

## Original Research Article

# Aqueous extract of *Aconitum carmichaelii* Debeaux attenuates sepsis-induced acute lung injury via regulation of TLR4/NF- $\kappa$ B pathway

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### Abstract

**Purpose:** To investigate the therapeutic effect of aqueous extract of *Aconitum carmichaelii* Debeaux (AEACD) on sepsis-induced acute lung injury (ALI), as well as explore the underlying mechanism of action.

**Methods:** C57BL/6 mice were treated with AEACD by gavage (4.0 g/kg/day) for 5 days before cecal ligation and puncture (CLP) challenge. After 24 h, the pathological morphology of lung tissue and the biochemical parameters in bronchoalveolar lavage fluid (BALF) were determined by H&E staining and enzyme-linked immunosorbent assay (ELISA). Furthermore, the total protein content and lactate dehydrogenase (LDH) level of BALF, as well as the oxidative biomarkers (malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD)) were evaluated in the lung homogenates by ELISA assay. The levels of pro-inflammatory cytokines, TNF $\alpha$ , IL-1 $\beta$ , and IL-6, in lung tissue were measured by qRT-PCR or ELISA. Finally, key proteins in Toll-like receptor 4 (TLR4)/nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway in lung tissue were evaluated by western blot.

**Results:** CLP challenge induced abnormal changes in the histological structures of lung tissue, lung wet-to-dry weight (W/D) ratio, protein content and LDH levels of BALF, which were remarkably reversed by AEACD. In addition, AEACD decreased MDA levels, and increased GSH levels and SOD activity in the lung tissue of CLP-treated mice ( $p < 0.05$ ). Furthermore, AEACD attenuated the CLP challenge-induced upregulation of TNF $\alpha$ , IL-1 $\beta$ , and IL-6. Finally, AEACD inactivated TLR4/NF- $\kappa$ B pathway by upregulating I $\kappa$ B $\alpha$  and downregulating TLR4 and phosphorylated-p65 levels.

**Conclusion:** AEACD administration protects mice against sepsis-induced ALI through the regulation of oxidative stress and inflammatory responses in lung tissues. The underlying mechanism occurs by inhibiting TLR4/NF- $\kappa$ B signaling pathway.

**Keywords:** *Aconitum carmichaelii* Debeaux, Acute lung injury, Sepsis, TLR4, NF- $\kappa$ B

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## INTRODUCTION

Acute lung injury (ALI) is a severe clinical illness that requires intensive care [1]. The pulmonary

infiltrates, pulmonary edema, and respiratory failure are main symptoms of ALI [2]. Though many studies have been performed to reduce symptoms and improve outcomes in ALI little

progress has achieved, and thus, the current mortality rate of ALI is still as high as 22 -58 % [3,4]. Therefore, revealing the pathogenesis and diagnostic strategy are necessary for the treatment of ALI. The underlying causes of ALI include infection, hypoxia and inflammation. In particular, inflammation is a promising breakthrough for the treatment of ALI [5].

Sepsis, a systemic inflammatory response, is one of the most common pathological symptoms in ALI [6]. It is usually caused by microbial infections, and exaggerated inflammatory responses are usually induced [7]. Generally, immune cells recognize various toxins released by microorganisms and produce inflammatory factors that contribute to the progress of ALI [8]. In innate immune cells, TLR4 is a common pathogen recognition receptor (PRR) that recognizes gram-negative bacteria [9]. The stimulation of TLR4 can activate the NF- $\kappa$ B pathway to promote expression of various pro-inflammatory cytokines and chemokines, such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 [10]. Importantly, the reduction of inflammatory responses induced by activating the TLR4/ NF- $\kappa$ B pathway can protect lung tissue from sepsis [11,12]. Therefore, TLR4 may be a promising therapeutic target for ALI.

*Aconitum carmichaelii* Debeaux belongs to the Ranunculaceae family. It has been historically reported that the lateral roots of *A. carmichaelii* Debeaux (Chinese: Fuzi) is a famous traditional Chinese medicine [13]. Fuzi is pharmacologically active and shows protective effects against inflammation, aging, and cardiovascular and immune system health [13,14]. A recent study revealed that aqueous extract from *Aconitum carmichaelii* Debeaux (AEACD) suppressed the inflammatory response and improved liver regeneration in acute liver failure via the TLR4/NF- $\kappa$ B and proliferating cell nuclear antigen (PCNA) signaling pathways [15]. Therefore, it seems that AEACD has a therapeutic effect on ALI through anti-inflammatory action.

The present study was aimed to further investigate whether the therapeutic effect of AEACD on sepsis-induced ALI was related to the suppression of oxidative stress and inflammation. Moreover, the inhibitory effects of AEACD on the TLR4/NF- $\kappa$ B pathway were evaluated.

## EXPERIMENTAL

### Reagents

Lactate dehydrogenase (LDH) activity assay kit was obtained from Solarbio Life Sciences

(Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kits for mouse TNF $\alpha$ , IL-1 $\beta$ , and IL-6 were from R&D Systems (Minneapolis, USA). Bicinchoninic Acid (BCA) protein assay kit, Lipid Peroxidation Assay Kit, Total Superoxide Dismutase Assay Kit, GSH Assay Kit, and RIPA lysis buffer were obtained from Beyotime (Shanghai, China). TRIzol reagent was purchased from Invitrogen (Carlsbad, USA). RNAprep pure tissue kit was provided by QIAGEN (Beijing, China). PrimeScript™ RT reagent kit and SYBR Green Plus reagent kit were bought from Takara (Shiga, Japan).

Polyvinylidene Fluoride (PVDF) membrane was purchased from Millipore (Billerica, USA). TLR4, I $\kappa$ B $\alpha$ , p-p65, p65, and  $\beta$ -actin antibodies and horseradish peroxidase (HRP)-labelled antibodies were purchased from Abcam (Cambridge, UK). Pierce Enhanced Chemiluminescence (ECL) Western Blotting Substrate was provided by Thermo Fisher (Waltham, USA). Tribromoethanol, chloral hydrate, paraformaldehyde, hematoxylin and eosin were obtained from Sigma-Aldrich (St. Louis, USA).

### Preparation of AEACD

50 g of *Aconitum carmichaelii* Debeaux, which was obtained from Sichuan Chinese Herbs Co., Ltd. (Sichuan, China), was soaked in 500 ml water for 1 h, all of which were then decocted in casserole for 3 times (500 ml water, 1 h each time). The decoctions were filtered and distilled in a rotary evaporator to 100 ml (0.5 g raw herbs in each milliliter).

### Animals

Male C57BL/6 mice (21 - 25 g, 6 - 8 weeks old, Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China)) were kept in the environment of specific pathogen-free (22  $\pm$  2 °C and 60  $\pm$  5 % humidity). All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committees of the United States (2011 edition) [16] and approved by the Ethics Committee of Anhui Medical University (approval no.LLSC20170070).

### CLP-induced sepsis model

Mice were randomly divided into four groups (n = 12 per group): (1) sham group, (2) sham + AEACD group, (3) CLP group, or (4) CLP + AEACD. Mice in the sham group were given saline by gavage for 5 days and then anesthetized with Tribromoethanol (2%, 15

$\mu\text{L/g}$ ). Mice in the sham + AEACD group were given AEACD by gavage (4 g/kg/day) for 5 days and then anesthetized with Tribromoethanol (2%, 15  $\mu\text{L/g}$ ). Mice in the CLP group were given saline by gavage for 5 days and then received CLP challenge. Mice in the CLP + AEACD group were given AEACD by gavage (4 g/kg/day) for 5 days and then received CLP challenge.

The CLP procedure was used to induce ALI as mentioned previously[17]. Briefly, mice were anesthetized using chloral hydrate (10 %, 3  $\mu\text{L/g}$ ), and a midline incision was performed in the mouse abdomen. The cecum was gently exteriorized and ligated (approximately 0.5-0.75 cm cecum was ligated). The ligated cecum was perforated with a 19-gauge needle then lightly squeezed to extrude feces. The cecum was returned and the incision was sutured. Finally, warm normal saline (1 mL) was subcutaneously injected into each mouse for fluid resuscitation.

#### Hematoxylin and eosin (H&E) staining

CLP challenge, mice in each group were euthanized 24 hours later. Fresh lung tissue was obtained and divided into five parts. One part of the lung tissue was fixed in 4% paraformaldehyde and embedded in paraffin. After sectioning into slices (6  $\mu\text{m}$ ), H&E staining was performed with hematoxylin (0.5 %, 3 min) and eosin (0.5 %, 2 min). A light microscope (Olympus, Tokyo, Japan) was used to observe and photograph the H&E-stained slides.

#### Lung W/D ratio

After euthanizing the mice, fresh lung tissues were collected immediately to acquire lung wet weight. Then, the lung tissues were dried at 80 °C for 48 h to obtain lung dry weight, finally, the lung W/D ratio was recorded.

#### BALF preparation

After CLP challenge, mice in each group were euthanized 24 hours later. The lung-gavage was performed with 0.5 mL normal saline for three times via endotracheal intubation, and 1.2-1.3 mL BALF was collected. Then the BALF was centrifuged (2000 g, 10 min, 4 °C), and total protein content (BCA protein assay kit), lactate dehydrogenase (LDH) activity (LDH activity assay kit), and cytokines (Enzyme-linked immunosorbent assay kits (ELISA) for mouse TNF $\alpha$ , IL-1 $\beta$  and IL-6 in the supernatant of BALF were detected.

#### Measurement of BALF malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD)

One part of fresh lung tissue was homogenized in normal saline (10%, w/v) with OMNI-PREP homogenizer (Omni, USA). The supernatant was extracted by centrifugation (2000 g, 15 min, 4°C), and then MDA levels (Lipid Peroxidation Assay Kit), GSH content (GSH Assay Kit Beyotime), and SOD activity (Total Superoxide Dismutase Assay Kit) in the supernatant were measured.

#### Quantitative polymerase chain reaction (qPCR)

One part of fresh lung tissue was homogenized in TRIzol, and total RNA was extracted with an RNeasy pure tissue kit. Then, a PrimeScript™ RT reagent kit (Takara Shiga, Japan) was used to reversely transcribe RNA. Quantitative polymerase chain reaction (qPCR) was performed with a SYBR Green Plus reagent kit using a QuantStudio 5 Real-Time PCR System (Thermo Fisher, Waltham, USA). The mRNA expression was normalized relative to GAPDH expression. The primers were shown in Table 1.

**Table 1:** Gene primers

Gene		Primer sequence
TNF $\alpha$	Forward sequence (5'-3')	CTTCTCATTCTGCTTGTG
	Reverse sequence (5'-3')	ACTTGGTGGTTTGCTACG
IL-1 $\beta$	Forward sequence (5'-3')	CCTGGGCTGCTCTGATGAGAG
	Reverse sequence (5'-3')	TCCACGGGAAAGACACAGGTA
IL-6	Forward sequence (5'-3')	GGCGGATCGGATGTTGTGAT
	Reverse sequence (5'-3')	GGACCCAGACAATCGGTTG
GAPDH	Forward sequence (5'-3')	CAATGTGTCCGTCGTGGATCT
	Reverse sequence (5'-3')	GTCCTCAGTGTAGCCCAAGATG

#### Western blotting

One part of fresh lung tissue was homogenized in RIPA lysis buffer. After centrifugation (12000 rpm, 15 min, 4°), the protein concentration was measured using BCA protein assay kit. Proteins

(50 µg) were separated with 10 % sodium dodecyl sulfate - polyacrylamide gel (SDS-PAGE) and transferred onto a PVDF membrane. Non-fat milk (5 %) was used to block the membranes, which were then blotted with primary antibodies (1:1000, 4 °C) overnight. After incubation with HRP-labelled secondary antibody (1:2000, 2 h), Pierce ECL Western Blotting Substrate was used to visualize the protein bands.

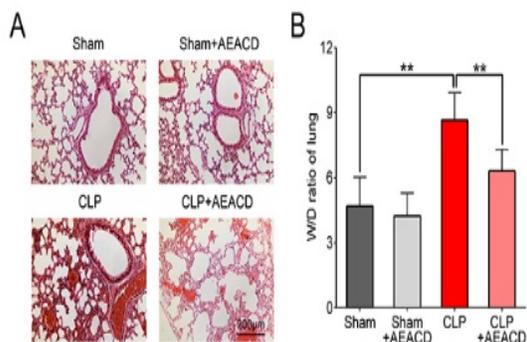
**Statistical analysis**

Data are shown as mean ± SD (standard deviation). Statistical analysis was performed by two-way ANOVA or Student's t-test using Graphpad Prism 8. Statistically significant difference was set at  $p < 0.05$ .

**RESULTS**

**AEACD alleviated sepsis-induced ALI**

To explore the therapeutic effect of AEACD on sepsis-induced ALI, CLP-induced mouse sepsis model was established and lung tissues were evaluated with H&E staining. As compared to the sham group, CLP treatment induced severe pathologic symptoms, including pulmonary hemorrhage, pulmonary edema, thickened alveolar septal, and inflammatory cell infiltration (Figure 1 A). Importantly, AEACD treatment markedly reduced the CLP-induced histological changes (Figure 1 A). AEACD treatment also decreased CLP-induced enhancement of lung edema, which was evaluated using the lung W/D ratio ( $p < 0.01$ , Figure 1 B). These findings demonstrated that AEACD could ameliorate sepsis-induced ALI.

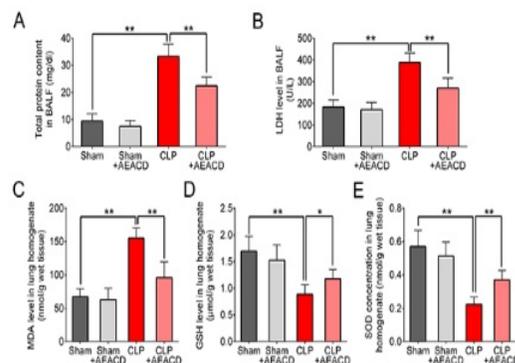


**Figure 1:** Effects of AEACD on sepsis-induced ALI. Mice were given normal saline/AEACD (4 g/kg/day) by gavage for 5 days before CLP treatment, and lung tissue was obtained 24 h later. (A) Morphology of lung tissue in each group. (B) Lung W/D ratio of each group (n = 5); \*\*  $p < 0.01$ .

**AEACD reduced sepsis-induced oxidative damage in lung tissue**

Bronchoalveolar lavage was used to determine the capillary leakage of lung tissue. Bronchoalveolar lavage fluid (BALF) of CLP-treated mice exhibited increases in both protein content and LDH levels as compared to that of sham mice ( $p < 0.01$ , Figure 2 A and B). However, AEACD treatment partially reversed the changes in total protein content and LDH level in the BALF of CLP-treated mice ( $p < 0.01$ , Figure 2 A and B).

Moreover, oxidative biomarkers were evaluated in the lung homogenates of each group. Compared to the sham group, CLP treatment significantly increased the MDA levels in lung tissue ( $p < 0.01$ , Figure 2 C). In contrast, AEACD intake reduced MDA levels in CLP-treated mice ( $p < 0.01$ , Figure 2 C). The GSH and SOD levels in lung homogenates were also determined. CLP treatment significantly decreased the GSH and SOD levels when compared with sham mice ( $p < 0.01$ , Figures 2 D and E). However, AEACD administration increased GSH and SOD levels in CLP-treated mice ( $p < 0.01$ , Figure 2 D and E). These results demonstrated that AEACD protected lung tissue against sepsis-induced oxidative damage.

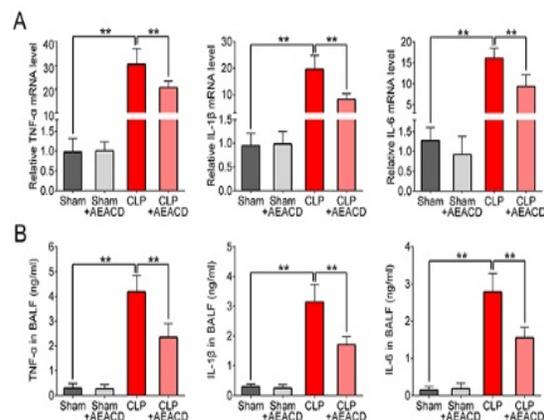


**Figure 2:** Effect of AEACD on sepsis-induced oxidative damage in lung tissue. Mice were given normal saline/AEACD (4 g/kg/day) by gavage for 5 days before CLP treatment and lung tissue/BALF were obtained 24 h later. (A) Total protein content in BALF. (B) LDH levels in BALF. (C) MDA levels in lung homogenate (n = 5). (D) GSH level in lung homogenate (n = 5). (E) SOD concentration in lung homogenate (n = 5); \*\*  $p < 0.01$

**AEACD reduced sepsis-induced levels of pro-inflammatory cytokines in lung tissue**

To investigate the effect of AEACD on sepsis-induced inflammatory responses, the levels of pro-inflammatory cytokines, TNFα, IL-1β, and IL-6, in lung tissue were measured. The mRNA

expressions of TNF $\alpha$ , IL-1 $\beta$ , and IL-6 were elevated in CLP treatment as compared to that of sham mice. However, AEACD treatment significantly attenuated CLP-induced increases in the mRNA levels of TNF $\alpha$ , IL-1 $\beta$ , and IL-6 ( $p < 0.01$ , Figure 3 A). Consistently, the elevated concentrations of TNF $\alpha$ , IL-1 $\beta$ , and IL-6 induced by CLP in BALF were also partially reversed by AEACD (Figure 3 B). These findings suggested that sepsis-induced inflammatory responses in lung tissue could be reduced by AEACD.



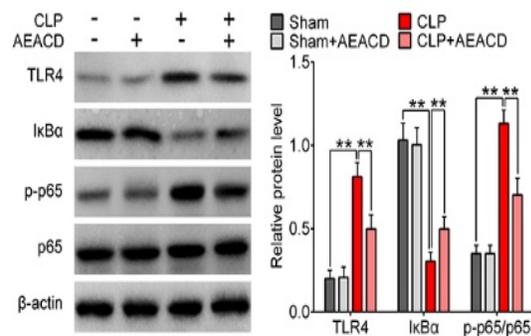
**Figure 3:** Effects of AEACD on sepsis-induced pro-inflammatory cytokines in lung tissue. Mice were given normal saline/AEACD (4 g/kg/day) by gavage for 5 days before CLP treatment, and lung tissue/BALF were obtained 24 h later. (A) Relative mRNA expression levels of TNF $\alpha$ , IL-1 $\beta$ , and IL-6 in lung homogenate (n = 5). (B) The concentrations of TNF $\alpha$ , IL-1 $\beta$ , and IL-6 in BALF (n = 5); \*\*  $p < 0.01$

### AEACD attenuated sepsis-induced activation of TLR4/NF- $\kappa$ B pathway in lung tissue

To further investigate the mechanisms underlying the therapeutic effect of AEACD in sepsis-induced ALI, key proteins in the TLR4/NF- $\kappa$ B pathway were determined by western blotting. The protein levels of TLR4 and p-p65 were increased, while the protein of I $\kappa$ B $\alpha$  was decreased in lung tissue after CLP treatment as compared to that of sham group ( $p < 0.01$ , Figure 4). Administration of AEACD reduced these protein levels induced by CLP ( $p < 0.01$ , Figure 4). These results demonstrated that AEACD could attenuate the sepsis-induced activation of TLR4/NF- $\kappa$ B pathway.

## DISCUSSION

ALI is a common complication associated with sepsis and often has a poor prognosis [18]. In the present study, the therapeutic effect of AEACD on sepsis-induced ALI, and the molecular mechanisms were investigated.



**Figure 4:** Effects of AEACD on sepsis-induced activation of TLR4/NF- $\kappa$ B pathway. TLR4, I $\kappa$ B $\alpha$ , p-p65, and p65 protein levels in lung tissue were measured using western blot (n = 5). Protein band intensity was evaluated by ImageJ; \*\*  $p < 0.01$

The pathological symptoms of sepsis-induced ALI include destruction of lung structure, pulmonary edema, and inflammatory cell infiltration [19]. Consistently, in the present study, H&E staining showed that CLP challenge induced alveolar structural damage. The increased lung W/D ratio also indicated that CLP challenge induced pulmonary edema [20]. Moreover, elevated protein content and LDH activity in BALF indicated that epithelial and endothelial permeability were enhanced in lung tissue of CLP mice [20]. Similar to previous study, these results suggested the successfully established ALI mice model induced by sepsis[17]. AEACD effectively reversed these CLP challenge-induced symptoms in lung tissue. Therefore, AEACD has therapeutic effects on sepsis-induced ALI.

It is well-known that oxidative stress is critical for the development of ALI [21]. For example, recent studies reported that ALI that induced by oxidative stress could occur via multiple mechanisms, including induction of DNA damage, promotion of lipid peroxidation, and upregulation of pro-inflammatory gene expression via NF- $\kappa$ B [22,23]. Sepsis can cause the alterations of a series of oxidative biomarkers.

Sepsis can cause the alterations of a series of oxidative biomarkers, including MDA (a biomarker of peroxidation damage), GSH (an antioxidant), and SOD (an oxygen radical scavenger) in lung tissue [24]. In the current study, CLP challenge enhanced MDA levels and reduced GSH levels and SOD activity. In lung tissue, sepsis-induced oxidative stress was markedly attenuated by AEACD treatment. These results were consistent with previous conclusion, showing that the lateral roots of A.

*carmichaelii* Debeaux strengthened the antioxidant function of tissues, including enhancement of SOD in red blood cell and reduction of MDA in blood and liver tissue [13,25].

Inflammation, the major cause of ALI, usually reflects an imbalanced state between anti-inflammatory and pro-inflammatory cytokines [5,26]. Pro-inflammatory cytokines are generated in immune cells and usually promote acute inflammatory injury [27,28]. In this study, the levels of TNF $\alpha$ , IL-1 $\beta$ , and IL-6 were markedly downregulated by AEACD in both lung tissue and BALF after CLP challenge. These findings demonstrated that AEACD protected lung tissue against inflammation-induced injury.

TLR4 recognizes lipopolysaccharide from gram-negative bacteria and activates downstream signaling pathways, including MAPKs and NF- $\kappa$ B pathways [9]. In this study, CLP challenge greatly elevated TLR4 levels in lung tissue, which were effectively reversed by AEACD treatment. Upon TLR4 activation, I $\kappa$ B $\alpha$  is phosphorylated and degraded, then triggers p65 phosphorylation and translocation into the nucleus, regulating the expression of various cytokines [9]. In CLP-challenge mice, AEACD inhibited p-65 expression and upregulated I $\kappa$ B $\alpha$  protein level, which could decrease translocation of p-p65 into the nucleus and reduce the production of cytokines. However, AEACD has wide-reaching biological activity, and there are likely multiple signaling pathways involved in the protection against ALI, which requires further analysis.

## CONCLUSION

The findings of the present study show that AEACD protects mice from sepsis-induced ALI via anti-oxidant stress and anti-inflammation effects through the TLR/NF- $\kappa$ B pathway. Therefore, AEACD is a promising traditional Chinese medicine for ALI treatment.

## DECLARATIONS

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### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. Qinghai You designed all the experiments and revised the paper. Jinmei Wang and Lijuan Jiang formed the experiments, while Yuanmin Chang and Wenmei Li wrote the paper.

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