Effect of penehyclidine hydrochloride on patients with acute lung injury and its mechanisms

LI Bai-qiang 李百强, SUN Hai-chen 孙海晨*, NIE Shi-nan 聂时南, SHAO Dan-bing 邵旦兵, LIU Hong-mei 刘红梅 and QIAN Xiao-ming 钱晓明

【Abstract】Objective: To assess the effects of penehyclidine hydrochloride on patients with acute lung injury (ALI), to observe the expression of Toll-like receptor 4 (TLR4) on the peripheral monocytes of ALI patients and changes of inflammatory & anti-inflammatory cytokines and to investigate the mechanism of TLR4 in ALI.

Methods: Forty-five patients with ALI were randomly divided into penehyclidine hydrochloride treatment group (P group, n=21) and conventional treatment group (control group, C group, n=24). Patients in both groups received conventional treatment, including active treatment of the primary disease, respiratory support, nutritional support and fluid management therapy, while those in P group were given penehyclidine hydrochloride (1 mg, im, q. 12 h) in addition. The TLR4 expression of 20 healthy volunteers were detected. The clinical effect, average length of stay in ICU and hospital, values of PaO$_2$ and PaO$_2$/FiO$_2$, expression of TLR4 on the surface of peripheral blood mononuclear cells and some serum cytokines were evaluated for 48 h.

Results: The general conditions of the two groups were improved gradually and PaO$_2$ increased progressively. Compared with 0 h, PaO$_2$ and PaO$_2$/FiO$_2$ at 6, 12, 24 and 48 h after treatment were significantly increased (P<0.05). The improvement in P group was obviously greater than that in C group (P<0.05). The average length of hospitalization showed no difference between the two groups, but penehyclidine hydrochloride significantly decreased the average length of stay in ICU (t=3.485, P<0.01). The expression of TLR4 in two groups were both obviously higher than that of healthy volunteers (P<0.01). It decreased significantly at 24 h (t=2.032, P<0.05) and 48 h (t=3.620, P<0.01) and was lower in P group than in C group. The patients who showed a higher level of TLR4 expression in early stage had a worse prognosis and most of them developed acute respiratory distress syndrome (ARDS). The incidence of ARDS was 23.8% in P group and 29.17% in C group at 24 h. Until 48 h, there were other two patients developing ARDS in control group. Serum IL-1, IL-8 and TNF-α expressions reduced after 24 h in both groups. The reduction in P group was more obvious than that in C group (P<0.05). IL-13 increased gradually from 0 h to 24 h, and decreased slightly at 48 h, which showed no difference between two groups (t=1.028, P>0.05).

Conclusions: Penehyclidine hydrochloride improves the arterial oxygen pressure, down-regulates the expression of TLR4 and restrains the inflammatory cytokines in the downstream of TLR4 signaling pathway. It prevents the development of ALI and can be considered as an important drug in ALI treatment.

Key words: Penehyclidine; Acute lung injury; Wounds and injuries; Toll-like receptor 4

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is a severe clinical syndrome caused by many pulmonary and extrapulmonary factors. The definition of this syndrome was clarified in the American-European Consensus Conference in 1992. The term “acute lung injury” has been used as an umbrella term for hypoxemic respiratory failure, a severe version of which is ARDS. Pulmonary alveoli epithelial cells and pulmonary capillary injury are the main pathophysiologic characteristics, but the pathogenesis is still unclear. At present, lots of researches show that over-expression of cytokines and their interactions are the root causes of ALI/ARDS. Regulating the expression of cytokines and blocking the cascade of inflammatory medium are thus critical. Recently, many studies find that the newly discovered Toll-like receptor 4 (TLR4) can regulate the cytokine
network in inflammatory response, which plays an important role in the process of ALI/ARDS. This study evaluated the protective effect of penehyclidine hydrochloride, a new type of receptor-selective anticholinergic medicine, on patients with ALI, investigated the expression of TLR4 on peripheral monocytes and expression change of inflammatory & anti-inflammatory cytokines, and explored the mechanism of TLR4 in the pathogenesis of ALI.

METHODS

Patient enrollment

From September 2007 to August 2008, 547 patients with a high risk of ALI were admitted in our hospital. The following international diagnostic criteria of ALI were used: (1) acute onset of symptoms, (2) PaO$_2$/FiO$_2$ $\leq$ 300 mm Hg, regardless of positive end-expiratory pressure level, (3) bilateral infiltrations on frontal chest radiograph, (4) pulmonary arterial wedge pressure $\leq$ 18 mm Hg or no clinical evidence of left atrial hypertension. Patients who met any of the following criteria were excluded from this trial: (1) diagnosed as having ARDS (PaO$_2$/FiO$_2$ $\leq$ 200 mm Hg); (2) age $>$ 65 years or $<$ 16 years, (3) with a history of chronic lung diseases or heart dysfunction, (4) liver and kidney functions damaged in the past, (6) having contraindication in using penehyclidine hydrochloride. After dynamic monitoring of physiological indexes, chest imaging and arterial blood gas analysis, a total of 45 patients were involved in this randomized prospective clinical trial. This research was approved by the Hospital Ethics Committee.

Grouping

First, we selected a starting number in the random number table, and then selected a set of random numbers after the starting number. The order of hospitalization of all patients was corresponded with the random numbers. The odd array was taken as penehyclidine hydrochloride group (P group), and the even array taken as the control group (C group). Patients who died within 48 h in the two groups were excluded. Finally, there were 21 patients, 16 males and 5 females with the mean age of 42.33 years, in P group and 24 patients, 18 males and 6 females with the mean age of 41.50 years, in C group. The main causes were trauma in 21 cases, cerebrovascular accidents in 8 cases, inhalation of vomitus in 6 cases, severe infection in 5 cases, severe acute pancreatitis in 3 cases, and ventilator-associated lung injury in 2 cases. Analysis of variance showed no significant difference in age, gender, heart rate (HR), distribution of causes and respiratory frequency at 0 h between the two groups ($P$>0.05).

Management

In C group, patients received the generally accepted conventional treatment for ALI: active treatment of the primary disease, respiratory support including oxygen therapy, noninvasive ventilation and invasive ventilation, nutritional support and fluid management therapy. Patients in P group received intramuscular injection of penehyclidine hydrochloride (1 mg, 1/12 h) besides the conventional treatment.

Parameters monitored

Acute physiological indexes, such as vital signs, blood routine, blood biochemical parameters, arterial blood gas analysis, electrocardiogram, inspired oxygen concentration and changes of general conditions were recorded within 48 h. For all patients, arterial blood gas analysis and serological test were performed at the 5 time points of 0, 6, 12, 24 and 48 h after ALI was confirmed. The venous blood was injected into the anticoagulated tube and centrifugated for 15 minutes at 3000 r/min. Then the supernatant was preserved at -70$^\circ$C to determine cytokines, including tumor necrosis factor-$\alpha$, IL-1, IL-8 and IL-13 by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA, Rapidbio Lab, USA) following the instructions strictly.

The expression of TLR4 on the surface of peripheral blood mononuclear cells was detected by flow cytometry (FCM) at 0, 24 and 48 h. Anti-human TLR4 (10 $\mu$l, eBioscience Company, USA) phycoerythrin (PE) was added into 100 $\mu$l anticoagulant blood. The mixture was incubated for 30 minutes in the dark at room temperature. And 10 $\mu$l mouse immunoglobulin G (IgG)-a-PE (eBioscience Company, USA) was added into 100 $\mu$l anticoagulant blood, acting as the control. Thereafter hemolytic agents of 0.5 ml (BD Company, USA) was added into the sample and stayed at room temperature for 10 minutes. Then vortex oscillator was used and the sample was centrifugated at a speed of 1500 r/min for 5 minutes. Then we washed it with phosphate buffered saline (PBS, 0.01 mol/L, pH=7.4) twice. By using the 488 nm exciting light from the FACSCalibur flow cytometer and setting an APC-CD14 positive gate, necrotic cells and debris were removed. Cells in the gate
were CD14 monocytes. The fluorescence intensity of the TLR4-PE located on the surface of monocytes was detected.

**Statistical analysis**

Data were expressed as mean±standard deviation (x±s). We used SPSS (Version 17.0) to analyze all the data. Student’s t test was used for measurement data and χ² test for count data and paired data. Fisher exact probability test was used to compare all the rates of two groups. Significance level was set at P<0.05.

**RESULTS**

**General conditions and prognosis**

Of all patients, HR, respiratory rate (RR) and acute physiology and chronic health evaluation (APACHE) II score were improved at 24 and 48 h, and there was no significant difference between the two groups (P>0.05). At 24 h, the incidence of ARDS in P group was 23.8 % (5/21), while 29.17% (7/24) in C group. The other two patients in C group developed ARDS between 24 h and 48 h. There was no newly-developed ARDS in P group during this time period (Figure 1). In C group, the average length of stay in hospital and ICU was (16.79±4.63) days and (5.54±1.29) days respectively, while in P group, it was (15.33±3.87) days and (3.95±1.76) days respectively. There was no significant difference in the average length of stay in hospital between two groups, but the data showed that penehyclidine hydrochloride shortened the period of ICU stay significantly (t=3.458, P<0.01, Figure 2).

**Arterial blood gas analysis**

At 0 h, patients presented with hypoxia, the oxygenation index PaO₂/FiO₂ was between 200-300 mm Hg and there was no statistical difference between two groups. Then PaO₂ increased gradually and the increase in P group was more obvious than that in C group at 6 h. At 24 and 48 h, PaO₂ was improved significantly, compared with 0 h (P<0.05, Figure 3). PaO₂/FiO₂ showed the same increase trend and it was improved more significantly in P group than in C group at 6, 24 and 48 h (P<0.05, Figure 4).

**Detection of TLR4 expression on the surface of peripheral blood monocytes**

Totally 1.0×10⁴ monocytes were obtained according to the CellQuest software (BD Company, USA, Figure 5). TLR4 expression of 20 healthy volunteers was (0.41±0.27) mean fluorescence intensity (MFI). TLR4 detected at 0 h showed no significant difference between P group and C group (P>0.05), but both of them differed significantly from that of healthy controls (P<0.01). Compared with 0 h, TLR4 of two groups decreased at 24 and 48 h (P<0.05), and the decrease in P group was greater than that in C group (t=2.032, P<0.05 at 24 h; t=3.620, P<0.01 at 48 h, Figure 6).

**Change of serum cytokines**

Serum inflammatory & anti-inflammatory cytokines showed no statistical difference between two groups at 0 h (P>0.05). Concentrations of TNF-α, IL-1, and IL-8 decreased gradually in both groups and there was a significant difference between the two groups at 24 and 48 h (P<0.05). IL-13, one anti-inflammatory cytokine, in two groups, increased from 0 to 24 h, and decreased slightly at 48 h. At 24 h and 48 h it was significantly different from that at 0 h (P<0.05), but there was no statistical difference between the two groups at the same time points (P>0.05) (Figure 7).
DISCUSSION

Nowadays ALI is usually regarded as a part of the systemic inflammatory process, particularly injuries of the chest and systemic sepsis. The lung and other tissues show widespread destruction of the capillary endothelium, extravasation of protein rich fluid and interstitial edema. In addition, the alveolar basement membrane is damaged and fluid seeps into the airspaces, which stiffens the lung and causes ventilation-perfusion mismatch.\(^3,5\)

TLR, a novel family of receptors, has been demonstrated to participate in the acute inflammatory process.\(^4\) Among the family members, TLR4 usually binds to lipopolysaccharide (LPS), mycobacterial components, cryptococcal capsule and lipoteichoic acid.\(^7-10\) It can activate innate immunity and take part in the specific immune response. TLR4 acts as an important connection between the innate and acquired immunity\(^10\) and promotes the body to secrete a large number of cathepsin, protease, TNF-\(\alpha\), IL-1, IL-6, IL-8 and other inflammatory cytokines to promote the pathological process of ALI/ARDS.

Effects of penehyclidine hydrochloride on patients with ALI

Penehyclidine hydrochloride is a new type of anticholinergic medicine which has both anti-muscarinic and anti-nicotinic activities and it retains a potent central and peripheral anti-cholinergic activities. Penehyclidine hydrochloride now has been widely used in China as an antagonist of organophosphate poisoning. It has the characteristics of M-cholinergic receptor subtype selectivity. Xiao and his colleagues\(^11\) found that penehyclidine hydrochloride had little or no effect on M2 subtypes receptors in isolated tracheal and lung smooth muscles of guinea pig and could be used in asthma and chronic obstructive pulmonary disease management.\(^12\)

When ALI/ARDS occurs, increase of cholinergic receptors density and airway resistance, hypertonia of vascular and airway smooth muscles, and airway hyperresponsiveness will appear. Patients usually present with increased RR and airway secretions, cyanosis, and restlessness in clinic. Shen et al\(^13\) found that a high dose of penehyclidine hydrochloride could significantly inhibit the increase of ALI pulmonary permeability using a LPS-induced ALI rat model. Electron
microscopy showed that damage of pulmonary microvascular endothelial cells and basement membrane were reduced obviously compared with the control group. Thus they speculated that penehyclidine hydrochloride could inhibit the increase of lung permeability by reducing injury of the microvascular barrier in rats. In recent years, more and more researches found that penehyclidine hydrochloride can be used to treat ALI because it can improve the microcirculation, reduce the permeability of capillary, exert a cell protective effect and reduce the lysosome release.

Our study observed that the HR, RR, and APACHE II score of patients in the two groups were improved after treatment for 48 h. PaO2 and oxygenation index of both groups increased gradually from 0 h, and the increase was greater in P group than in C group at 6 h. This suggests that the application of penehyclidine hydrochloride can improve the anoxia and respiratory function in patients with ALI at an early stage. The HR and RR decreased gradually, and there was no significant difference between the two groups, suggesting that penehyclidine hydrochloride does not increase the HR in ALI patients.

Traditional cholinergic receptor antagonists would increase HR, and thus increase the myocardial oxygen consumption, which limits their application in the treatment of ALI. Penehyclidine hydrochloride, which does not have this side effect, is expected to be a potential choice for ALI management. In this study totally 14 patients developed ARDS and the incidence was higher in C group than in P group at 24 and 48 h but there was no statistical difference between the two groups. This may be due to the small size of sample. In the end, 4 patients in C group died and only one in P group died. The main causes of death were severe hypoxemia and multiple organ failure.

Changes of serum anti-inflammatory & inflammatory cytokines and TLR4 expression on the peripheral blood mononuclear cells in ALI patients after treatment

TLR4 was found by Janewary and Medzzhitov in 1997. It is a type-I transmembrane protein and mainly expressed in the placenta and lung tissues. After binding with exogenous and/ or endogenous ligand, TLR4 activated NF-κB-induced inflammatory cytokine secretions through a series of signal transduction pathways. We found that TLR4 expression in peripheral blood mononuclear cells was much lower in healthy people than in ALI patients (P<0.01). In this trial, the TLR4 expression in two groups declined after treatment, and the decrease in P group was greater than that in C group at 24 h and 48 h. This showed that penehyclidine hydrochloride intervention reduced TLR4 expression in the peripheral blood of ALI patients, but whether it enhanced the negative regulation of the body or there existed other mechanisms needs to be confirmed by further studies.

Our trial found that the concentrations of inflammatory cytokines including TNF-α, IL-1 and IL-8 decreased after 0 h in two groups. The highest values of inflammatory cytokines were detected at 0 h. Because the second time point was set at 6 h, the peak of inflammatory cytokines would be between 0 h and 6 h. Decline of TLR4 expression and inflammatory cytokines was consistent with the changes of clinical manifestations, which proves that the inflammatory response is the core of ALI. This provides a more solid clinical evidence for the theory of excessive inflammatory response. Penehyclidine hydrochloride inhibited the expression of TLR4 and inflammatory cytokines in the downstream of TLR4 signaling pathway, but the expression of anti-inflammatory cytokines did not show differences between the two groups. Thus we can infer that penehyclidine hydrochloride inhibit the expression of inflammatory cytokines by down-regulating TLR4 but not by increasing anti-inflammatory IL-13.

Mechanisms of TLR4 in the pathogenesis of ALI

The relationship between TLR4, an endotoxin receptor, and LPS-induced ALI has been explored by several studies. Togbe and his colleagues found that TNF-α expression, microvascular and alveolar epithelial injury, respiratory protein leakage, lung micro-structural damage and TLR4 gene dosage were positively correlated through an animal experiment of LPS-induced ALI on rats. It is reported that the degree of inflammatory response depends on the level of TLR4 expression. Our finding is in accordance with this view: TLR4 expression declined gradually with the improvement of ALI. We also found that patients who showed high expression of TLR4 at early stage (0 h) had poor prognosis. Among them, ALI progressed rapidly; the typical clinical manifestation of ARDS and hyaline membrane formation could be observed by bronchoscopy. Five pa-
tients with TLR4 higher than 20.8 MFI at 0 h all developed ARDS within 48 h and finally 4 died. Many foreign scholars have reported that TLR4 is closely related with various diseases because it has lots of ligands, which are mainly related to inflammatory responses mediated by TLR4. TLR4 can recognize not only the Gram-negative bacteria lipopolysaccharide but also the mycobacterium tuberculosis, aspergillus, cryptococcus neoformans, candida albicans and other pathogenic micro-organisms with molecular patterns, as well as endogenous molecules.

Apart from this, when severe non-infectious pulmonary inflammation happens, though without infection or invasion of pathogenic bacteria, abundant endogenous ligands could still trigger TLR4, activate macrophages and release large number of inflammatory mediators, thus bring the body into a state of excessive inflammatory response.

At present, mechanical ventilation is still the main clinical treatment of ALI/ARDS and the medication is disappointing. Some drugs that were once reported to have a potential protective effect on ALI, such as glucocorticoid, pulmonary surfactant, NO, prostaglandin E1, cytokine antagonists, monoclonal antibodies, etc, are not recommended now. In this study, we used penehyclidine hydrochloride, a new type of receptor-selective cholinergic receptor antagonist, to treat ALI and achieved a good clinical effect. We found that penehyclidine hydrochloride could increase the vascular smooth muscle spasm, inhibit respiratory gland secretion and improve oxygenation at an early stage, inhibit the expressions of TLR4 and inflammatory cytokines in the downstream of TLR4 signaling pathway significantly, and have a protective effect on patients with ALI.

TLR4 is only a member of the Toll-like receptor family, so we cannot preclude that other TLRs may play an uncertain role in the process of ALI. Maybe there exists coordination or antagonism among those TLRs in the ALI pathogenesis, but their specific distribution in the lung and the variety of ligands make TLR4 play an indispensable role in this process. We recommend TLR4 as a marker to determine the prognosis of ALI.

**Conclusion**

Penehyclidine hydrochloride can improve the arterial oxygen pressure, down-regulate TLR4 expression and restrain inflammatory cytokines in the downstream of TLR4 signaling pathway. This inhibitory action is accomplished by down-regulating TLR4, but not by heightening the anti-inflammatory factor of IL-13. Penehyclidine hydrochloride can prevent the development of ALI, so we suggest that it be considered as an important medicine in ALI treatment. TLR4 plays a significant role in the pathogenesis of ALI, and it is recommended that TLR4 expression be a prognostic sign.

**REFERENCES**


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