Acta Pharmaceutica Sinica B 2018;8(2):218–227

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

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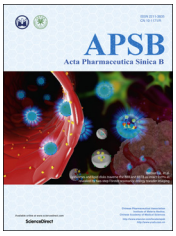

ORIGINAL ARTICLE

Beneficial effects of *Houttuynia cordata* polysaccharides on "two-hit" acute lung injury and endotoxic fever in rats associated with anti-complementary activities

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Received 23 June 2017; received in revised form 18 September 2017; accepted 1 November 2017

KEY WORDS

Houttuynia cordata;
Polysaccharides;
Fever;
Acute lung injury;
Anti-complementary

Abstract *Houttuynia cordata* Thunb. is a traditional herb used for clearing heat and eliminating toxins, and has also been used for the treatment of severe acute respiratory syndrome (SARS). *In vitro,* the crude *H. cordata* polysaccharides (CHCP) exhibited potent anti-complementary activity through both the classical and alternative pathways by acting on components C3 and C4 of the complement system without interfering with the coagulation system. This study was to investigate the preventive effects of CHCP on acute lung injury (ALI) induced by hemorrhagic shock plus lipopolysaccharide (LPS) instillation (two-hit) and LPS-induced fever in rats. CHCP significantly attenuated pulmonary injury in the "two-hit" ALI model by reducing pulmonary edema and protein exudation in bronchoalveolar lavage fluid (BALF). In addition, it reduced the deposit of complement activation products in the lung and improved oxidant-antioxidant imbalance. Moreover, CHCP administration inhibited fever in rats, reduced the number of leukocytes and restored serum complement levels. The inhibition on the inappropriate activation of complement system by CHCP may play an important role in its beneficial effects on inflammatory diseases. The anti-complementary polysaccharides are likely to be among the key substances for the heat-clearing function of *H. cordata*.

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Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

https://doi.org/10.1016/j.apsb.2017.11.003

1. Introduction

The complement system is composed of more than 30 plasma and membrane-bound proteins, and is considered to be a nonspecific host immune response. It is activated immediately after injury and to a greater degree during resuscitation[1]. However, the inappropriate activation of the complement system has been demonstrated to play a role in the pathogenesis of a wide range of auto-immune disorders, acute inflammatory diseases and tissue injuries[2,3]. C3a, C4a and C5a, the anaphylatoxins cleaved respectively from the complement components C3, C4 and C5 during complement activation, may trigger degranulation of endothelial cells, mast cells or phagocytes, and induce inflammatory responses as well as the fatal shock-like syndrome[4]. Modulation of the complement activity could be beneficial in treating various complement-associated diseases[5,6].

Acute lung injury (ALI) is characterized with respiratory dysfunction, hypoxemia with presence of diffuse pulmonary infiltrates, widespread lung inflammation[7], increased pulmonary vascular permeability and accumulation of activated neutrophils in the lung[8]. Acute respiratory distress syndrome (ARDS) is the most severe form of ALI[9]. Complement activation is an early step in ALI[3] through both the classical pathway and the alternative pathway[10]. The loss of endothelial integrity in the process of ALI/ARDS also causes injured pulmonary cells to release some tissue factors or enzymes, which would lead to the initiation of complement cascade[10,11]. A cycle of complement activation and lung injury is consequently formed. Hence, the application of complement inhibitors should be a novel treatment strategy for infectious fever and ALI.

Fever is another characteristic symptom of infectious diseases. It is one of complex and nonspecific host defense responses to infections[12]. It is reported that hyper-activated complement system also involves in fever[13].

As a traditional Chinese medicine with actions of clearing heat and eliminating toxins, the whole plant of *Houttuynia cordata* Thunb. (Saururaceae) is commonly used for the treatment of pulmonary symptoms (including lung abscess, dyspnea, phlegm and cough), as well as infectious diseases, anaphylaxis, cancer and viral infection[14–17]. A Chinese multiherb remedy with *H. cordata* as the principal ingredient had been used for the treatment and prevention of severe acute respiratory syndrome (SARS) in 2003[18] and was found to have significant anti-complementary activity[19]. Our previous study indicated that the crude *H. cordata* polysaccharides (CHCP)[20] and flavonoids[21] were the major anti-complementary principles of this plant. Some anti-complementary polysaccharides showed potent effects on complement-associated diseases *in vivo*[22,23]. The presence of lipopolysaccharide (LPS), a prototypical endotoxin, in blood can cause the immediate activation of the complement cascade. We had reported that CHCP could ameliorate LPS-induced ALI in mice and reduce the complement activation products deposited in the lung tissue[20]. However, it is still unclear whether this effect was a direct result of CHCP's anti-complementary activity *in vivo*. Herein, the beneficial effect of CHCP on ALI was further evaluated using a "two-hit" ALI rat model induced by hemorrhagic shock plus LPS instillation, which is more susceptive to lung injury[24]. Specifically, the *in vivo* anti-complementary activity of CHCP was also assessed. Besides pulmonary infections, fever is another typical indication for heat-clearing traditional Chinese medicine (TCM). Therefore this paper investigated *in vivo* the anti-pyretic effects and anti-complementary activity of CHCP on a febrile rat model induced by LPS.

2. Materials and methods

2.1. Agents and animals

The dried whole plant of *H. cordata* was purchased from Shanghai Hua-Yu Chinese Materia Medica Co., Ltd. (Shanghai, China) and was identified by one of the authors (Dr. Yan Lu). A voucher specimen (DFC-YXC-2006072601) has been deposited at the Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, China.

CHCP was prepared through the process of water extraction, alcohol precipitation and deproteinization as previously reported[20]. The dried whole plant of *H. cordata* was grounded and defatted with 95% ethanol. The residue was extracted with hot water. The water extract was concentrated and precipitated with trichloroacetic acid to remove proteins. The supernatant was dialyzed in running water for 3 days, and then precipitated by adding 4 volumes of 95% ethanol. The precipitate was lyophilized to yield the polysaccharides (CHCP). The total carbohydrate content was determined with the phenol-sulfuric acid using D-galactose as the standard. The *m*-hydroxybiphenyl method was used to test the uronic acid content with D-galacturonic acid as the standard. The concentration of total protein was evaluated using the Coomassie brilliant blue method, with bovine serum albumin as the standard. High performance gel permeation chromatography (HPGPC) was carried out to analyse the molecular weight of CHCP on a TSKgel GMPWxl column (300 mm × 7.6 mm, TOSOH, Japan). Gas chromatography (GC) was used to analyze the monosaccharide composition of the completely hydrolyzed CHCP on a HP6890 (Hewlett-Packard, Wilmington, USA) fitted with a capillary column DB-225 (30 m × 0.25 mm).

Male Sprague–Dawley (SD) rats (300 to 350 g, SPF II Certificate; No. SCXK 2008-0016), were provided by the Sino British SIPPR/BK Lab Animal Ltd. (Shanghai, China). Male Wistar rats (210 ± 20 g, SPF II Certificate; No. SCXK 2007-0005) were purchased from Slaccas-Shanghai Lab Animal Ltd. (Shanghai, China). All the experimental procedures described in this study were previously approved by the Animal Ethics Committee of School of Pharmacy, Fudan University.

Sheep blood cells were collected in Alsevers' solution. Normal human serum (NHS) was obtained from healthy male donors (at the age of 21–25 years old). Heparin (sodium salt, 160 IU/mg) was from Shanghai Aizite Biotech Co., Ltd. (Shanghai, China). Antisera of C1q, C2 and C9 were from Merck Biosciences (Darmstadt, Germany). Antiserum of C5 was from Shanghai Shengsuo Reagent Co., Ltd. (Shanghai, China). Antisera of C3 and C4 were from Shanghai Taiyang Biotech Co., Ltd. (Shanghai, China). LPS (*Escherichia coli* O111:B4 endotoxin) was from Sigma–Aldrich (St. Louis, USA). Malondialdehyde (MDA) and superoxide dismutase (SOD) kits were from Nanjing Jiancheng Biotech Co., Ltd. (Nanjing, China). Complement components C3 and C4 kits were from Taiyang Biotech Co., Ltd. (Shanghai, China).

Buffers: barbitol buffer solution (BBS), containing 0.5 mmol/L Mg^{2+} and 0.15 mmol/L Ca^{2+}. GVB-Mg-EGTA, veronal buffer saline, containing 5 mmol/L Mg^{2+} and 8 mmol/L Ca^{2+}.

2.2. Assays of anti-complementary activities of CHCP

Anti-complementary activity through the classical pathway was studied based on Mayer's modified method[25] with BBS as the buffer. As the complement source, the 1:80 diluted serum of guinea pig was chosen to give sub-maximal lysis in the absence of complement inhibitors. CHCP and heparin, used as the positive control, were dissolved in BBS. Different dilutions of samples (200 μL) were incubated with 200 μL guinea pig serum, 100 μL sheep erythrocytes (4.0×10^8 cells/mL) and 100 μL rabbit anti-sheep erythrocyte antibody at 37 °C for 30 min. Then the reaction mixture was centrifuged at $1800 \times g$ for 5 min. Optical density (OD) of the supernatant was measured at 405 nm. The sample control of each dilution, the blank control and 100% lysis control were all prepared under the same conditions. The corrected absorbance of each dilution of CHCP sample was obtained by subtracting the absorbance of the sample control from each value. The activity was determined by means of triplicate measurements and expressed as the 50% inhibition (CH_{50}).

According to the method of Klerx et al.[26], the samples were dissolved in GVB-Mg-EGTA for the anti-complementary assay through alternative pathway. 150 μL sample was mixed with 150 μL 1:10 diluted NHS, and 200 μL rabbit erythrocytes were added. Following the incubation at 37 °C for 30 min, the cell lysis was measured as described above. The 50% inhibition was presented as AP_{50}.

2.3. Identification of the targets of CHCP in the complement activation cascade

The identification of the targets of CHCP was conducted using complement-depleted (C-depleted) sera as described in the literatures[21,27]. Six components of the complement system (C1q, C2, C3, C4, C5 and C9) were selected as possible targets. Different dilutions of each antiserum (anti-C1q, C2, C3, C4, C5 and C9) were incubated with the same volume of NHS (1:8) at 37 °C for 15 min. After centrifugation, 200 μL supernatant was incubated with 200 μL erythrocyte (EA) and 200 μL BBS. Then cell lysis was measured and the antiserum dilution against the NHS hemolytic capacity was thus determined. The optimal dilutions for antisera are 1:128 for C1q and C2, 1:1 for C3 and C4, 1:32 for C5, 1:64 for C9. The antisera at the above dilutions were incubated with the same volume of NHS (1:8) at 37 °C for 15 min. After centrifugation, the supernatants were stored at −70 °C in aliquots as the C-depleted sera.

Target identification is performed by evaluating the capacity of various C-depleted sera to restore the hemolytic capacity of the CHCP-treated serum. The optimal concentration of CHCP was 1.4 mg/mL, being just sufficient to completely inhibit the hemolytic activity of 1:8 diluted NHS through CP. 200 μL sensitized erythrocytes and 200 μL individual C-depleted serum were added to 200 μL sample-treated NHS, and the mixture was incubated at 37 °C for 30 min. After centrifugation, the percentage of hemolysis was calculated. The capacity of C-depleted sera to lyse erythrocytes was also assessed by incubating C-depleted serum directly with erythrocytes under the same conditions. All controls (vehicle, 100% lysis, complement and sample control) were treated in the same way.

2.4. Influence of CHCP on coagulation

Influence of CHCP on the coagulation system was examined by determining the recalcification time (RT) and thrombin time (TT)[21,27]. Platelet poor plasma (PPP) was prepared from the anticoagulant blood of guinea pigs. Guinea pigs were anesthetized with 1 g/kg urethane intraperitoneally (i.p.). The right common carotid artery was separated and cannulated with a 24-gauge angiocath (Shanghai Puyi Medical Instruments Co., Ltd., Shanghai, China) filled with 3.8% (*w/w*) trisodium citrate for blood sampling. The whole blood of guinea pig was mixed with 3.8% trisodium citrate (9:1, *v/v*) and centrifuged for 10 min at $1000 \times g$. The supernatant obtained was used as PPP. Samples (15 μL) were incubated with 150 μL PPP at 37 °C for 5 min. A total of 150 μL $CaCl_2$ (0.025 mol/L) or 150 μL thrombin was added for the assay of RT or TT, respectively. Heparin (5.5 mg/L) was used as the positive control.

2.5. ALI experiment

2.5.1. Model and grouping

Before the experiment, all male SD rats were fasted overnight (8 h), with free access to water. The "two-hit" ALI animal model induced by hemorrhagic shock/resuscitation and LPS instillation[24] was used in this study. The rats were randomly divided into six groups: Sham, ALI model and prednisone (70 mg/kg) groups, as well as CHCP groups of 25, 50 and 100 mg/kg. All animals were anesthetized with 1 g/kg urethane (i.p.). The right common carotid artery was cannulated with a 24-gauge Angiocath indwelling needle filled with 0.4% heparin for the monitor of mean arterial pressure (MAP), blood sampling and resuscitation. The heparinization was performed by injecting 0.1 mL 0.4% heparin through the angiocath to prevent clotting. Hemorrhagic shock was initiated by blood withdrawal, leading to a reduction of the MAP to 40 ± 5 mmHg in 20 min. This blood pressure was maintained by further blood withdrawal if MAP was higher than 45 mmHg and by infusion of 0.5 mL normal saline (NS) if the MAP was less than 35 mmHg. After a hypotensive period of 60 min, rats were resuscitated by transfusion of the withdrawn blood in 1 h. Then, either LPS (1 mg/kg in 200 μL NS) or NS alone was administered intratracheally (i.t.). CHCP (100 mg/kg, 50 mg/kg and 25 mg/kg, dissolved in saline) was given intragastrically (i.g.) at 30 min before the LPS instillation. The rats of the positive control group received a single dose of prednisone 70 mg/kg i.p. Sham animals underwent the same surgical procedures but without the hypotensive process, and received intratracheal instillation of NS. Serum samples of all animals were obtained from the right carotid artery at 0, 2 and 4 h after the administration of LPS. All animals were breathing spontaneously during the experiment and sacrificed at 4 h after LPS administration.

2.5.2. Lung wet/dry weight ratio

The lower lobe of the right lung was used for the determination of wet-dry weight (W/D) ratio. It was harvested, weighed immediately and dried at 80 °C for 48 h, then reweighed.

2.5.3. Histologic examination and immunohistochemistry

The upper lobe of right lung was fixed immediately in 4% formaldehyde. After the routine paraffin embedding, the sample was cut into 5-μm sections and stained with haematoxylin and eosin (H&E) for the detection of lung histopathological alteration. The severity of the lung injury was blindly quantified by a pathologist based on microscopic changes, including extent of disease, alveolar wall thickness, exudation, pulmonary congestion and inflammation degree. The injury was graded from 0 (normal)

to 4 (severe) in the above categories. The deposit of complement activation products was determined using immunohistochemical staining with rabbit anti-human C3c[20].

2.5.4. BALF collection and analysis

Bronchoalveolar lavage fluid (BALF) was collected immediately after sacrifice. The lobes of left lung were lavaged with a total volume of 4 mL saline at 4 °C. BALF was centrifuged at 111 × *g* for 10 min. The cell-free supernatant was collected and stored at −20 °C for further assays. Total cell counts were performed on the Neubauer's counting chamber. The leukocyte classifications were examined by counting at least 100 cells/smear prepared using cytospin and Wright-Giemsa staining. The obtained values represented the percentage of neutrophils or lymphocytes in total leukocytes[28]. The concentration of the total protein was determined by the Coomassie brilliant blue method[29]. The assays of superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were carried out using commercial kits.

2.5.5. Complement activity assay

The sera collected at different times were tested for their hemolytic activity according to Mayer's method[25]. Serum complement activities at 2 and 4 h were quantified as relative changes normalized to their initial values at 0 h. The contents of complement components C3 and C4 in the sera collected at 4 h were measured through immunonephelometric method under the instructions of the commercial kits.

2.6. Fever experiment

2.6.1. Model and grouping

Adult male Wistar rats (200–230 g) were housed at least 3 days before experiments under room temperature at 18 ± 2 °C, relative humidity at 55%–60% and a 12-h light-dark cycle. The rectal temperature was determined twice every day. On the day before experiments, each two rats were housed in one cage, fasted for 10 h with free access to water. The rats with stable baseline temperatures were selected for experiment by determining their rectal temperature three times every 20 min, and divided into six groups ($n = 6$): sham group, LPS-induced model group, CHCP (at different doses of 50, 100, and 200 mg/kg) groups, and aspirin (150 mg/kg) group. The drugs were diluted in normal saline for oral administration. After drug administration for 30 min, LPS (60 μg/kg) was injected intraperitoneally (i.p.) to induce fever. The same volume saline (1 mL/kg) without LPS was injected in the sham group. The body temperature was recorded every 30 min from 0 to 6 h after LPS injection. The change of body temperature was calculated as ΔT. The thermal response index (TRI, °C × h) of each group was evaluated as the area under the temperature–time curve, which was calculated by summing the area of the trapezoids under every two data points. The area of each trapezoid from t_i to t_{i+1} under the curve was calculated as $(t_{i+1}-t_i) \times (\Delta T_i + \Delta T_{i+1})/2$. After the rats were anesthetized with 1 g/kg urethane i.p., the serum and blood samples were collected for complement activity assay and leukocyte counts, respectively.

2.6.2. Complement activity assay and the number of leukocytes in peripheral blood

The complement activities of the collected sera, diluted by BBS (1:10), were measured according to Mayer's method[25] as shown in Eq. (1):

$$\text{Hemolysis}\ (\%) = \left[\left(OD_{sample} - OD_{0\%\ lysis}\right) / \left(OD_{100\%\ lysis} - OD_{0\%\ lysis}\right)\right] \times 100 \quad (1)$$

The total number of leukocytes in peripheral blood was detected on the Neubauer's counting chamber.

2.7. Statistical analysis

All quantitative data were expressed as mean ± SD. Statistical analysis was carried out by using SPSS 13.0 (IBM, Chicago, USA). Differences among groups were assessed by a one-way analysis of variance (ANOVA) followed by Dunnett's Test. Non-parametric data were evaluated using the Mann-Whitney test. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Characterization of CHCP and its anti-complementary activities in vitro

CHCP was a dark brown powder and soluble in water. The total carbohydrate, uronic acid and protein contents of CHCP were 77.21 ± 2.84%, 36.69 ± 4.94% and 6.17 ± 1.93%, respectively. HPGPC analysis showed that CHCP contained one major polysaccharide and several minor ones, with molecular weight ranging from 1000 to 5000 kDa (Supporting information Fig. S1). GC analysis indicated that the complex macromolecules were linked by multiple monosacchrides, mainly including glucose, galactose, arabinose and rhamnose in the ratio of 3.40:2.14:1.17:1, together

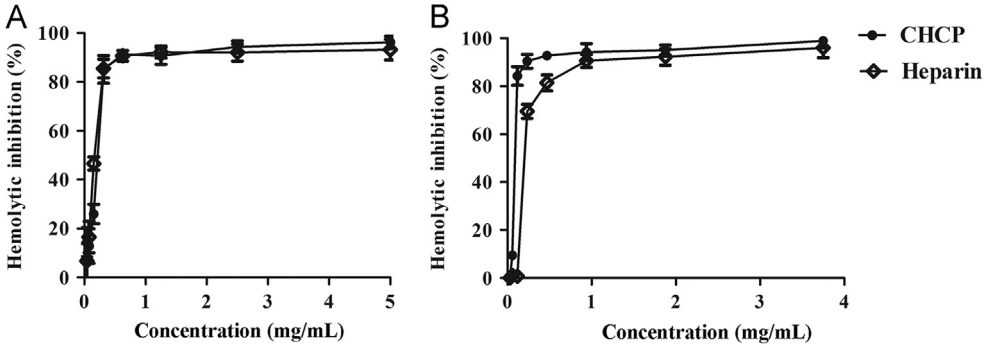

Figure 1 Inhibition of CHCP on the classical pathway (A) and alternative pathway (B) of complement system. Heparin was used as the reference. Results are expressed as percent inhibition of hemolysis. Data were listed as mean ± SD, $n = 3$.

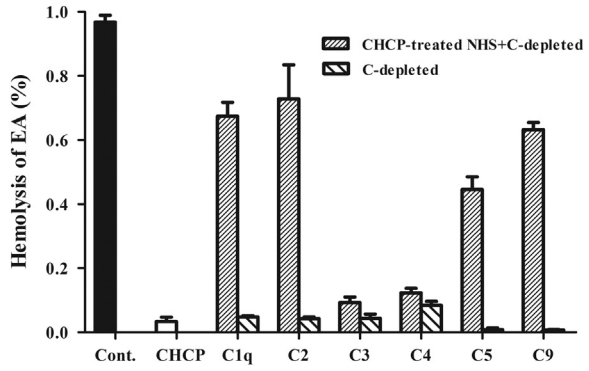

Figure 2 Hemolytic assays for individual components utilizing C-depleted serum. CHCP-treated serum was mixed with various depleted sera, and the capacity of these depleted sera to restore the classical pathway hemolysis was estimated by adding sheep antibody-sensitized erythrocytes. Results were expressed as hemolytic percentages. Data were listed as mean ± SD, $n = 3$.

Table 1 Effects of CHCP on coagulation system.

Sample	Concentration (mg/L)	RT (s)	TT (s)
Vehicle	–	67.88 ± 2.62	121.18 ± 2.87
Heparin	5.5	93.34 ± 4.33***	171.31 ± 3.47***
CHCP	2000	64.14 ± 4.07	121.57 ± 2.72
	1000	67.56 ± 2.87	125.38 ± 3.44
	500	63.57 ± 3.03	124.11 ± 4.92

–Not applicable. Data were expressed as mean ± SD, $n = 3$.
***$P < 0.001$ significantly different from the vehicle.

with trace amounts of mannose and xylose (Supporting information Fig. S2).

CHCP showed stronger inhibitory effect than heparin through classical pathway ($P < 0.001$), while its inhibition on alternative pathway was a little weaker compared with heparin ($P < 0.01$) (Fig. 1). Its CH_{50} and AP_{50} values were 0.092 ± 0.020 mg/mL and 0.209 ± 0.036 mg/mL ($n = 3$), respectively.

The targets of CHCP in the complement activation cascade are indicated in Fig. 2. The 1:8 NHS led to 96.77 ± 2.15% of hemolysis in the complement-control group. CHCP at the concentration of 1.4 mg/mL exhibited a strong inhibitory effect on the hemolysis (3.29 ± 0.67%). The hemolytic percentages induced by all the C-depleted sera were less than 10%, indicating that the C-depleted sera would not lyse erythrocyte independently. After treating with CHCP, the serum depleted of C1q still restored the hemolytic activity to 67.40 ± 4.79%, suggesting that CHCP did not block the complement cascade at C1q. C2-depleted and C9-depleted sera also restored the hemolysis markedly (72.82 ± 10.61% for C2 and 63.21 ± 2.27% for C9). However, CHCP nearly abolished the hemolysis when C3- or C4-depleted serum was added (9.29 ± 1.69% for C3, 12.34 ± 1.39% for C4). For C5, the hemolysis was only partly restored (44.54 ± 3.92%). These results suggested that CHCP mainly block C3 and C4, and may interact with C5.

As shown in Table 1, heparin of 5.5 mg/L markedly prolonged recalcification time (RT = 93.34 ± 4.33 s) and thrombin time (TT = 171.31 ± 3.47 s) compared with the vehicle control (RT = 67.88 ± 2.62 s, TT = 121.18 ± 2.87 s; both $P < 0.001$). However, CHCP, even at the concentration of 2000 mg/L, did not have any effect on the coagulation system (RT = 64.14 ± 4.07 s; TT = 121.57 ± 2.72 s).

3.2. *Effect of CHCP on "two-hit" ALI*

3.2.1. *Histopathological observation and complement activation products deposition*

Histopathological findings of lung tissues with H&E staining showed clear pulmonary structure and little cellular influx in the Sham group, whereas the ALI animals exhibited severe lung injury, including marked interstitial edema, septal thickening, hemorrhage and inflammatory cell infiltration (Fig. 3A). Prednisone was effective in suppressing lung damage. Administration of CHCP (50 mg/kg and 100 mg/kg) significantly attenuated the neutrophil accumulation, alveolar wall thickening, and intra-alveolar exudation. However, serious pulmonary inflammation was still seen in the low-dose group of CHCP (25 mg/kg). The above results were confirmed by the total lung injury scores listed in Table 2.

The result of immunohistochemistry showed very low level of yellow-stained deposit of C3c in the lungs of sham group, but very intense C3c deposit in the model group. Treatment with CHCP (50 and 100 mg/kg) significantly reduced the complement activation products deposit in the lungs of ALI rats (Fig. 3B).

3.2.2. *CHCP attenuated severity of inflammation in the lung and BALF*

As shown in Table 2, the lung W/D ratio in ALI model group was notably higher than that in the Sham group. The lung W/D ratios were significantly reduced in 100 and 50 mg/kg CHCP groups ($P < 0.01$ and $P < 0.05$, respectively), as well as in prednisone group ($P < 0.05$), compared with ALI rats.

Elevated protein concentration in BALF is an important marker of injury to the pulmonary alveolar–capillary barrier. The pulmonary protein exudation was significantly reduced by CHCP at the dose of 50 and 100 mg/kg and by prednisone (Fig. 4), indicating that the treatment ameliorated the alveolar–capillary barrier damage during ALI.

The cells in BALF were classified and counted to estimate the inflammatory responses during ALI. BALF from the Sham animals contained the minimal number of leukocytes, the lowest percentage of neutrophils and the highest proportion of lymphocytes. The development of ALI elicited a massive recruitment of total leukocytes and neutrophils into the lungs. As shown in Fig. 4, the numbers of leukocytes and the percentages of neutrophils in BALF were apparently higher in ALI animals ($P < 0.001$) than those in Sham animals, while the proportion of lymphocytes in BALF was much lower ($P < 0.001$). Administration of CHCP (50 and 100 mg/kg) and prednisone apparently suppressed the accumulation of leukocytes and attenuated lung neutrophilia, as indicated by the reduction in total leukocyte counts and neutrophil percentages in BALF, while they all had good ability in inhibiting the decrease of lymphocytes ($P < 0.001$).

3.2.3. *Effect on SOD activity and MDA content*

As shown in Fig. 4, the pathogenesis of ALI resulted in significant decrease of SOD activity and marked increase of MDA content in comparison with the Sham group ($P < 0.001$). Treatment with prednisone or CHCP at the dose of 50 and 100 mg/kg significantly

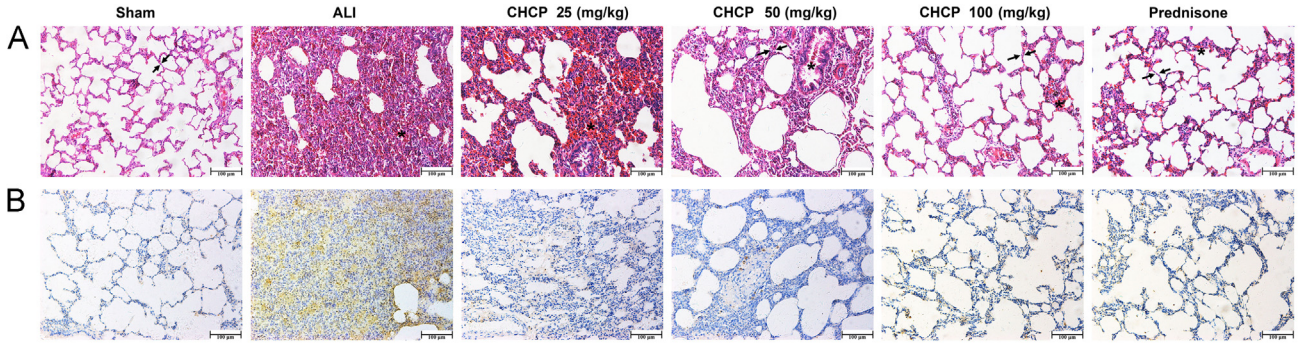

Figure 3 Effect of CHCP on pathological changes of lung tissues in ALI rats (200×). The upper lobe of the right lung was fixed in 4% formalin and 5-μm sections were prepared. **(A)** Stained with hematoxylin and eosin. Alveolar wall was labelled using a pair of arrows. Congestion in lungs was labeled using asterisks. **(B)** Immunochemistry microscopic images of the lung of each group indicated the complement activation products deposit. Representative photomicrograph of each group: (a) Sham group, (b) ALI group, (c) CHCP 25 mg/kg, (d) CHCP 50 mg/kg, (e) CHCP 100 mg/kg and (f) prednisone group.

Table 2 Effects of CHCP on lung wet/dry weight ratio (W/D), and total lung injury scores in ALI rats.

Group	Dose (mg/kg)	W/D ratio	Total lung injury score[a]
Sham	–	$3.62\pm0.78^{**}$	$0.5\ (0–4)^{***}$
ALI	–	5.05 ± 0.73	14.5 (10–15)
CHCP	100	$3.99\pm0.72^{**}$	$4\ (0–9)^{***}$
	50	$4.04\pm0.95^{*}$	$8.5\ (4–15)^{***}$
	25	4.70 ± 0.98	11.5 (9–14)
Prednisone	70	$4.20\pm0.90^{*}$	$4.5\ (4–8)^{***}$

–Not applicable.
Data were expressed as mean±SD, $n=9$ in ALI group, $n=8$ in other groups. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* ALI group, analyzed by ANOVA and Dunnett's Test.
[a]Median (minimum–maximum) tested by Mann-Whitney U-test.

upregulated SOD activity and depressed MDA content in BALF compared with the ALI model group ($P<0.01$ or 0.001).

3.2.4. *Complement activity in sera*

ALI caused a sharp reduction in serum complement activity at 2 h (62.57±10.84%) and at 4 h (40.49±7.92%). Sham animals showed only a slight decrease (86.32±6.56% at 4 h, $P<0.001$) during the experiment. CHCP at the dose of 50 and 100 mg/kg significantly diminished the decrease of serum complement activity both at 2 h ($P<0.05$ and $P<0.01$, respectively) and at 4 h (both $P<0.001$), indicating that CHCP reduced the consumption of the complement components (Fig. 5).

3.3. *Effect of CHCP on LPS-induced fever*

3.3.1. *Body temperature and the TRI*

During the experiment, the body temperature of rats in sham group was maintained at a normal range with minor changes. The body temperature and TRI of the model group were significantly higher than those of the sham group at 30 min and from 2 to 6 h ($P<0.01$ or 0.001), except for a normal period from 60 to 90 min (Fig. 6A).

As shown in Fig. 3A, the CHCP (50, 100 and 200 mg/kg) treatment significantly inhibited the fever induced by LPS from 30 min to 6 h ($P<0.05$, 0.01 or 0.001). Aspirin also inhibited the temperature increase. Similar results were found in TRI (Fig. 6B).

3.3.2. *Complement activity in sera and number of leukocytes in peripheral blood*

As shown in Fig. 6C, complement levels of the model group were significantly decreased compared with those of the sham group ($P<0.001$). LPS injection increased the number of leukocytes in peripheral blood ($P<0.001$) in the model rats (Fig. 6D).

The CHCP (100 and 200 mg/kg) treatment partly restored complement levels in serum ($P<0.05$), but no significant effect was observed in the low-dose group of CHCP (50 mg/kg) and aspirin group. All the CHCP groups (50 mg/kg, $P<0.01$; 100 and 200 mg/kg, $P<0.001$) and aspirin group ($P<0.001$) significantly inhibited the increase in leukocytes in peripheral blood induced by LPS.

4. Discussion

The heat-clearing Chinese medicines are well known for their effects on fever and inflammation of infectious diseases. Our previous studies led to the isolation of a series of complement inhibitors from these medicines, indicating the inhibition on the excessively activated complement system would be a key mechanism for TCM to clear heat[20–23]. In this study, the anti-complementary properties of CHCP were elucidated both *in vitro* and *in vivo*.

The *in vitro* experiments clearly revealed that CHCP is a potent anti-complementary agent affecting both classical pathway and the alternative pathway by selectively blocking the key components C3 and C4 in the complement activation cascade. As demonstrated in the literature, C3 functions to activate both the classical and alternative pathways and makes up 70% of the total protein in the complement system[30]. C4, the second most abundant complement protein in serum, is a glycoprotein which functions as the limited activation step in the activation of the classical pathway[2]. It suggested that CHCP could be effective in treating the complement-associated diseases. ALI is a life-threatening complication of

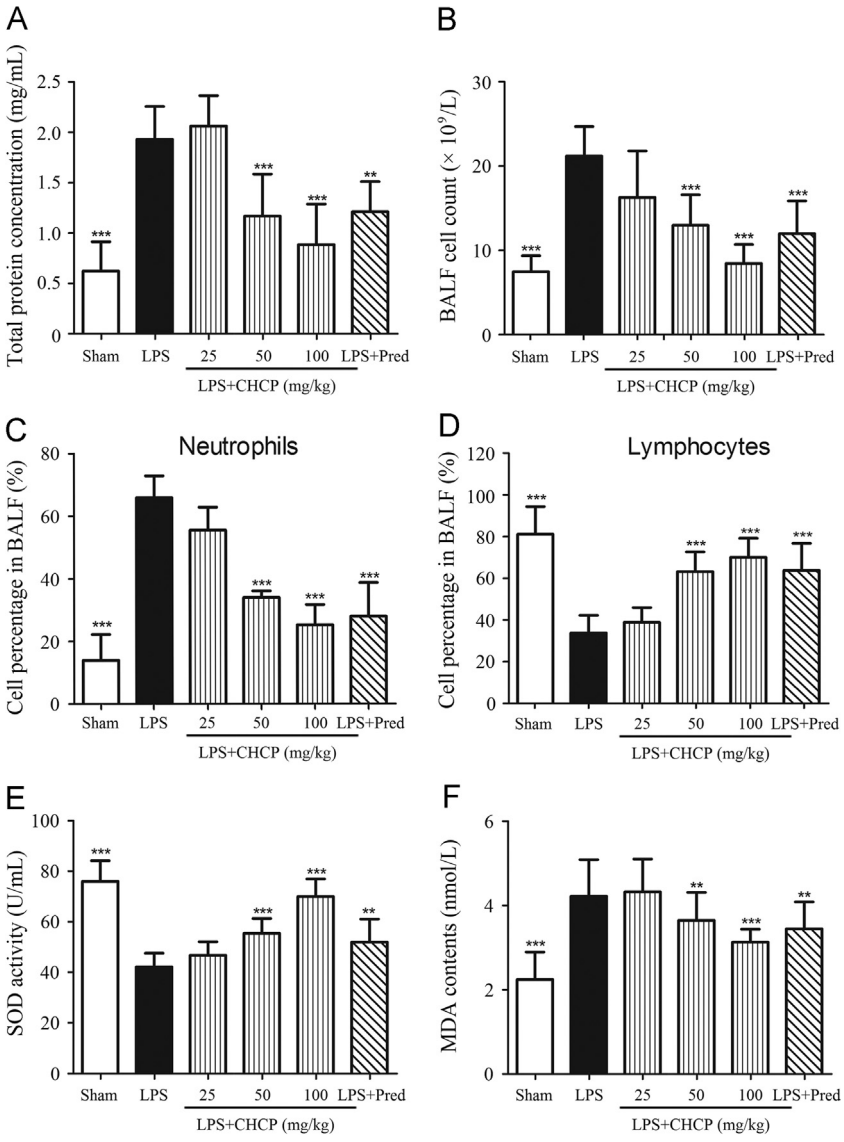

Figure 4 BALF analysis. (A) Protein concentrations in BALF. (B) Number of leukocytes in BALF. (C) Neutrophils percentage in BALF. (D) Lymphocytes percentage in BALF. (E) SOD activity in BALF. (F) MDA contents in BALF. Data were expressed as mean ± SD, $n=9$ in ALI group, $n=8$ in other groups. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with ALI group, analyzed by ANOVA and Dunnett's Test.

various systemic inflammations and of pulmonary disorders that can cause a mortality rate of 30–40%[9]. The current main treatment against ALI has been the application of glucocorticoids, which often lead to severe adverse reactions during the long treatment. Hence, other choice of agents with less adverse effects is crucial to the recovery of ALI. Based on the important role of complement activation in the induction of inflammatory response in ALI/ARDS[3,12], the *in vivo* anti-complementary activity of CHCP and its preventive effect on ALI were examined in the "two-hit" ALI rat model of ischemia/reperfusion (I/R) injury followed by LPS instillation. This "two-hit" ALI rat model had been shown to induce an exaggerated response for more susceptibility to lung injury[24]. I/R is believed to augment neutrophil-mediated lung injury by priming circulating neutrophils for increased superoxide production[31]. The activation of complement system is known to be necessary for the full development of inflammatory responses and tissue injuries associated with I/R in pulmonary injury[3]. Based on I/R, intratracheal instillation of LPS will induce an ideal experimental model of ALI by resulting in pulmonary injury with great BALF neutrophil infiltration[32].

Histological examination revealed marked inflammation, extensive alveolar wall thickness, congestion as well as diffused interstitial infiltration in ALI. The immunohistochemistry showed over-activation of complement in ALI by the abundantly increased complement activation products deposition. The administration of CHCP (especially 50 and 100 mg/kg) significantly improved the histologic changes combined with the inhibition of the over-activation of complement system, the regulation of the oxidant-antioxidant imbalance, as well as reducing the numbers of leukocytes and neutrophils in BALF. The protective effect of CHCP against ALI was also accompanied with a decrease in pulmonary edema indicated by lung W/D weight ratio, and a reduction in protein concentrations in the BALF, which represented a preserved pulmonary epithelial–endothelial barrier function[11].

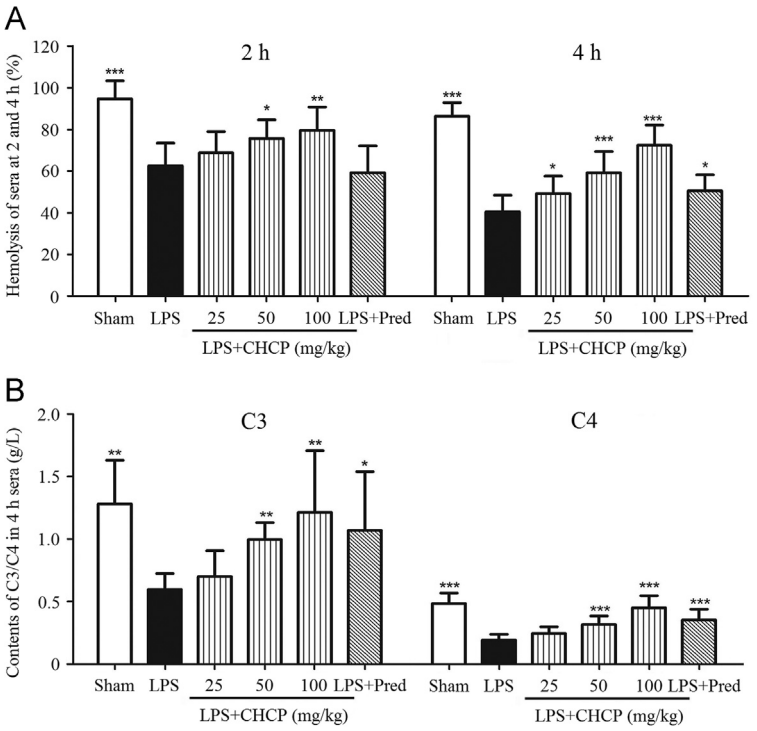

Figure 5 Effect of CHCP on complement activity in the ALI rats. **(A)** The hemolytic activity of the sera obtained at 2 and 4 h. **(B)** Contents of complement components C3 and C4 in serum collected at 4 h. Data were expressed as mean ± SD, $n=9$ in ALI group, $n=8$ in other groups. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with ALI group, analyzed by ANOVA and Dunnett's Test.

Activation of the complement system decreases complement levels in serum, which can be accounted for by the reduction in C3 or C4 concentrations[33]. In the Sham group, minor surgical injuries caused a slight activation of the complement cascade, represented by the minimal decrease of hemolysis of serum. The "two-hit" ALI caused significantly more complement consumption. Oral administration of CHCP (100 and 50 mg/kg) apparently reduced the consumption of complement system. The effects of CHCP on C3 and C4 were consistent with the results of the *in vitro* complement-target test.

The systemic activation of the complement system generates C5-derived chemotactic activity. This causes blood neutrophils to sequester in pulmonary capillaries and dramatically result in the release of toxic oxygen species, which can destroy pulmonary endothelial cells as well as basement membranes[34]. And an oxidant-antioxidant imbalance is thought to underlie as one of the important pathogenesis of lung injury[10]. These findings provide strong evidence that ALI following the over-activation of complement system is closely linked to the production of oxygen metabolites by lung-sequestered neutrophils. In the experiment, the proportions of neutrophils were actually suppressed with the administration of CHCP, suggesting that CHCP potently attenuated the neutrophil respiratory burst. SOD is an important antioxidant enzyme that scavenges the superoxide free radicals[35]. MDA is recognized as a standard measurement for the degree of oxidation[36]. The pathogenesis of ALI resulted in the decreased SOD level and the elevated MDA content. Taking CHCP could reduce the pulmonary oxidative stress to modulate the oxidant-antioxidant balance.

LPS-induced fever is a classic inflammatory heat model, characterized with high body temperature, increased leukocyte and hyper-activated complement system[37]. In this study, both CHCP, the complement inhibitor, and aspirin, the cyclooxygenase inhibitor, significantly decreased the body temperature and leukocytes in peripheral blood of the LPS-induced fever rats. Remarkably, only CHCP (100 and 200 mg/kg) but not aspirin could inhibit the decreased complement level of the fever rats, indicating the characteristic effect of CHCP on complement system *in vivo*.

Our recent bioactivity-guided fractionation on CHCP led to the isolation of three anti-complementary homogeneous polysaccharides with CH_{50} and AP_{50} values not more than 0.3 mg/mL, and similar monosaccharide composition to that of CHCP (data not published). These results further confirmed that the anti-complementary polysaccharides should be the key material basis of the beneficial effects of CHCP on ALI and fever. Composed of large amount of monosaccharides through glucosidic bonds, polysaccharides are too complex to accurately control the quality by one specific analytical method. Multiple methods were used to comprehensively characterize the structure of CHCP, including contents of carbohydrate and uronic acid, molecular weight, and monosaccharide composition.

In conclusion, this study demonstrated that CHCP can ameliorate not only the "two-hit" ALI, but also the LPS-induced fever in rats, both of which are associated with inhibition of the excessively activated complement system. The anti-complementary polysaccharides were shown to play an important role in the heat-clearing effect of *H. cordata*. Moreover, CHCP has no interference with the coagulation system. CHCP could be a promising natural complement inhibitor to be applied in the treatment of complement-associated diseases.

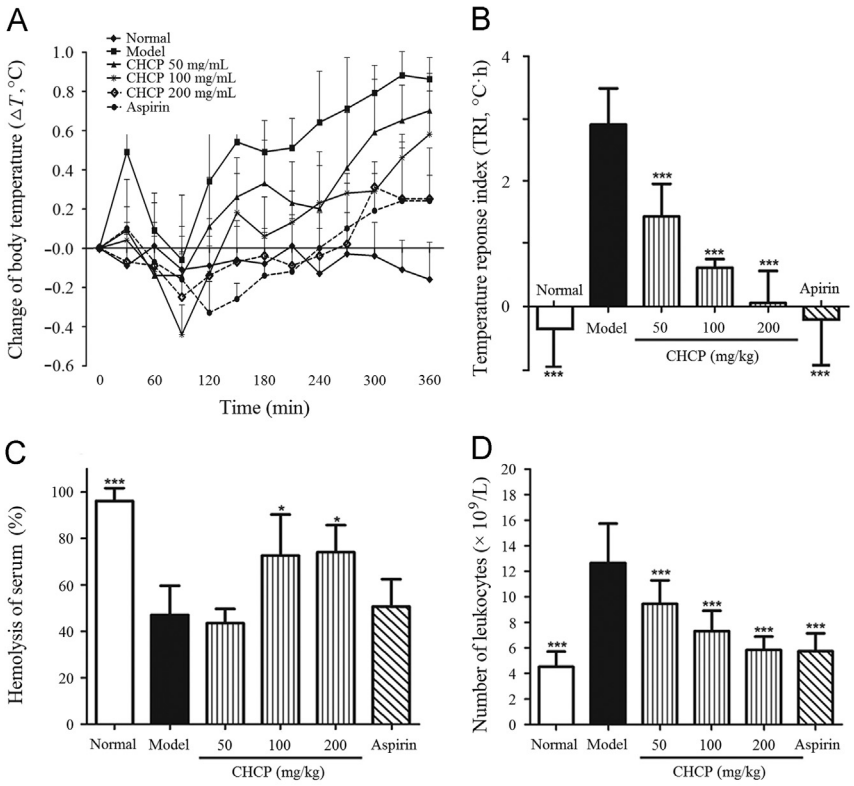

Figure 6 Effect of CHCP on LPS-induced febrile rats. (A) and (B) The change of body temperature and the temperature response index. (C) Serum total hemolytic activity. (D) Count of leukocytes in peripheral blood. Data were expressed as mean ± SD, $n=7$ for CHCP 100 mg/kg and aspirin group, and $n=8$ for other groups. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with Model group, tested by ANOVA and Dunnett's Test.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (81330089, 30925042, and 30801508), and the Ministry of Science and Technology of China (2012ZX 09301001-003).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2017.11.003.

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