inhibited expression of caspase-8 at 2 h time point ($p < 0.05$ vs CPB) (Fig. 5B). These findings reflected that the inhibition of apoptosis by EAc is caspase-8 dependednt.

3.6. EAc inhibits ROS generation in vitro

The oxidative stress-related indices such as malondialdehyde (MDA) and superoxide dismutase (SOD) had already been investigated in our previous work [4], CPB treatment notably increased the MDA concentration along with reduced SOD level, and both were attenuated by EAc treatment. ROS plays pivotal role to mediate the activation of Nrf2, and NLRP3 [26]. From the context of ROS, we set to investigate the ROS production to affirm the activity of EAc by 2′,7′-dichlorofluorescin diacetate (DCF-DA) staining. Here, H_2O_2 was used as ROS representative which is generated from superoxide by SOD and NAC as negative control scavenges ROS. As shown in Fig. 6, the intracellular accumulation of ROS increased significantly in NR8383 cells treated with serum from CPB group at 2 h ($p < 0.05$ vs negative control). While treatment with the serum from CPB + EAc group at 2 h decreased ROS level by 5.35 folds ($p < 0.05$ vs CPB). However, there was no significance difference between CPB + EAc group and CPB group at 0.5 h, concomitant with the p-Nrf2 expression *in vivo* and *in vitro*. These findings suggested that ROS may mediate the up-regulation of p-Nrf2.

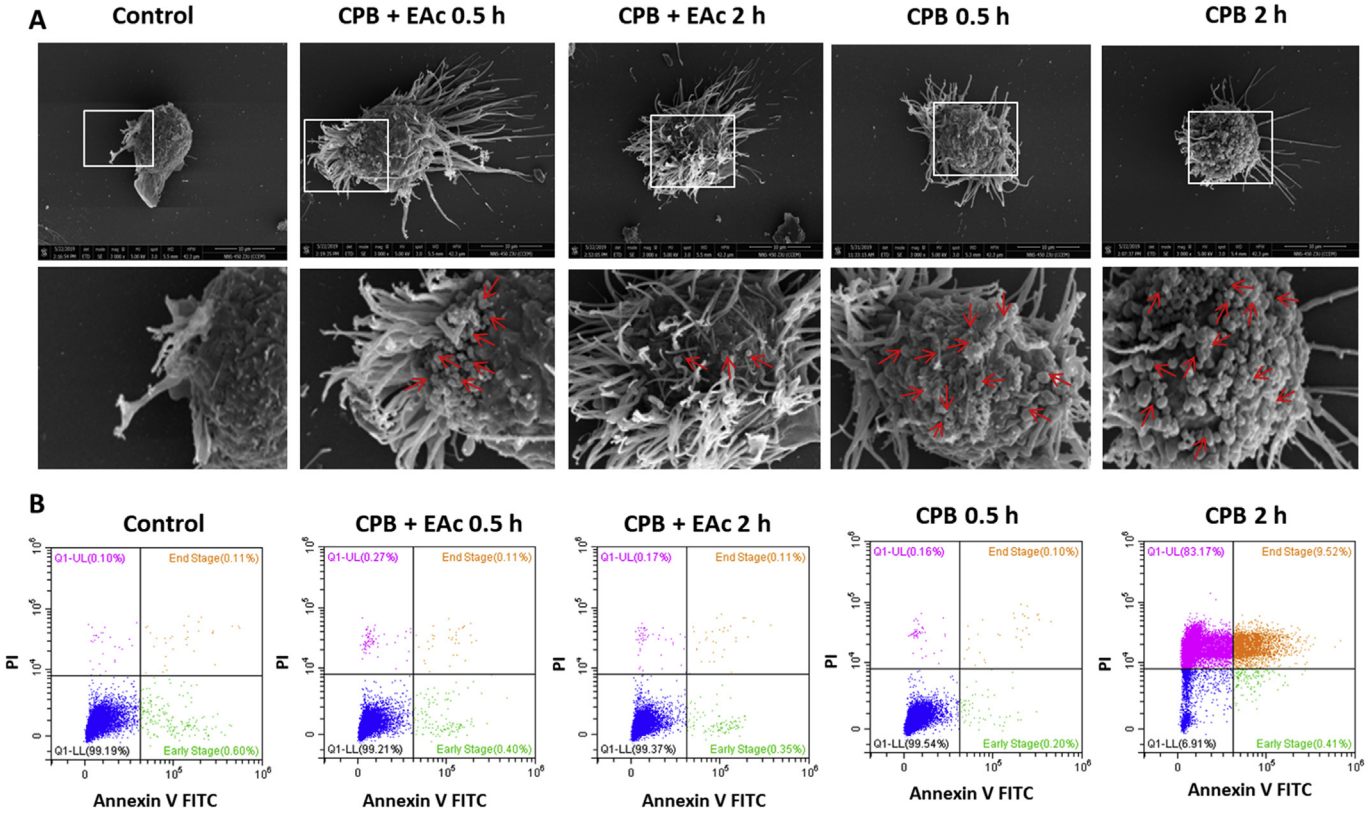

Fig. 4. Effects of EAc on apoptosis in NR8383 cell. (A) Apoptotic morphological changes detected by scanning electron microscope. Red arrows indicated the examples of membrane bubbles on the surface of cells. The numbers of bubbles in CPB groups were higher than that in the CPB + EAc groups, while control group had no bubbles. (B) Early apoptosis and late apoptosis determined by flow cytometry following AnnexinV-FITC and PI staining. Cells in the right lower quadrant are undergoing early apoptosis; Cells in the right upper quadrant are undergoing late apoptosis. NR 8383 cells were treated with CPB and CPB + EAc serum for 10 h. The numbers of early apoptosis and late apoptosis cells in CPB 2 h groups were higher than that in the CPB + EAc groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

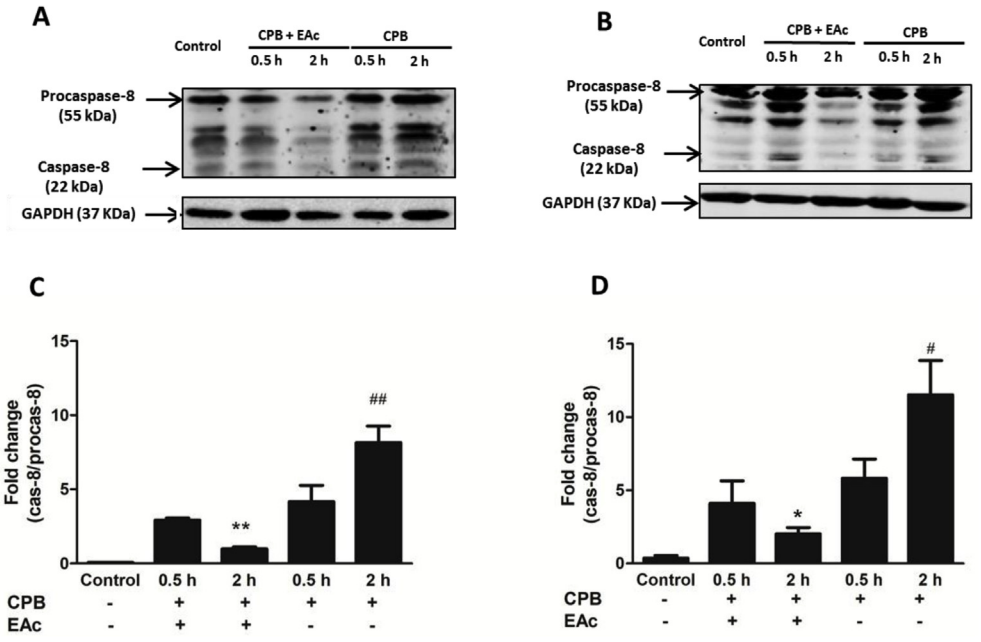

Fig. 5. Induction of the activation of caspase-8 in NR8383 cells by CPB, CPB + EAC serum and CPB treated lung tissues. Expression of caspase-8 in lung tissues (A) and NR8383 cells (B) were determined by Western blot. GAPDH was used as a normal control. Relative protein levels were quantified by densitometry. Each value indicates the mean ± SEM of two independent experiments. $^{**}p < 0.01$, versus CPB-stimulated group, n = 2. $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ versus control group; $^{*}p < 0.05$, and $^{**}p < 0.01$ versus CPB at same time point.

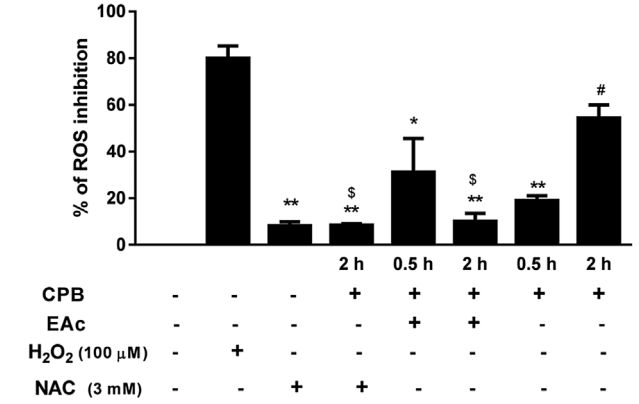

Fig. 6. EAc Suppressed the ROS generation in NR8383 cells. The cells were divided into 8 groups, treated with the CPB or CPB + EAc serum at 0.5 h or 2 h, and NAC or H_2O_2 for 2 h, then and added 100 μL DCFH-DA, incubated for 45 min at 37 °C. The fluorescence intensity was measured on a Varioskan Flash microplate reader by excitation and emission wavelengths of 485 and 530 nm, respectively. The results are expressed as the mean ± SEM (n = 2). $^{\#}p < 0.05$ versus NAC negative control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus H_2O_2 positive control group and $^{\$}p < 0.05$ versus CPB group at 2 h.

4. Discussion

In our study, CPB treatment induced lung injury both in *in vivo* and *in vitro*. We demonstrated that EAc could reduce pulmonary neutrophils infiltrations and inflammatory cytokines (TNF-α, IL-18 and IL-1β) level in CPB-treated rats model. The possible underlying protective mechanisms of EAc are inhibition of the expression of NLRP3, caspase-1, modulation the ROS-mediated Nrf2 phosphorylation and caspase-8 mediated apoptosis process in both *in vitro* and *in vivo* model. The result indicated that EAc is a potential therapy to ameliorate the lung inflammation and reduces the cell apoptosis process against CPB.

Previous studies have demonstrated that pre-treatment of EAc prevents heart injury in rats model by regulating β-adrenergic receptors [27] or modulating β1-AR-Gs-protein–cAMP pathway [28]. In addition, EAc possess plethora of pharmacological benefits, since the ancient period, EAc therapy has been implied to reduce the various kinds of diseases [29,30]. But the protective mechanism of EAc is yet to fully underline. Thus, it would be the beneficial to find out the possible mechanism of the EAc therapy, especially in prevention of lung injury. It is well noted that foreign particles coming from CPB treatment contributes to inflammation in the lung via activating inflammatory cascades [31]. After the CPB treatment, the architecture of lung tissues had changed with alveolar septal thickening, interstitial edema and neutrophil infiltration [32], whereas our previous work identified that EAc prevented CPB induced the morphological changes of lung injury and suppressed the neutrophil infiltration [4]. Growing evidences suggested that stimulators can bind with Toll-like receptor-4 (TLR-4) and activates different signalling pathways such as Nrf2 and NF-κB that results in the activation of NLRP3 protein complex [33,34]. Hence, NLRP3 inflammasome complex is associated with various inflammatory disorders [35], as well as a key role player in the pathogenesis of environmental toxicants [36]. Notably, CPB also induces the activation of inflammasome in rats, and EAc can inhibit this process [37]. While our previous study had identified CPB induce inflammation involved in MAPK and apoptosis, and EAc can rescue these process [4]. In this study, we confirmed that EAc inhibits NLRP3, cleaved caspase-1 expression both in *in vitro* and *in vivo*. Besides, crucial inflammatory cytokines such as IL-18, IL-1β and TNF-α were markedly repressed by EAc. These findings confirmed that the EAc possess significant anti-inflammatory activities in agreement with our previous study [4].

Evidence supports that CPB activates the apoptosis via ROS overproduction and possible activation of complement system [1]. Accumulation of ROS can also stimulates other pro-inflammatory mediators such as IL-6, TNF-α, IL-1β etc. following the CPB treatment [38]. ROS up-regulates activation of MAPKs and the expression of PKC family which results in phosphorylation of Nrf2 whereas PKC phosphorylates Nrf2 at Ser 40 [9]. In our previous work, CPB treatment led to the apparent increase in the oxidative stress-related indexes, such as MDA content and SOD activities [4], which were reversed by EAc treatment. The further ROS determination provides the direct evidence of oxidative stress. Concomitant with our previous findings, current study reveals that CPB increased the ROS production as well as enhanced the expression of p-Nrf2, while CPB + EAc attenuated the abundance of ROS and p-Nrf2. These findings supported that the ROS/Nrf2 pathway mediated by CPB [4].

NLRP3 inflammasome activates different inflammation mediated programed cell death. Activated caspase-1 also cleaves gasdermin D, which leads to a particular form of cell death called pyroptosis More over, NLRP3 inflammasome activates downstream MLKL pore, which

initiates another form of cell death called necroptosis [39]. Indeed, CPB induces activation of NLRP3 inflammasome, but the form of cell death is obscure. Therefore, we used SEM to confirm morphological changes of NR8383 cells. We found that CPB induced apoptosis, no pyroptosis or necroptosis. This results was consistent with our previous findings [4].

Caspase-8 is an apoptotic protein which is required to activate inflammasome-dependent apoptosis process [40]. Apoptosis follows two major pathways, the death receptor and the mitochondrial pathway. In the death receptor pathway, the death-inducing signalling complex consists of a Fas-associated death domain and a initiator caspases such as caspase-8 that shape death receptor. Besides, activated caspase-8 can directly activated caspase-3, and in our previous study we have shown that EAc can inhibit the expression of caspase-3 [4]. The pro-apoptotic proteins Bax and Bak are required for induction of apoptosis by the mitochondrial pathway [41]. According to this study, we found that EAc inhibited the expression of caspase-8 and thereby hampered the activation of apoptosis, so EAc maybe caspase-8 dependently inhibit the death receptor pathway. About the effect of EAc on mitochondrial pathway may be essential for further study. In addition, CPB + EAc only inhibit late stage of apoptosis at 2 h, as confirmed by Annexin V/PI apoptosis detection kit. However, involvements of other caspases to initiate apoptosis are not excluded in this study, maybe need further study.

In summary, ROS mediates apoptosis process as well as activation of inflammasome and the pathogenesis of CPB-treated acute lung injury via the Nrf2 axis. The EAc therapy effectively attenuates all the aberrant conditions related to inflammasome activation and apoptosis through inhibiting ROS generation. Therefore, EAc may represent a feasible therapeutic approach for managing CPB-induced lung injury but extensive studies are needed to imply clinically.

Declaration of competing interest

No potential conflict of interest was reported by the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116962.

Author's contributions

RD, LJZ, YJL performed the research, RD and MNR wrote the manuscript and prepared the figures. ZQH and ZGL analysed the data. HSC and HFT designed and supervised the manuscript.

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