Free Radical Research, July 2007; 41(7): 798–805

informa
healthcare

Protection of echinacoside against acute lung injury caused by oleic acid in rats

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Accepted by Professor F. Kelly

(Received 6 November 2006; in revised form 24 March 2007)

Introduction

Acute lung injury/acute respiratory distress syndrome (ALI/ARDS) manifests as non-cardiogenic pulmonary edema, respiratory distress and refractory hypoxemia, which is caused by increased permeability of damaged pulmonary capillary endothelia. ARDS is the ultimate stage clinical syndrome of ALI [1] and the leading cause of death in intensive care units with a very high mortality rate of 40% or more [2]. The common predisposing causes of ALI/ARDS are trauma, aspiration and sepsis, which can trigger a series of pathologic events including aggregation and activation of neutrophils, release of proteases and lipid mediators, production of free radicals and cytokines, finally leading to occurrence of ALI/ARDS [3].

However, there are no specific pharmacologic intervention for prevention and treatment by now [4]. The present strategies and future perspectives of the treatment for ALI/ARDS, focus on the ventilatory, pharmacological and cell therapies [5–7]: (1) improving ventilation, such as lung protective mechanical ventilation, positive end expiratory pressure ventilation, inverse ratio ventilation and high frequency ventilation; (2) drug therapy, such as inhaled nitric oxide, aerosolized prostacyclin, surfactant, anti-inflammatory drugs and antioxidants; and (3)cell and cell-based gene therapy, such as endothelial progenitor cells (EPCs) and embryonic stem (ES) cells. The markedly high mortality of ARDS indicates that the key mechanism of the pathogenesis is not very clear. Thus, future studies should focus on newer forms of therapy and early phase of the pathogenesis of the disease.

OA-induced ALI is a well-known model of chemical ARDS and extensive studies on it have been made for decades [8]. Oxidative stress has been shown to play an important role in this model in recent several studies [9,10]. The reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase-dependent pathway from activated polymorphonuclear leukocytes and endothelial cells was considered to contribute much to the generation of reactive oxygen species (ROS) in OA-induced ALI [11]. One of the mechanisms of OA-induced ALI is that the intravenous administration of OA can induce neutrophil activation through aggregation and attachment to endothelial cells [12]. Then the activated neutrophils may generate much free radicals including ROS and reactive nitrogen species (RNS) [13]. The free radicals may cause tissue injury directly, such as lipid peroxidation, destroying of construction integrity and disfunction of the biomembranes. Moreover, the free radicals can serve as signals to activate both neutrophils and endothelial cells in a positive feedback

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2007 Informa UK Ltd.
DOI: 10.1080/10715760701376422

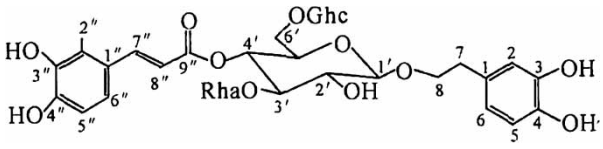

Figure 1. Chemical structure of ECH (major caffeic acid derivative in *E. angustifolia* and *E. pallida*).

mode [14]. As a result, the uncontrolled inflammatory responses will result in a rapid increase of alveolar-capillary permeability, pulmonary edema and severe hypoxia at last [15]. Several studies have demonstrated that antioxidants can obviously improve ALI through scavenging ROS [16,17].

Echinacoside (ECH) is the main phenolic compound found in *Echinacea angustifolia* and *Echinacea pallida* [18], both of which have been used to treat infectious and traumatic diseases for long time [19]. Recently, several studies reported that ECH might possess antioxidant and anti-inflammatory properties [20–22]. In this study, we investigated whether ECH could protect against ALI caused by OA through scavenging free radicals in rats (Figure 1).

Materials and methods

Animals

All experimental protocols of this study were approved by the Institutional Animal Care and Use Committee of Health Sciences Center, Peking University. Male Sprague-Dawley (SD) rats (220–240 g) were purchased from the Experimental Animal Center of Peking University (Peking, China) and allowed to acclimate upon arrival for 3 days before experimentation. Animals were fed rodent chow and water *ad libitum*.

Reagents

ECH from *E. angustifolia* was kindly supplied by Dr Ai Tie-Min (School of Pharmaceutical Sciences, Peking University), the purity of which was shown to be more than 98% on high-performance liquid chromatography (HPLC), dissolved in a phosphate buffered solution (PBS). α-Phenyl-*N*-*tert*-butyl nitrone (PBN) was purchased from Sigma-Aldrich (St Louis, MO, USA). OA was purchased from Golden Dragon Chemical Co. (Peking, China). Diethylenetriaminepentaacetic acid and ethyl acetate were the products of Peking Chemical Agent Manufactory (Peking, China), which were at the analytical reagent level. Myeloperoxidase (MPO) detection kit was purchased from Jiancheng Biological Co. (Nanjing, China).

Study design

In initial dose-response experiments, ECH was administered at four different doses (2, 10, 50 and 125 mg/kg body weight) by intraperitoneal (ip) injection. The orthogonal test was used to obtain the optimum experimental condition and the 50 mg/kg ECH dose was chosen.

To watch the change of the biological parameter with time course, the sampling was done at five time points (0.5, 1, 2, 6 and 24 h) respectively after the intravenous (iv) injection of OA (0.15 ml/kg body weight). At each time point, there were four groups (six rats per group): (1) control group, (2) ECH group, (3) OA group, and (4) OA–ECH group. The animal model of ALI was verified by blood gas analysis and pathological examination of the lung tissue.

After being anesthetized by inhalation of isoflurane, the rats from the OA and OA–ECH group were injected OA via femoral vein, whereas other rats were only administered the equivalent PBS intravenously. Then the rats from the ECH and OA–ECH group were immediately given ECH once (50 mg/kg ip) after OA injection, whereas other rats only received the equivalent PBS intraperitoneally. Animals were allowed to recover on a heated pad and permitted access to food and water. At the setted time points, rats were anesthetized by urethane (1.0 g/kg ip) and surgery was carried out with rats in the supine position in a sterile setting.

Blood gas analysis

A carotid catheter with heparinized saline was inserted for measurement of blood gas and the blood sample was analyzed at 37°C with a Ciba Corning-170 blood gas analyzer (Ciba Corning, Canada).

MPO measurement of lung tissue

After the experimental protocol was completed, lung samples were rapidly obtained from the left lower lobe for measurement of MPO. The samples were homogenized with 0.9% normal saline, centrifuged, and then detected by 1601-UV–Visible Spectrophotometer (Shimadzu, Japan) at 460 nm wavelength with MPO detection kit.

Analysis of pulmonary edema formation

After the experimental protocol was completed, the samples of rat lung tissues were rapidly obtained from the right upper lobe and rinsed in PBS to remove contaminating blood. After removal of excessive PBS by careful drying on tissue paper, the lung tissues were weighed (wet weight). Then the samples were dried in an oven at 60°C for 72 h, followed by a second weighing (dry weight). The ratio of lung wet/dry

weight was chosen as a marker for the degree of pulmonary edema.

Lung histopathology

Samples from the right lower lobe were fixed into 10% buffered formalin for 24 h. Lung tissues were embedded in paraffin and 4 μm sections were stained with hematoxylin and eosin. The stained sections were scored by pathologists who were blinded to the experimental protocol. The degree of microscopic injury was scored based on the following variables: hemorrhage, interstitial edema, necrosis, neutrophil infiltration and atelectasis. The severity of injury was judged by the previous criteria [23]: no injury, 0; injury to 25% of the field, 1; injury to 50% of the field, 2; injury to 75% of the field, 3; and diffuse injury, 4. Three microscopic visual fields were selected randomly for each lung section. The sum of all scores was combined to calculate a composite score.

Electron spin resonance (ESR) spectra of free radicals from lung tissues

Spin trap agent dispensing: PBN and DETAPAC were dissolved in PBS buffer (pH 7.4) at a final concentration of 100 and 2 mM, respectively. The samples of lung tissues from the left upper lobe were removed and homogenized. To 2 g of lung tissues 1 ml of spin trap agent was added. Lung homogenates were centrifuged at 14,000 rounds per minute (rpm) for 10 min after adding 0.6 ml ethyl acetate. Finally, the ethyl acetate extracts were taken in a test tube and stored at 0 ~ 4°C in the dark for 2 h. The specimen was then transferred into a quartz tube for ESR measurement. An X-band ESR spectrometer (Varian E-109; Varian, Inc., Palo Alto, CA, USA) was used to detect the levels of free radicals in lung tissues directly at room temperature (25°C) with measurement conditions as: X-band, central magnetic field (3445 G), scan width (200 G), microwave power (20 mW), frequency (100 kHz), modulation amplitude (2.5 G), and time constant (0.128 s) at 37°C [24]. Before measurement, ESR spectrometer was standardized with diphenyl-picri-hydrazyl to make sure that the equipment was in the same condition every time the experiment was performed.

Statistical analysis

Statistical analyses were performed by SPSS 12.0 software, and the results were presented as mean ± SD. Statistical significance of differences among groups was determined by analysis of variance, followed by the Student–Newman–Keuls test for between groups difference. The nonparametric Kruskal–Wallis test was used when comparing pathological scores among groups. A p-value < 0.05 was regarded as significant.

Results

General situation of the rats

There were 128 male SD rats in our study, eight of which died before sampling. Four rats died 2 h after injection of OA, three at 6 h and one at 24 h, respectively, in OA group, while the rats of other groups all survived. The rat body weights were similar among groups. All animals tolerated either the vehicle or the ECH without obvious adverse events.

Blood gas analysis

After administration of OA, hypoxemia was much more evident in the OA group than in the control group and ECH group as Figure 2 showed, especially

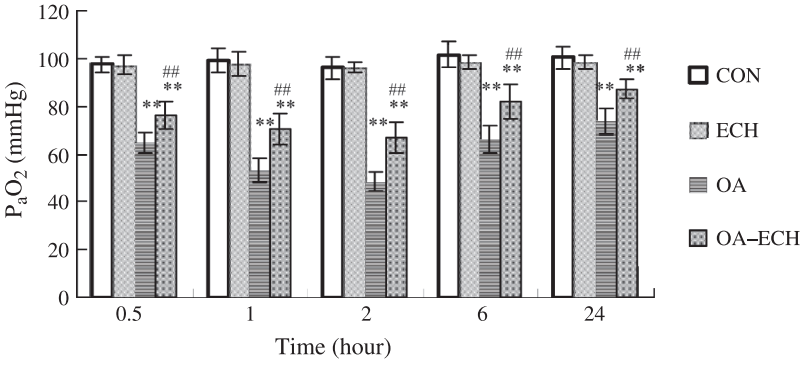

Figure 2. The effect of ECH on P_aO_2 in SD rats with OA induced ALI. The rats were randomized into four groups (thirty rats per group): control group, ECH group, OA group, and OA–ECH group. The sampling was done at five time points (0.5, 1, 2, 6 and 24 h) respectively after injection of OA in each group. There was no statistical difference between the control and ECH group. The statistical difference between the OA and control group was very significant, so did the statistical difference between the OA and OA-ECH group at all setted time points. $*p < 0.05$ vs. the control group, $**p < 0.01$ vs. the control group, $\#p < 0.05$ vs. the OA group, $\#\#p < 0.01$ vs. the OA group.

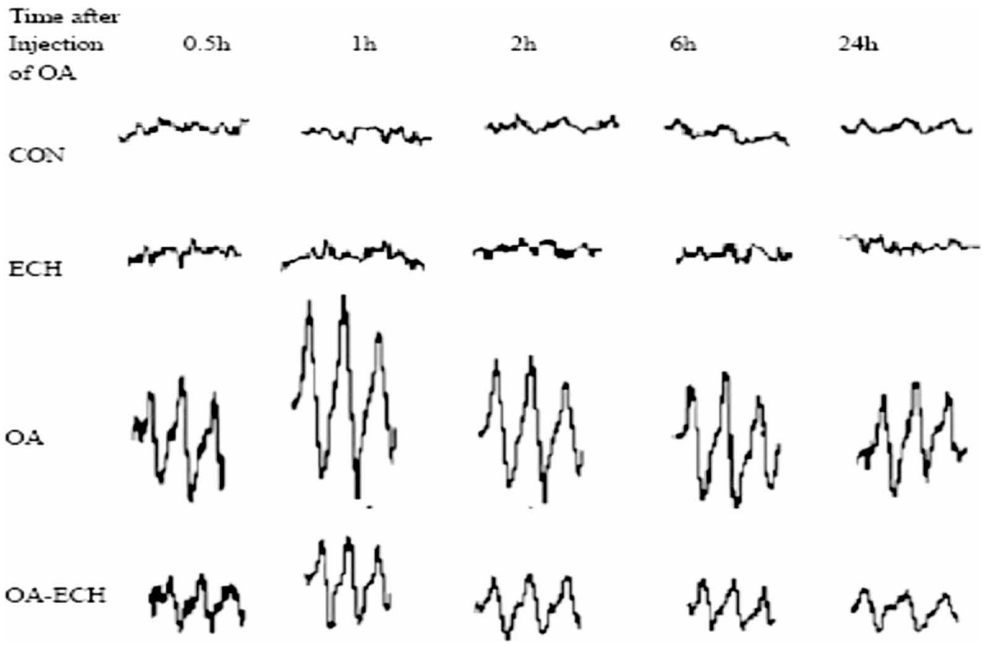

Figure 3. Effect of ECH on free radicals detected by an X-band ESR spectrometer. The peak height of spectrum represented relative concentration of free radicals (Gauss). The figure showed that nearly no signal of oxygen radicals could be seen in normal lung tissues. A significant release of free radicals in the OA group was detected at 1 h after intravascular injection of OA. Free radical levels in the OA group rose considerably over the control group at each time point. Free radicals in OA–ECH group were sharply lower than that in the OA group at all time points.

2 h after OA injection. The changes of P_aO_2 were corresponding with the diagnostic criterion of ALI established by the meeting of the American–European Consensus Conference, suggesting that the animal model of ALI was successful. P_aO_2 in the OA–ECH group was significantly more elevated than in the OA group. There was statistical difference between the OA–ECH group and the OA group when they were compared at the same time points. P_aCO_2 and pH nearly did not change among different groups at each time point (P_aCO_2 and pH data were not shown).

ESR spectra of free radicals from lung tissues

The ESR spectra of the spin trapping adduct of free radicals extracted from the lung tissue with PBN were shown in Figures 3 and 4. The peak height of the spectrum represented relative concentration of ROS. The result showed that nearly no free radicals signals

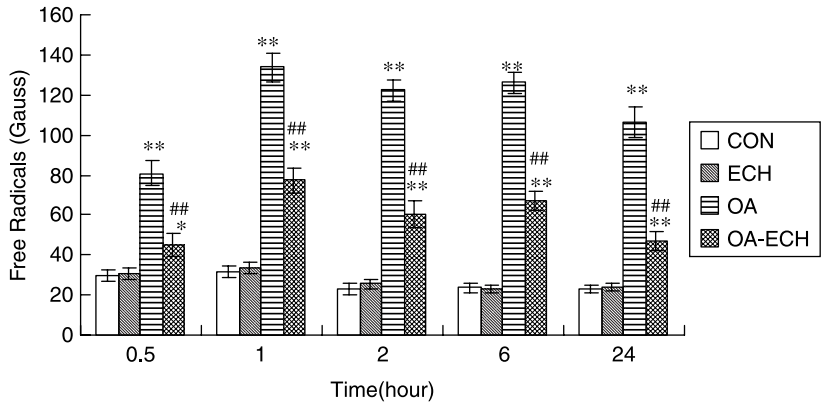

Figure 4. The effect of ECH on the oxygen radicals in SD rats with OA-induced ALI. There was no statistical difference between the control and ECH group. The statistical difference between the OA and control group was very significant at all setted time points. The statistical difference between the OA and OA–ECH group was also very significant. *$p < 0.05$ vs. the control group, **$p < 0.01$ vs. the control group, #$p < 0.05$ vs. the OA group, ##$p < 0.01$ vs. the OA group.

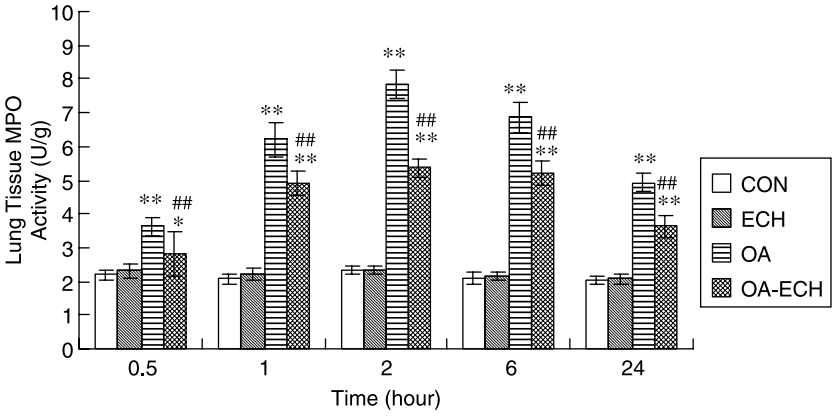

Figure 5. The effect of ECH on the activity of MPO in SD rats with OA-induced ALI. There was no statistical difference between the control and ECH group. The statistical difference between the OA and control group was very significant, so did the statistical difference between the OA and OA–ECH group at all setted time points. $*p < 0.05$ vs. the control group, $**p < 0.01$ vs. the control group, $\#p < 0.05$ vs. the OA group, $\#\#p < 0.01$ vs. the OA group.

could be seen in normal lung tissues, whereas the free radicals signals notably increased after injection of OA. The significant signals of free radicals were detected 1 and 6 h after injection of OA, especially reaching the summit at 1 h, then returned to the normal level 24 h after the OA injection. The concentration of free radicals in the OA group enhanced considerably over the control and ECH group at each time point. Free radicals in the OA–ECH group were sharply lower than those in the OA group at all time points.

Analysis of MPO of lung tissue

In the pathogenesis of ALI, the MPO is mainly released from neutrophils. The activity of MPO may represent the degree of inflammatory response to some extent. After administration of OA, the activity of MPO in lung tissue was much more evident in the OA group than that in the control and ECH group as Figure 5 showed, especially 2 h after OA injection. The activity of MPO in the OA–ECH group was much lower than that in the OA group, while higher than that in the control and ECH group. There was statistical difference between the OA–ECH and the OA group when they were compared at the same time points.

Evaluation of lung water

The pulmonary edema is viewed as a hallmark of ALI, so lung wet/dry weight ratio may indicate the degree of ALI. Figure 6 showed that lung wet/dry weight ratio was much more pronounced in the OA group than in the control and ECH group after injection of OA. The summit of lung wet/dry weight ratio in the OA group was found 2 h after OA injection. Lung wet/dry weight ratio in the OA–ECH group was significantly lower than that in the OA group at all time points. There was statistical difference between the OA–ECH group and the OA group when they were compared at the same time points.

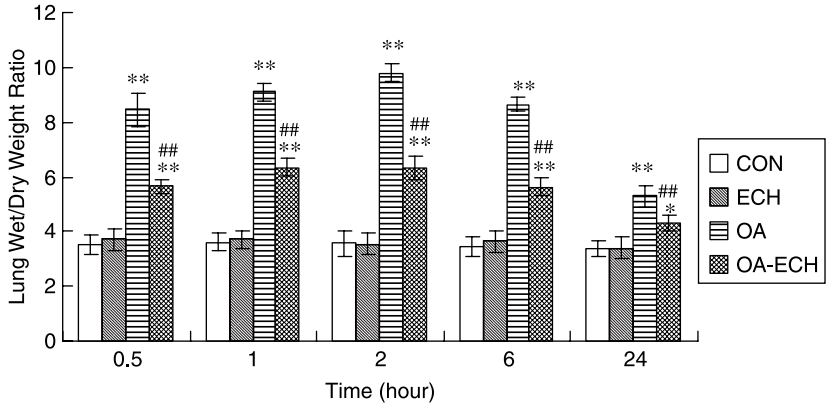

Figure 6. The effect of ECH on lung wet/dry weight ratio in SD rats with OA induced ALI. There was no statistical difference between the control and ECH group. The statistical difference between the OA and control group was very significant at all setted time points. The statistical difference between the OA and OA–ECH group was also very significant. $*p < 0.05$ vs. the control group, $**p < 0.01$ vs. the control group, $\#p < 0.05$ vs. the OA group, $\#\#p < 0.01$ vs. the OA group.

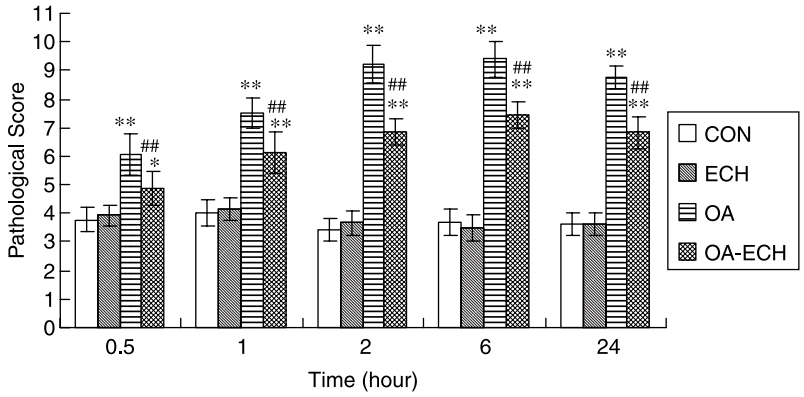

Figure 7. The effect of ECH on the pathologic score in SD rats with OA induced ALI. There was no statistical difference between the control and ECH group. The statistical difference between the OA and control group was very significant, so did the statistical difference between the OA and OA–ECH group at all setted time points. $\star p < 0.05$ vs. the control group, $\star\star p < 0.01$ vs. the control group, $\# p < 0.05$ vs. the OA group, $\#\# p < 0.01$ vs. the OA group.

Lung histopathology

There were obvious differences among the groups in histological sections of the lung (supplemental data). There was patchy hemorrhage observed grossly and pathological findings observed by light microscope in the OA group, including hemorrhage and edema, thickened alveolar interstitial, and the existence of inflammatory cells in alveolar spaces. It is obvious that lung histological changes were already present 0.5 h after injection of OA and were most serious 6 h after OA challenge. In OA–ECH group, these changes and pathologic score were far less serious than those in the OA group as Figure 7 showed.

Discussion

The previous studies in our laboratory have shown that ROS plays a pivotal role in the initiation of ALI/ARDS [13], so preventing the damage associated with free radicals might be a key measure to block the pathogenesis of ALI [25].

As the results showed in our study, the signals of free radicals increased in 30 min, reached the peak value at 1 h and decreased at 2 h after OA exposure, whereas the serious reduction of PaO_2 started from 2 h after OA injection, much later than the change of free radical production. The activity of MPO, the lung wet/dry weight ratio and the pathological score were most severe at 2, 4 and 6 h, respectively, after OA injection, also later than the free radicals. So it might indicate that the change of free radicals was earlier than that of pathophysiology.

We also found that the degree of ALI was corresponding with the relative concentration of free radicals in all groups. The samples from the control and ECH group showed nearly no signals of free radicals because the rats had not been injected with OA. In OA group, notable signals of free radicals were detected and the rats suffered from severe symptoms associated ALI. The results gave a new confirmation on our previous studies and supported the conclusion that the free radicals play a critical role in the initiation of ALI caused by OA injection [26].

The results showed that the signals of free radicals in ECH-treated ALI rats were much lower than that of ECH-untreated at all time points, while PaO_2, activity of MPO, lung wet/dry weight ratio and pathologic score were all better. Owing to the change of free radicals was earlier than that of the pathophysiological changes and the degree of ALI was corresponding with the relative concentration of free radicals, it was strongly suggested that ECH might reduce the severity of experimental ALI by scavengering the excess free radicals.

As to the protective effects of ECH on OA-induced ALI, there seems to be two possible ways:

(1) The aromatic rings in the chemical structure of ECH has hydroxyl groups, which can clean out free radicals, thereby preventing the lung injury associated with free radicals such as superoxide anion, hydroxyl radical, and hydrogen peroxide [27]. In addition, ECH can chelate the transition metal ions such as Fe, Cu and Zn to reduce the lipid peroxidation [28]. ECH might also modulate oxidative stress by regulating enzymatic activities of prooxidant and antioxidant enzymes, although the study in this field is still underway.
(2) ECH can reduce organ injury caused by the inflammatory cascade associated with free radical signals as well. Firstly, as reported previously, ECH could modulate lipopolysaccharide (LPS) and interferon-gamma induced expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in peritoneal macrophages, so as to inhibit directly inflammation [29]. Additionally, ECH also possesses the effect of antihyaluronidase activity, which reduces

the extension and intensity of inflammation [30]. Secondly, ECH can inhibit the inflammatory cascade caused by free radicals. Free radicals may act as an intracellular signal molecule to activate the redox-sensitive nuclear factor-κB (NF-κB) and the activator protein-1 (AP-1) complexes, which may coordinate the induction of multiple genes that encode inflammatory mediators such as cell adhesion molecules, enzymes, cytokines and chemokines. The interplay among above inflammatory mediators leads to the trapping and activation of inflammatory cell in lung, pulmonary parenchymal cellular injury, activation and release of additional inflammatory mediators. Thus, an initial small quantity of free radicals can initiate a cascade of inflammatory events. So, free radicals seem to be a real molecular event leading to the rapid activation of intracellular pathways for ALI [31,32]. It seems to suggest that ECH can suppress the production of free radicals and the activation of inflammatory cascade, thereby ameliorating pulmonary edema and improving gas exchange during ALI [33].

Our initial dose-response experiments showed that the anti-oxidative and -inflammatory property of ECH was dose-depended. If the dose of ECH exceeded the effective dose range, it might activate other signal pathways and impacted its protective effect. In our study, there were no statistical differences in signal of free radicals, activity of MPO, lung wet/dry weight ratio and pathological score between control and ECH group. $PaCO_2$ and blood pH also had similar change between them. All of them suggested ECH had no obvious adverse effects within the dose range.

In summary, our results are the first to report the protective effect of ECH on OA-induced ALI. The study found that ECH could improve ALI and animal survival without causing adverse effects. Its antioxidant property played an important role in the course of anti-inflammation and anti-injury in ALI. Because this paper is stemmed from an animal experiment of ALI caused by OA, it must be extremely cautious in extrapolating these data to complex clinical situation. We consider ECH should be a promising drug for the protection of lungs against acute injury by chemicals, especially used at early phase.

Acknowledgements

This work was supported by grant from the National Science Foundation Committee (NSFC, No. 30571551) of China.

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Supplemental data

Light microscopic photomicrograph of pulmonary histological structure (H.E. × 100).

CON group. There were nearly no hemorrhage, interstitial edema, inflammatory cells infiltration, microthrombus formation or epithelium necrosis.

ECH group. The results were similar with the control group.

OA group. Pulmonary hemorrhage, pulmonary interstitial edema, inflammatory cells infiltration, microthrombus formation and epithelium necrosis could be seen. It was obvious that lung histological changes were already present 0.5 h after injection of OA and were most serious 6 h after OA challenge.

OA-ECH group. There was less pulmonary hemorrhage, pulmonary interstitial edema in the lung after OA injection. The lung was less damaged than that of OA group.

Note: CON group only received phosphate buffered solution (PBS); ECH group received PBS (0.15 ml/kg iv) and ECH (50 mg/kg ip); OA group was administered OA (0.15 ml/kg iv) and PBS (equivalent to ECH solution ip); OA-ECH group was administered OA (0.15 ml/kg iv) and ECH (50 mg/kg ip). 0.5, 1, 2, 6 and 24 h represent the time points of OA exposure, respectively. Hematoxylin-eosin stain; Original magnification × 100.