Effects of early administration of a novel anticholinergic drug on acute respiratory distress syndrome induced by sepsis

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Summary

Background:
Acute respiratory distress syndrome (ARDS) is the inflammatory disorder of the lung most commonly caused by sepsis. It was hypothesized that treating the lung with penehyclidine hydrochloride (PHC), a new type of hyoscyamus drug, early in the development of sepsis could diminish the lung dysfunction.

Material/Methods:
Sprague-Dawley rats were divided into 4 groups: 1) a control group; 2) a sham-operated group; 3) a cecal ligation and puncture (CLP) group; 4) a PHC-treated group. One hour after CLP surgery, rats were either untreated or treated with PHC via intraperitoneal injection. Lung wet/dry weight ratio, bronchoalveolar lavage fluid (BALF), serum tumor necrosis factor (TNF-α), interleukin 6 (IL-6), interleukin 10 (IL-10), total nitrite/nitrate (NOx), superoxide dismutase (SOD), malondialdehyde (MDA) in lung tissues, and pulmonary functions were examined 24 hour after surgery. Another 60 rats were randomly assigned to 4 equal groups to observe survival status 96 hours after surgery.

Results:
Treatment of PHC markedly decreased TNF-α, IL-6, NOx, SOD, MDA content, protein concentration in BALF, and lung wet/dry weight ratio and enhanced SOD activity (p<0.05), which are indicative of PHC-induced suppression in the pathogenesis of ARDS caused by sepsis. In comparison to group CLP/saline, plasma IL-10 level markedly increased in group CLP/PHC. In PHC-treated groups, the administered PHC had a significant protective effect on the lung dysfunction induced by sepsis.

Conclusions:
We conclude that administration of PHC at the time of a systemic insult can protect the lung from the damaging effects of sepsis.

key words: acute respiratory distress syndrome • penehyclidine hydrochloride • sepsis • inflammatory responses • intervention

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**BACKGROUND**

Acute respiratory distress syndrome (ARDS) is a common, devastating clinical syndrome of acute lung injury that affects both medical and surgical patients. The commonly associated clinical disorders of ARDS can be divided into those that are associated with direct injury to the lung and those that cause indirect lung injury in the setting of a systemic process. Overall, sepsis is associated with the highest risk (approximately 40%) of progression to acute lung injury or ARDS [1].

Oxidative stress is thought to be central to the pathogenesis of ALI/ARDS. Reactive oxygen species (ROS) released from alveolar macrophages and recruited and activated neutrophils can cause injury through interactions with proteins, lipids, and DNA [2]. There is increasing experimental and clinical evidence that a number of cytokines play a major role in the response to injury and infection and in the development of ARDS [3]. Cholinergic signals provide tonic or continuous neurological modulation of cytokine synthesis, functioning as a governor on an engine, that limits the magnitude of the immune response [4]. The efferent arm of the inflammatory reflex, now termed the cholinergic anti-inflammatory pathway, is a highly robust mechanism for cytokine control [5]. Stimulation of the cholinergic anti-inflammatory pathway by pharmacological methods can attenuate the systemic inflammatory response to endotoxin-induced sepsis [6]. Anticholinergics have been used for the treatment of sepsis because they have a pharmacologic action of lifting small blood vessel spasm, improving microcirculation, inhibiting biomembrane lipid peroxidation, and decreasing cytokines and oxyradicals in septic patients [7–9].

However, these drugs exhibit classical antimuscarinic adverse effects, such as dry mouth and accelerated heart rate (HR). Penehyclidine hydrochloride (PHC), a new type of hyoscyamus drug that was developed by the Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, PR China, has both antimuscarinic and antinicotinic activities and retains potent central and peripheral anticholinergic activities [10,11]. Recently, experimental results demonstrated that PHC can inhibit lipid peroxidation, attenuate the release of lysosomes, and depress microvascular permeability [12]. Additionally, it can minimize M2 receptor-associated cardiovascular side effects, owing to selective blocking of M1 M3 receptors and N receptor [11,12].

In an endeavor to elucidate the complex process of ARDS and seek solutions for successful medical or pharmacological intervention, we hypothesized that PHC might have some therapeutic value against ARDS following sepsis. To test this hypothesis, we used an ARDS rat model of sepsis induced by cecal ligation and perforation (CLP). The specific objectives of the current study were to examine the effects of administering PHC early on to influence survival, production of inflammatory mediators, plasma nitrate/nitrite (NOx) levels, tissue superoxide dismutase (SOD) activity, lipid peroxidation, and modulation of survival status.

**MATERIAL AND METHODS**

**General preparations**

We obtained adult male Sprague-Dawley rats that weighed 250–300 g from the Department of Laboratory Animal Center of Centre-South University. We housed the rats in the rooms of the University Laboratory Animal Research Center. The room was maintained at 22±1°C under a 12/12-hr light/dark regimen. Food and water were provided *ad libitum*. All animal experiments were approved by the University Committee for Animal Care and performed in accordance with the national legislation and with the National Institutes of Health Guide regarding the care and use of animals for experimental procedures. PHC (Chengdu List Pharmaceutical Co. Ltd.) was dissolved in sterile pyrogen-free water (1 mg/ml).

**CLP/sham surgery and experimental groups**

ARDS was induced into rats via CLP as previously described [13]. It has been shown that a larger cecal puncture produces a far more severe model of sepsis [14]. The above procedures resulted in a total of 4 experimental groups into which the rats were randomized: 1) a non-anesthetized non-operated control group (group control); 2) a sham-operated group (group sham); 3) a CLP group (group CLP/sham); 4) a PHC-treated group (group CLP/PHC). Briefly, male Sprague-Dawley rats were anesthetized with an intraperitoneal injection of ketamine/xylazine (0.25 and 0.025 mg/kg, respectively). A laparotomy was performed to expose the entire cecum, which then was ligated distally to the ileocecal valve and punctured twice by an 18-gauge needle. The cecum was gently manipulated to extrude a small amount of fecal material and placed back into the abdomen. The abdominal incision was then closed with 4-0 silk suture. Sham-operated control groups consisted of animals that underwent identical anesthetic and laparotomy procedures, but with no manipulation of the cecum. Normal saline solution, 20 ml, was administered subcutaneously as volume resuscitation. The sham/CLP surgeries were performed with the surgeon blinded to the subsequent treatment. In PHC groups, rat were intraperitoneally injected with 0.45-mg/kg dose of PHC 1 h after performing CLP [12]. Sham controls and CLP-alone rats received the same volume of saline.

A femoral vein was catheterized for intravenous administration of fluid or drugs. After the operation, the rats were placed in a special metabolic cage and allowed time for awakening and stabilization. The rats were free to move their heads and had access to food and water but could not escape from the cage. The operation was of short duration. The animals appeared comfortable and were only minimally restrained [15,16].

**Measurement of HRs**

During the period of anesthesia, a femoral artery was cannulated and connected to a pressure transducer (Xinmin Instruments, Xian, Shanxi, China) to record the arterial pressure and heart rate on a polygraph recorder (Shanmei Instruments, Jinan, Shandong, China).
Sample preparation

Blood plasma and lung tissue samples were collected at 24 h after surgery. All blood samples were centrifuged immediately at 2500 rpm for 15 min at +4°C. Plasma supernatants were collected and stored at −20°C. Tissue samples were frozen and stored at −70°C for index assessment.

Measurement of lung wet/dry weight ratio

Wet/dry weight ratios, indicating fluid accumulation, were measured as indexes of lung injury. Lung tissues were taken from the upper and lower lobes, weighed, and then dried to a constant dry weight in an oven that was kept at 80°C for 24 h and weighed again. Lung wet/dry weight ratios were calculated and expressed as relative values.

Bronchoalveolar lavage fluid (BALF) collection and its total protein concentration assay

At 24 h after surgery, rats were anesthetized and exsanguinated, and their tracheas were exposed. Each animal’s trachea was cannulated with a blunt needle that was secured with a silk ligature. BALF collection was performed with 4 ml PBS that was infused and aspirated into each lung 3 times. The lavage fluid was recovered (average fluid recovery was 3.2 ml) and centrifuged at 1000 g for 10 min. The supernatants were removed and stored at −70°C until the total protein concentrations were determined by the method of Lowry [17].

Cytokine analysis

Sixty rats were divided into 4 experimental groups of 15 rats each and blood samples of each group were collected at defined time points of 8 h, 16 h and 24 h. Plasma TNF-α, IL-6 and IL-10 were measured with antibody enzyme-linked immunosorbent assays with a commercial antibody pair, recombinant standards, and a biotin-streptavidin-peroxidase detection system (Boshide, Wuhan, Hubei, China). All agents, samples, and working standards were prepared according to the manufacturer’s directions. The optical density was measured at 450/540 nm wavelengths by automated enzyme-linked immunosorbent assay readers. Cytokines are expressed in ng/mL.

Nitrite/nitrate (NOx) analysis

Blood samples collected at above-mentioned time points were centrifuged at 1000 g for 15 min, and supernatants were stored at −80°C until assayed. NOx level was measured using the Griess reaction [18]. Results are expressed as mmol/L.

Measurement of MDA, SOD

Whole lung was homogenized in 0.9% saline solution using a homogenizer. The homogenate was then centrifuged at 3000 rpm for 10 min at 4°C. The supernatant that was obtained was used for assays of malondialdehyde (MDA) and superoxide dismutase (SOD) activities. MDA content was determined was used for assays of malondialdehyde (MDA) and superoxide dismutase (SOD) activities. MDA content was determined by the thiobarbituric acid method, whereas SOD activity is expressed as units per milligram of protein.

Pulmonary functions: Blood gases and acid-base status

Arterial blood samples (0.5 ml) were taken before surgery and at 24 h after PHC administration. We determined the pH, PaO₂, PaCO₂, arterial oxygen saturation, and PaO₂/FIO₂ with a pH and blood gas analyzer (178 pH, Blood Gas Analyzer, Corning, Essex, UK).

Survival analysis

Another 45 rats were randomly assigned to 3 equal groups to observe survival status at 96 h after CLP. The cumulative survival curve was depicted using the Kaplan-Meier method.

Statistical analysis

Data are expressed as mean ±S.D. Statistical analysis was performed using the SPSS statistical software package for Windows, version 13.0 (SPSS, Chicago, IL, US). Results between the groups were compared using the Kruskal-Wallis test and, if statistically significant, followed by Mann-Whitney U test. Survival data were measured using a Kaplan-Meier model, and overall strata comparisons were made using log rank tests. A probability level of p<0.05 was considered statistically significant.

RESULTS

Heart Rate

Changes in HR in 3 groups of rats over the observation period are presented in Figure 1. The baseline HR was 394.88±23.69 bpm. Sham controls during the first 2 h had no significant differences in HR. CLP/saline rats had significantly higher HR values than corresponding sham controls at 4 h after surgery (p<0.05), whereas fluctuations in HR were relatively stable following the administration of PHC. Rats of the CLP/PHC group increased their heart rate slightly but significantly versus sham controls (Figure 1).

Lung wet/dry weight ratio, Protein Concentration in Bronchoalveolar Lavage

Results of lung wet/dry weight ratio measurements are presented in Figure 2. Treatment with PHC produced a greater effect on nullifying the sepsis-induced changes (Figure 2). Markedly elevated levels of wet/dry ratio and protein in BALF were lowered after administration of PHC (4.34±0.05 vs. 4.68±0.09; 4.60±0.26 vs. 7.36±0.48 mg/dl, each p<0.001). There was no significant difference in lung wet/dry weight ratio (p=0.168) and BALF (p=0.508) between the Control group and the Sham group.

Plasma TNF-α, IL-6, and nitrate/nitrite

TNF-α and IL-6 levels were significantly elevated in the CLP/saline group when compared with Sham group controls at 3 time points (each p<0.001) (Figure 3). PHC treatment effectively inhibited this elevation in systemic cytokine levels. There was a significant difference in plasma
levels of TNF-α (p<0.001) and IL-6 (p<0.001) between the CLP/saline group and the CLP/PHC group as mentioned above. IL-10 levels were significantly lower in CLP/saline rats compared with the CLP/PHC group at all defined time points (p<0.001).

Plasma NOx concentration was significantly decreased in the CLP/PHC group when compared with the CLP/saline group (37.51±3.17 vs. 46.95±6.19 mmol/l, p<0.001) (Figure 3).
The role of PHC on MDA content and SOD activities in lung tissues was investigated, and these data are summarized in Table 1. The lung MDA level in CLP/saline rats was significantly higher than in sham controls (p<0.001). Superoxide dismutase activities were significantly lower in CLP/saline rats compared with sham control rats (p<0.001). Treatment with PHC prevented the marked elevation in MDA levels and reduction in SOD activities (p<0.05).

Blood gases and acid-base status

CLP reduced pH, PaO\textsubscript{2}, arterial oxygen saturation, and PaO\textsubscript{2}/FiO\textsubscript{2}, indicating that CLP caused impairment of pulmonary functions. Treatment with PHC improved the pulmonary functions (Table 2).

Survival status

After 4 days of observation, only 1 rat died from hemorrhage in the sham control group. In the CLP/saline group and CLP/PHC group, mortality rates were 53.3% (8/15) and 40% (6/15), respectively, and the difference was significant compared with sham controls (log rank=8.117, p=0.004; log rank=4.778, p=0.029) (Figure 4). At the same time, mortality was dramatically elevated in the CLP/saline group, while PHC that was administrated 1 h after surgery decreased the mortality rate (40.0% vs. 53.3%). However, there were no significant differences between the PHC-treated group and the CLP/saline group (log rank=0.722, p=0.395) (Figure 4).

### Discussion

ARDS is defined by severe lung dysfunction that involves decreased lung compliance and hypoxemia [19]. The pathophysiology of ARDS is complex and evolves after a variety of possible initiating events, including direct insults to the lung such as acid aspiration and pneumonia, and indirect insults such as sepsis. Extensive research has focused on this issue, and recent studies have shown that additional drugs may improve the outcome of ARDS patients [20,21]. Despite these promising results, however, the morbidity and mortality of patients with ARDS remain high, particularly when it is associated with sepsis. Several studies have shown that one of the mechanisms by which sepsis may contribute to progressive lung dysfunction is via the M, N receptor system [22,23]. Cholinolytics mainly block muscarinic acetylcholine receptors, which show a wide range of biological activities, including reduction of lipid peroxides in vital organs, stabilization of the biomembrane, and protection of cell structure [24,25]. As a new type of anticholine agent, penehyclidine hydrochloride has both antimuscarinic and antinicotinic activities and retains potent central and peripheral anticholinergic activities [10]. Compared with other anticholine agents, the notable advantage of PHC is that it has few M2 receptor-associated cardiovascular adverse effects [10,11].

### Table 1. Changes in MDA content and SOD activity in experimental groups at 24 h after operation.

| Groups       | MDA (nmol/mg protein) | SOD (U/mg protein) |
|--------------|-----------------------|--------------------|
| Control      | 1.13±0.05             | 113.27±5.53        |
| Sham controls| 1.16±0.07             | 109.80±8.90        |
| CLP/saline   | 1.70±0.12*,**         | 74.53±5.76*,**     |
| CLP/PHC      | 1.52±0.13*,**,#       | 81.26±8.38*,**,#   |

Control n=15; Sham, n=15; CLP/saline, n=12; CLP/PHC, n=13; mean ± S.D.). Compared with non anesthetized non-operated controls, * p<0.001; compared with sham controls ** p<0.001; compared with CLP/saline group, *** p<0.05 and # p<0.001.

### Table 2. Pulmonary functions: blood gases and acid-base status.

|            | pH     | PaO\textsubscript{2} (mmHg) | PaCO\textsubscript{2} (mmHg) | SaO\textsubscript{2} (%) | PaO\textsubscript{2}/FiO\textsubscript{2} (mmHg) |
|------------|--------|-----------------------------|-----------------------------|--------------------------|--------------------------------------------------|
| Control    | 7.33±0.03 | 96±2                        | 38±3                        | 98.3±3.0                  | 466±40                                           |
| Sham Controls | 7.33±0.03 | 95±2                        | 40±4                        | 96.4±2.8                  | 453±33                                           |
| CLP/saline | 7.11±0.07*,** | 64±7*,**                   | 53±6*,**                   | 73.9±15.4*,**             | 287±66*,**                                      |
| CLP/PHC    | 7.21±0.06***,1 | 69±8***,1                   | 49±5***,1                   | 82.6±12.1*,**             | 321±51***,1                                     |

(Control n=15; Sham, n=15; CLP/saline, n=12; CLP/PHC, n=13; mean ± S.D.). * p<0.001 vs. group control; ** p<0.001 vs. group sham; *** p<0.05 and ¹ p<0.001 vs. group CLP/saline.
Sepsis-induced ARDS that results from CLP in animals is an accepted animal model that closely mimics the physiological changes that are observed during the progression of systemic sepsis in humans; many investigators believe that this simulates clinical sepsis-induced ARDS more closely than intravenous injection of endotoxin [26]. This animal model revealed that CLP caused systemic sepsis and ARDS, as evidenced by the increases in lung wet/dry weight ratio and protein in BALF and attenuation of pulmonary functions. The sepsis syndromes together represent the leading cause of death in ICUs, with an associated mortality of 30–45%, rising even higher in those who develop ALI/ARDS.

Uncontrolled generation of proinflammatory and potentially autotoxic mediators have been noted in experimental models of ARDS and in clinical settings. Early release of macrophage-derived proinflammatory cytokines, such as TNF-α and IL-6, is important in the pathogenesis of septic shock and ARDS [27–29]. In general, the plasma concentration of TNF-α is correlated both with the severity of sepsis and with the extent of subsequent multiple organ dysfunction syndrome (MODS) [30]. In our study, PHC treatment resulted in significant decreases in proinflammatory (TNF-α, IL-6) levels following CLP. These data suggest that the ability of these rats to produce fewer inflammatory cytokines in response to CLP-induced sepsis may in part account for a significant increase in the survival of ARDS rat. It has been shown [31] that PHC decreases TNF-α generation that is induced by LPS through blocking the activation of NF-kB, one of the most ubiquitous eukaryotic transcription factors, which regulates expression of genes that are involved in controlling inflammatory responses [32]. PHC can significantly decrease brain nuclear factor-kB expression in cerebral I/R injury [33]. The mechanisms by which PPC treatment exerts an inhibitory effect on proinflammatory cytokine levels may involve the suppression of proinflammatory cytokine expression. Our studies found that plasma TNF-α and IL-6 were markedly increased after CLP, whereas after treatment with PHC, plasma TNF-α and IL-6 were significantly lower in CLP/saline rat (p<0.001), demonstrating that PHC can inhibit TNF-α and IL-6 generation.

Severe sepsis and ARDS is associated with increased plasma levels of the NO bioreaction products nitrite and nitrate [34,35]. Mediators that are associated with sepsis-induced ARDS, such as endotoxin and the proinflammatory cytokines IL-1β, IL-2, IL-6, TNF, and interferon have been shown to induce the enzyme-inducible NO synthase and thereby increase NO production in vivo [36]. A small quantity of NO has been shown to be critical to normal physiology, maintaining tissue microcirculation and endothelial integrity, whereas excessive NO has been shown to play a major role in the pathogenesis of MODS in septic shock [37]. Animal experiments suggest that inhibiting the generation of NO can decrease mortality rates of CLP rats [38], indicating that keeping NO low ameliorates organ function in sepsis. In our study, PHC treatment resulted in decreased NOx levels in rats with ARDS. A possible explanation for this observation was that PHC treatment reduced the amount of proinflammatory cytokines in septic rats, and hence, NOx production was reduced.

There now exists a considerable body of evidence for redox imbalance and oxidative stress in human sepsis, demonstrating increased markers of free radical production [2,39]. In ARDS, bacteria and endotoxin directly act on phagocytes and result in lipid peroxide formation and membrane damage in tissues of experimental animals, causing tissue injury [40,41]. Oxidicals can induce lipid peroxidation in cellular and subcellular organelle membranes, causing serious damage to cellular structure and function and, eventually, ARDS. Superoxide dismutase is an enzyme that exists in cells removing oxiradicals, whose variations in activity may represent degrees of tissue injury [42,43]. MDA levels (a marker of lipid peroxidation) also are increased in ARDS patients [44]. In our study, SOD activity in different lung tissues significantly increased, and MDA levels significantly decreased in PHC-treated ARDS rats when compared with the untreated group. These results show that PHC treatment increases antioxidants and has a cytoprotective effect and, hence, could lessen the lung tissue damage that results from sepsis-induced oxidative stress.

It has been shown that a larger cecal puncture produces a far more severe model of sepsis [14]. Mortality in this model is very high (80%), with animals succumbing within 48 h. In our study, the untreated group of animals suffered a mortality rate of 53.3% (8/15 animals). In contrast, PHC treatment was not able to remarkably reduce CLP-induced death during a 96-h observation period (40%, 6/15; P=0.395). Despite the effect of PHC to lower some inflammatory mediators, there is no apparent effect on survival.

The significance of the clinical use of PHC in treatment of ARDS induced by sepsis needs detailed investigation. Therefore, we will further extend our study, especially with respect to the mechanisms of action of PHC on systemic inflammatory responses in the rat model. Studies that are evaluating such mechanisms are currently underway.

**Conclusions**

In conclusion, this preliminary investigation in ARDS rats that were treated with PHC shows an inhibition of inflammatory factor production and suppression of NO expression and lipid peroxidation. PHC might have a potential therapeutic effect to ameliorate the clinical symptoms of ARDS following acute septic complications.

**Conflict of interest**

The authors declare there is no conflict of interest in this study.

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