PROTECTIVE EFFECTS OF NICOTINE AGAINST SEPSIS IN MICE AFTER A CLP VIA ACTIVATING CHOLINERGIC ANTI-INFLAMMATORY PATHWAY

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ABSTRACT

Objective: *To investigate the effects of nicotine on sepsis in mice.*

Methods: *Sepsis was induced in female Kunming mice with cecal ligation and puncture operation (CLP). The experiment group were administered nicotine (400μg/kg) 30 minutes before and three times daily for 3 days after sepsis induction. Survival of mice was observed up to 7 days after CLP in a total of 65 mice. In a separate experiment, the severity of the sepsis was evaluated and lung and liver histopathological examinations were performed (n=18). In the third experiment (n=120), we examined the temporal levels of proinflammatory cytokines (TNF-α and IL-1β) in mouse plasma corresponding to varying doses (40-400 μg/kg) of nicotine administered.*

Results: *Nicotine (400μg/kg) significantly increased the survival rate of CLP mice compared to those of the control group (56.7% vs. 20%; P<0.01). It could attenuate sepsis severity (15.00±3.58 in experiment group vs. 21.00±2.37 in CLP group; P<0.05) and decrease the lung wet/dry weight ratio (2.30±1.03 vs. 2.71±1.58 in CLP group; P<0.05). It could also ameliorate hepatic and lung damage. Six hours after administration, nicotine (400μg/kg) significantly decreased the serum levels of TNF-α (531.79 ± 8.14 vs. 731.11 ± 42.56 in CLP group; P<0.01) and IL-1β (245.19 ± 35.03 vs. 556.12 ± 7.08 in CLP group; P<0.01).*

Conclusions: *Nicotine has protective effects against sepsis in mice, and it could be attributed to its activation of cholinergic anti-inflammatory pathway to inhibit the production of inflammatory cytokines.*

Keywords: *Nicotine, Cholinergic anti-inflammatory pathway, Sepsis, Tumor necrosis factor, Interleukine-1β.*

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Introduction

Systemic inflammatory response syndrome (SIRS) is the major pathophysiologic basis of sepsis. The over-response of the innate immune system to infection results in the production of excessive proinflammatory cytokines, including tumor necrosis factor- α(TNF-α) and interleukin-1β （IL-1β). High levels of these cytokines contribute to the damage of kidney, liver, lung and other organs[(1)]. The blockade or antagonist of some inflammatory mediators have successfully attenuated sepsis in established animal models, such as antagonists of HMG1[(2)]. However, the majority of clinical trials of so-called mediator-directed therapy in critically ill patients with sepsis failed to demonstrate the protective effect of this method[(3)]. Thus, further experimentations will be required to determine the pathogenesis of sepsis.

Recent studies confirmed a close relationship between cholinergic nervous system and innate immune system. The vagus nerve releases acetyl-

choline, a neurotransmitter which will combine with α7 nicotinic acetylcholine receptor (α7nAChR) to activate intracellular signal transduction, which in turn inhibits the release of pro-inflammatory cytokines[4-5]. Therefore, researchers have proposed the concept of "nicotinic anti-inflammatory pathway"[6]. Accumulating evidence indicated that nicotine significantly reduced blood proinflammatory cytokines levels to enhance the survival of model animals[7]. However, Leite et al.[8] indicated that repeated nicotine administration did not alter the survival of sepsis rats. The exact molecular mechanisms are still controversial. In this study, we activated cholinergic anti-inflammatory pathway to investigate the effects of nicotine on sepsis mice induced by CLP.

Materials and methods

Kunming mice (female, 20-25 g) was provided by Laboratory Animal Center, Medical College of Nanchang University. Nicotine was purchased from Sigma-Aldrich (Munich, Germany). TNF-α(m) ELISA Kit (EK0527) and IL-1β (m) ELISA kit (EK0394) were from Boster (Wuhan, China).

Cecal Ligation and Puncture(CLP)

CLP was performed as previously described[9]. In short, female Kunming mice had been fasting for 12 hours before operation, and were intraperitoneally anesthetized with mass fraction of 7% chloral hydrate (350 mg/kg). The cecum was exposed and isolated through a 1.0- to 1.5-cm incision in the left lower abdomen, ligated with a 4-0 silk suture just distal to the ileocecal valve, and punctured twice with a 12-gauge needle puncture about 1.0-cm away its opposite ends, then pressed lightly to extrude a little amount of feces from the perforation site. Following this, the cecum was returned to abdominal cavity, and the abdominal incision was closed in two layers.

Afterward, the mice received normal saline solution (3 ml/100 g) subcutaneously to compensate fluid loss meanwhile, was injected intramuscularly with penicillin (2×104 U) to prevent incision infection, and then were allowed to eat and drink freely. Sham-operative mice served as a control group that had laparotomy but no ligation or puncture was performed. This study was approved by Institutional Animal Care and Use Committee.

Experimental protocols

There are three experiments. The experiment number 1, at 30 minutes prior to CLP, A total of 65 mice received nicotine (400 μg/kg, ip) or saline vehicle of the same voloume at random, and were randomly assigned into three groups: sham (n=15), CLP (n=20), nicotine 400 ug/kg (n=30). The mice receiving nicotine prior to CLP continued to received nicotine (400 μg/kg, 3 time daily, for a duration of 3 days). The sham group of mice not receiving CLP was included as an additional control. The second experiment, a total of 18 mice also assigned into three groups averagely: sham (n=6), CLP (n=6), nicotine 400 ug/kg (n=6). The third experiment, a total of 120 mice were randomly assigned into five equal groups: Sham, CLP, nicotine 40 μg/kg, nicotine 200 μg/kg, and nicotine 40 μg/kg. Nicotine was administered intraperitoneally 30 minutes before sepsis induction. Mice in Sham and CLP groups received equal volume of 0.9% saline 30 minutes before operation.

Survival analysis

In the first experiment, survival was observed up to 7 days after CLP to analyze the mean survival time and the overall survival rates.

Observation of general condition and gross examination

In the second experiment, after the surgery, the diet and activities of mice were recorded and whether they had some clinical signs (for example: malaise, chills, piloerection, diarrhea, purulent urine) were observed. 20 hours after surgery, the mice were sacrificed so as to observe the pathological changes of critical organs.

Measurement of sepsis severity

In the second experiment, the right lung tissue was taken out of thoracic cavity to weigh, and dried to a constant weight in an oven for 24h at 65°C and weighed again. Then wet/dry weight ratios of lung were calculated and were used to indicate lung injury. Severity of the sepsis was evaluated by the score scale of sepsis severity(10) in mice from seven aspects of the conditions including general, intestines, ascites, the cecum, encapsulated lesions, the measurement of liver volume, and lung wet/dry weight ratio.

Histopathological examinations

The liver and lung tissue samples were collected at 20 hours after surgery, were frozen and stored at -70°C for histopathological examination. All the tissues were fixed in 4% formaldehyde, processed routinely for paraffin embedding, and stained with hematoxylin-eosin stains. Then, a light microscope was used for observing the histopathology feature.

Enzyme-linked immunosorbent assay (ELISA)

After surgery, the mice were sacrificed at different time intervals (1, 2, 4, and 6 h) respectively, the blood sample was collected from mice by pick of eyeball, and immediately separated by centrifugation (2000g, 20min). Then the plasma were divided into aliquots and stored at -70°C until being analyzed. The plasma levels of TNF-α and IL-1β was measured according to the manufacturer's instructions, respectively. Each test specimen and standard product was performed in duplicate. CurveExpert 1.3 software was used to draw the standard curve and calculate the concentration of plasma cytokines TNF-α and IL-1β (pg/ml).

Statistical analysis

Data were presented as mean±SD. By using SPSS version 17.0, comparison in multiple groups was performed with one-way ANOVA, and pairwise comparison among groups was conducted with SNK-q test. The overall survival rate, a percentage of survivors base on the total number of mice at a specified point in time after treatment, was analyzed to compare the inter-group differences by using survival analysis, and the survival curve was depicted by Graphpad prism v5.0. A significant difference for the test was presumed at $P<0.05$.

Results

Survival status

Survival curve, as presented in Figure 1, shows the overall survival rate in three groups of mice after 7 days. 7 days after surgery, all mice in sham group survived. However, 16 of 20 mice died in CLP group and 13 of 30 mice died in nicotine(400 μg/kg) group, respectively. The overall survival analysis showed that nicotine (400 μg/kg) significantly increased survival rate (56.7% vs. 20% in CLP group; P=0.0026). Compared to CLP group, nicotine (400 μg/kg) lowered the mortality rate to 43%. Within the observation of 7 days, the mean survival time in sham group was 168 hours; as compared to 18 hours in CLP group and 144 hours in nicotine (400 μg/kg) group.

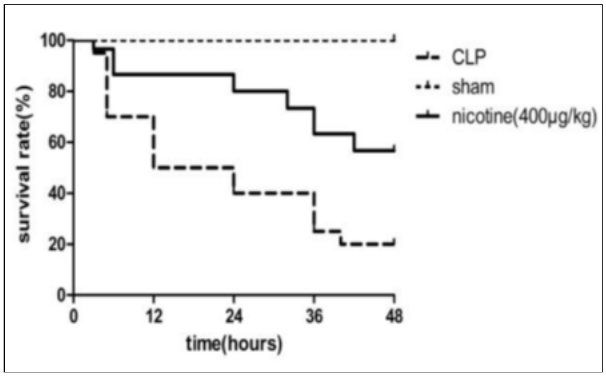

Fig. 1: Survival curve in three groups of mice over the observation of 7 days. At 24, 36, and 48 hours after surgery, the overall survival rate of sham group were 100%, 100%, 100%; CLP group, 40.0%, 25.0%, 20.0% ; Nicotine (400 μg/kg) group, 80.0%, 63.3%, 56.7%, respectively.

General condition and gross examination

Compared to mice in sham group, mice in CLP group exhibited symptoms of prolonged reviving time after anaesthesia, less activity, lethargy, malaise, poor appetite, weight loss, diarrhea, pyuria, and increased canthal secretion. Before dying, some mice demonstrated symptoms of subcutaneous hemorrhage, hemorrhagic secretion inside nasal cavity, shivering, respiratory distress, muscular hypertonia, or even neck rigidity. Gross examination in CLP mice showed lots of turbid purulent excreta with foul smell in peritoneal cavity, swelling cecum with gangrene and adhesion, flatulent jejunum, and dark red lungs with points of blood and increased volume. The liver and the remaining organs had no obvious abnormalities with visual inspection. Compared to CLP mice, the general condition and gross morphological changes of mice in nicotine (400 μg/kg) group were significantly better.

Lung wet/dry weight ratio and sepsis severity

Mice received CLP had significantly increased ratio of the lung wet/dry weight (2.71±1.58vs. 5.10±0.39 in sham group; $P<0.01$), and mice treated with nicotine (400μg/kg) demonstrated decreased ratio of the lung wet/dry weight (2.30±1.03vs. 2.71±1.58 in CLP group; $P<0.05$). This indicated that nicotine (400 μg/kg) could reduce the edema of lung induced by CLP. In addition, score was counted using the score scale of sepsis severity, and it

showed that the higher the score, the worse the sepsis. Hence, nicotine (400 μg/kg) reduced sepsis severity (15.00±3.58vs. 21.00±2.37 in CLP group; P<0.05).

Pathological changes

As shown in Figure 2, under light microscope, pathological examination of livers from CLP mice showed focal abscess in portal area, dilated sinusoids, infiltrating neutrophils , swollen hepatocytes, as well as punctiform cellular necrosis (Figure 2-A). In comparison, CLP mice treated with nicotine (400μg/kg) showed few swollen hepatocytes and infiltrating neutrophils, or no obvious necrosis (Figure 2-B).

Lungs from CLP mice had significant inflammation, with capillary congestion,micro thrombus formation, and pulmonary interstitial edema, as well as a lot of alveolar wall thickening and neutrophil infiltration (Figure 2-C). In contrast, lungs from mice treated with nicotine (400 μg/kg) showed mild pulmonary interstitial edema, and a little of alveolar wall thickening and neutrophil infiltration (Figure 2-D).

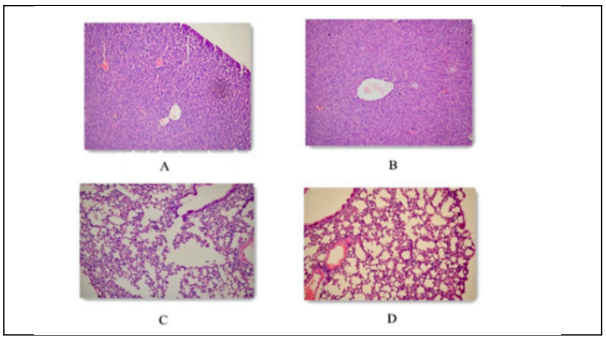

Fig. 2: **A**, CLP group liver. **B**, nicotine(400μg/kg) group liver. **C**, CLP group lung. **D**, nicotine (400μg/kg) group lung (HE×200).

The concentration of plasma cytokines, TNF-α and IL-1β

As shown in Figure 3, the concentration of plasma IL-1β was significantly increased in CLP mice (P<0.05) as compared to nicotine 200μg/kg mice at 2, 4 and 6 hours after surgery (P<0.01). In all mice group except the sham group, the peak level of TNF-α was observed at 4hours. Compared to CLP group, mice treated with three dose of nicotine (20, 200 and 400 μg/kg) had significantly decreased concentration of plasma IL-1β at 2, 4 and 6 hours after surgery (P<0.01). Compared to mice in nicotine (40μg/kg) group, mice in nicotine 200μg/kg group(at 2h, P<0.05; at 4h, P<0.01) and mice in nicotine 400μg/kg group (at 2 and 6h, P<0.01) had significant decrease in the concentration of TNF-α. At 6h, the concentration of plasma TNF-α was significantly decreased in nicotine 400μg/kg mice (P<0.05) as compared to nicotine 200μg/kg mice.

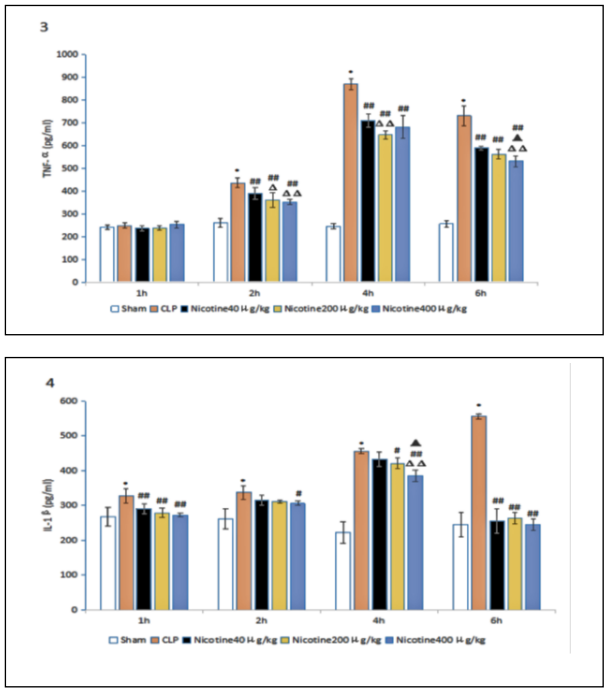

Fig. 3-4: *P < 0.01 compared to the corresponding time points of the sham group; #P < 0.05 and ##P < 0.01 and compared to the corresponding time points of the CLP group; ΔP < 0.05 and ΔΔP < 0.01 compared to the corresponding time points of the nicotine 40μg/kg group; ▲P < 0.05 and ▲▲P < 0.01 compared to the corresponding time points of the nicotine 200μg/kg group.

As shown in Figure 4, compared to the sham mice. The concentration of plasma IL-1β in CLP mice was significantly increased at 1, 2, 4 and 6 hours (P<0.01), with the peak level of IL-1β being observed at 6 h. But mice treated with three dose of nicotine (20, 200 and 400 μg/kg) showed the peak level of IL-1β at 4 hours which decreased subsequently. At 1 and 6 hours after surgery, the concentration of plasma IL-1β in mice treated with nicotine 40μg/kg significantly decreased when compared to CLP group (P<0.01). Compared to CLP mice, significant decrease in the concentration of TNF-α was observed in nicotine 20μg/kg mice (at 1 and 6h, P<0.01), in nicotine 200μg/kg mice (at 1 and 6h, P<0.01; at 4h, P<0.05), and in nicotine 400μg/kg group mice (at 1, 4 and 6h, P<0.01; at 2h, P<0.05). Moreover, at 4 hours after surgery, the concentration of plasma IL-1β in mice treated with

nicotine 400μg/kg significantly decreased when compared to nicotine 40μg/kg mice ($P<0.01$) and nicotine 200μg/kg mice ($P<0.05$).

Altogether, lots of TNF-α and IL-1β cytokines were produced after CLP induction and were released into the blood, which were significantly reduced if the CLP mice were treated with nicotine.

Discussion

At the early stage of infection, the proinflammatory TNF is produced by immune cells. It stimulates the release of a subset of cytokines such as interleukin-1 (IL-1) and high mobility group B 1 (HMGB1), which in turn recruit inflammatory cells to the infection site. However, if bacteria enter circulation and cannot be eradicated immediately, sepsis can develop quickly which contributes to damages to the kidney, liver, lung and other organs and eventually results in the host death.

Neural-immune pathways play a pivotal role in stimulating the body's defense response. Among them, the vagus nerve are considered to play a crucial role as it mediates afferent effects triggered by inflammatory mediators, and also interacts with visceral organs by efferent activity. Borovikova et al.[4] found that acetylcholine could significantly inhibit the synthesis of TNF-α in cells cultured in vitro . Moreover, electrical stimulation of the vagus nerve can reduce systemic inflammatory response induced by endotoxin. The physiological mechanism is that, when bacteria invading, the body's vagus nerves and its neurotransmitter acetylcholine interact with the body's inner immune system to combat inflammation, thus termed "cholinergic anti-inflammatory pathway". Sato et al.[11] confirmed the existence of the two kinds of acetylcholine receptor in peripheral blood mononuclear cells: M receptors and N receptors. Binding of acetylcholine to N receptors inhibits synthesis of pro-inflammatory cytokine TNF, so the anti-inflammatory effect of acetylcholine is achieved mainly by N receptor.

Furthermore, Bernik et al.[12] discovered the molecular basis of the close link between the cholinergic nervous system and the innate immune system is α7 nicotinic acetylcholine receptor (α7nAChR) that presented in macrophages and other cells. with confocal microscopy and RT-PCR. Besides, the release of TNF-α, IL-1, and IL-18 was inhibited when human macrophages was exposed to nicotine or acetylcholine. Thus, LPS-stimulated macrophages release a variety of cytokines (such as TNF-α, IL-1), and binding of IL-1 to IL-1 receptors in vagus nerves stimulate its-efferent pathway to release acetylcholine, the neurotransmitter which interacts with the α7 subunit of the nicotinic AChR (α7nAChR) which inhibits the production of the inflammatory cytokines.

Previously, we treated myocardium ischemia-reperfusion injury rat that induced by the method of left anterior descending coronary artery ligation-open with nicotine, and found that nicotine could significantly reduce myocardial cell damage and decrease ischemic area, thus had a protective effect on cardiac tissue[13]. We found that the mechanism is that activation of cholinergic anti-inflammatory pathway reduce the levels of inflammatory cytokines in vivo and then exert anti-inflammatory effects[14]. The pathophysiological process evoked by cecal ligation and puncture (CLP) is similar to sepsis caused by acute intestinal perforation or diffuse peritonitis in clinic. Previously we established the animal model of sepsis using cecal ligation and puncture (CLP), and confirmed that the liver and lung tissues of mice with sepsis were severely damaged[14].

In this study, sepsis was induced in mice with a standard method of cecal ligation and puncturethe, and the effects of nicotine on the overall survival rate, general conditions, the severity of sepsis, and the pathological changes of major organs of mice were observed and examined. The results showed that nicotine significantly increased the survival rate of sepsis mice, prolonged its survival time, reduced the degree of sepsis severity, and alleviated the pathological damage of hepatic and lung tissues as well as down-regulated the expression of serum proinflammatory cytokines TNF-α and IL-1β. Therefore, nicotine has a protective effect on sepsis in mice.

In summary, the results of this study showed that the concentration of plasma TNF-α and IL-1β increased significantly and liver and lung tissue inflammatory pathology changed obviously in sepsis mice. In sepsis mice treated with nicotine, the concentrations of plasma TNF-α and IL-1β decreased significantly, and liver and lung tissue inflammatory pathology reduced significantly, which indicated that nicotine can reduce the systemic inflammtory pathology changed obviously in sepsis mice. In sepsis mice treated with nicotine, the concentrations of plasma TNF-α and IL-1β decreased significantly, and liver and lung tissue

inflammatory pathology reduced significantly, which indicated that nicotine can reduce the systemic inflammatory response during sepsis and have protective effects on liver and lung. Nicotine stimulates the cholinergic anti-inflammatory pathway and prevents excessive inflammation by inhibiting the release of inflammatory cytokines from macrophages and thus antagonize the damage of systematical inflammation to liver and lung tissue. The results is in accordance with the research of Zabrodskii et al[16].

Therefore, the fundamental mechanism of protective effects of nicotine on animals is to inhibit the release of inflammatory cytokines TNF-αand IL-1β.That is to say, nicotine has anti-inflammatory effects[17]. Vagus nerve exerts anti-inflammatory effects in endotoxin-stimulated macrophages through an α7 nicotinic acetylcholine receptor (α7nAChR)-dependent mechanism, a molecular link between the acetylcholine nerve system and the innate immune system. In addition, our study further showed that the protective effects of nicotine and its effect on the inflammatory mediators may be dose-dependent. So, further research in this area is needed.

In conclusion, appropriate application of nicotine is beneficial to the treatment of sepsis. Moreover, in order to expound the mechanism of interaction between the receptors and drugs at the molecular level, we need to investigate further the role of nicotine in inflammatory mediators and its upstream regulatory molecules. to Here we demonstarte the pathogenesis of sepsis, which will be significantly helpful for researchers to explore effective prevention and control measurements of sepsis, as well as to develop novel therapies with better efficacy and lower side-effects, which could be realized by targeting the α7 subunit of nicotinic acetylcholine receptor specifically.

References

1) Bonaz B, Sinniger V, Pellissier S. Anti-inflammatory properties of the vagus nerve: potential therapeutic implications of vagus nerve stimulation(J). J Physiol, 2016, 594(20): 5781-5790.
2) Tracey K J and Wang H. Antagonists of HMG1 for treating inflammatory conditions: US, US7572446(P). 2009.
3) Di Giovangiulio M, Bosmans G, Meroni E, et al. Vagotomy Affects the Development of Oral Tolerance and Increases Susceptibility to Develop Colitis Independently of α-7 Nicotinic Receptor(J).Mol Med, 2016, 22: 464-476.
4) Maria C. Maldifassi, Gema Atienza, Francisco Arnalich, et al. A new IRAK-M-mediated mechanism implicated in the anti-inflammatory effect of nicotine via α7 nicotinic receptors in human macrophages (J). PLoS One, 2014, 9(9): e108397.
5) Willenberg I, Rund K, Rong S, et al. Characterization of changes in plasma and tissue oxylipin levels in LPS and CLP induced murine sepsis(J). Inflamm Res, 2016, 65(2): 133-142.
6) Jing F, Li M, Ren H, et al. Effects of atorvastatin combined with low-molecular-weight heparin on plasma inflammatory cytokine level and pulmonary pathophysiology of rats with sepsis(J). Exp Ther Med, 2016, 12(2): 1048-1054.
7) Zabrodskii PF, et al. Effects of reversible inhibition of cholinesterase and nicotine on mouse mortality and blood levels of proinflammatory cytokines during the early phase of sepsis. Bull Exp Biol Med, 2012, 152 (5): 600-602.
8) Leite FB, et al. Role of nicotine on cognitive and behavioral deficits in sepsis-surviving rats. Brain Res, 2013, 1507: 74-82.
9) Stéphanie R, Fanny V B, Virginie M D, et al. Sepsis modeling in mice: ligation length is a major severity factor in cecal ligation and puncture:(J). Intensive Care Medicine Experimental, 2016, 4(1): 22.
10) Lee W, Ku S K, Dong H N, et al. Anti-Inflammatory Effects of Lysozyme Against HMGB1 in Human Endothelial Cells and in Mice(J). Inflammation, 2015, 38(5): 1911-1924.
11) Halpin D M, Vogelmeier C, Pieper M P, et al. Effect of tiotropium on COPD exacerbations: A systematic review.(J). Respiratory Medicine, 2016, 114:1. 12. Kanashiro A, Sônego F, Ferreira R G, et al. Therapeutic potential and limitations of cholinergic anti-inflammatory pathway in sepsis(J). Pharmacological Research, 2016, 117:1-8.
13) JIA BH, et al. Experimental study of nicotine against myocardium ischemia-reperfusion injury in rat by cholinergic anti-inflammatory pathway(J). Chin J Crit Care Med, 2013, 33(2): 168-171.
14) JIA BH, et al. The expressions of MOD, SOD and inducible nitric oxide synthase in hepatic and lung tissues of sepsis mice(J).Chin J Mod Med, 2012, 22(7): 17-21.
15) Inci A, Demiraran Y, Erus S, et al. Comparison of procalcitonin value of patients in intensive care unit with systemic and localized bacterial infections [J]. Acta Med Mediterr, 2016, 32:249-253.
16) Zabrodskii PF, et al. Effect of α7n-acetylcholine receptor activation and antibodies to TNF-α on mortality of mice and concentration of proinflammatory cytokines during early stage of sepsis[J]. Bull Exp Biol Med, 2015,159(6):740-742.
17) Savran Y, Duran Y, Comert B. Impactoftime to antibiotics on mortality in severe sepsis antibiotics on mortality insevere sepsis and septic shock [J]. Acta Med Mediterr, 2016, 32: 17-22.

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