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A systematic investigation on animal models of cyclosporine A combined with *Escherichia coli* to simulate the immunosuppressive status of sepsis patients before onset

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ABSTRACT

Immunosuppression is an important mechanism for the development of sepsis pathology, and is the key to the high mortality of sepsis. However, patients appear to be immunocompromised before sepsis onset due to lack of enough attention. Present sepsis models cannot fully mimic the onset of sepsis in patients. Hence, effective treatments in animal experiments could not be transformed into clinical application. In the present study, we improved the animal model of sepsis and used cyclosporine A immunosuppressive mice to make it closer to immune status before the onset of sepsis, followed by the intraperitoneal injection of *Escherichia coli* (*E. coli*) CMCC (B) 44,102 standard strain to produce the immunocompromised sepsis model. This trial systematically evaluates the new immunosuppressive sepsis model. Compared with routine sepsis models, the release of inflammatory factors in the new sepsis model was insufficient, blood bacteria were more cultured, diffuse intravascular coagulation (DIC) was more severe, lung, liver and kidney damage were heavier, and mortality rate was higher. In conclusion, the new sepsis model can mimic the patient's pre-onset immunocompromised state, is suitable for the development and evaluation of new methods of sepsis, and solves the controversy of sepsis treatment, providing new ideas and direction.

1. Introduction

Sepsis has been a major challenge in modern intensive care medicine, and remains as the main cause of death [1,2]. According to the latest Global Burden of Disease report, 10 million people die of infection every year, and this number is higher than the number of people who die of cancer [3]. Furthermore, it has been reported that the incidence of sepsis continue to increase, which reflects the complications of population aging [4]. To date, the pathogenesis of sepsis remains unclear, and there is no gold standard indicator for the diagnosis of sepsis in clinic. Studies have revealed a sepsis hospital mortality of 55% in the Intensive Care Unit (ICU) at the national level [5]. Therefore, there is an urgent need for the study of sepsis.

Despite the advances in modern medicine, the absence of a sepsis drug approved by the FDA remains as a concern at present, which is due to the poor clinical efficacy of the treatments for sepsis. Drotrecogin alfa (a human recombinant activated protein C) [6] and eritoran (a toll-like receptor 4 blocker) were withdrawn [7,8]. Furthermore, drug development and clinical trials for sepsis continue to face great risks. Part of the problem may be the use of an animal model as a substitute for human sepsis, which cannot completely mimic the pathogenesis of sepsis [9]. The incidence of sepsis increases with age, which is due to the increase in the elderly population with the disease, malnutrition and other factors that alter immune function [10]. Therefore, this increases the probability of infection [11,12]. Several large cohort studies have found low $CD4^+$ cell counts in the Nordic population, and the $CD4^+/CD8^+$ ratio of lower populations has a relatively weak T cell proliferation response and increased mortality rate of infection [13]. At

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the same time, the $CD4^+$ T cell maintenance function of induced transcription factor NF-KB reduces in the elderly, and the immune system of the elderly reduces its functional response [14]. In the early onset of sepsis, the severity decrease in most patients with lymphocytes. Furthermore, upon entering the ICU, the apoptosis of T cells and B cells usually occurs at an early period [15]. Basal and clinical trials have revealed that CD4+ T cells proliferate in elderly sepsis patients and mice, more immunosuppressive cytokines are released, the number of elderly patients infected with gram-negative bacteria increases, and persistent inflammatory response and T cell failure reduce the survival rate of elderly patients and mice [16]. Present sepsis models do not take into account the factors of immunocompromised subjects before onset, resulting in the present sepsis model. This renders the clinical sepsis pathological process to be quite different. Hence, effective treatments in basic research fail to show promising results for human sepsis.

The commonly used sepsis animal models include the cecal ligation and puncture (CLP), colon ascendens stent peritonitis (CASP) model, and intraperitoneal injection of lipopolysaccharide sepsis (LPS) model. The severity between the CLP and CASP models is different due to the length of bowel, size of the needle and amount of fecal release [17,18]. The sepsis model induced by lipopolysaccharide is the acute endotoxemia model, but this model cannot mimic sepsis patients against the microbial infection process and immune response [19]. The sepsis animal model remains to be inadequate and needs to be improved. Regardless of whether animal models are close to clinical applications, these would directly depend on the clinical success or failure of the basic research results. In the present study, immune cytokines, inflammatory factors, bacterial culture, coagulation factors and lung, liver and kidney pathologies were observed to comprehensively assess the new immunosuppressive sepsis model, and determine whether the new model can imitate the state before the onset of sepsis. This would allow it to be closely similar to clinical applications and enable sepsis drug research and development to be more conducive to clinical transformation.

2. Materials and methods

2.1. Reagents

Cyclosorin A was purchased from MedChem Express (HY-B0579, Shanghai, China); the *Escherichia coli* (*E. coli*) CMCC (B) 44,102 standard strain was purchased from National Center for Medical Culture Collections (Beijing, China); mice TNF-α and IL-6enzyme-linked immunosorbent assay (ELISA) kits were purchased from 4A Biotech (CME0004, CME0006, Beijing, China); the erythrocyte lysate was purchased from Solarbio (R1010, Beijing, China); mice CD3-FITC monoclonal antibody, mice CD4-PE monoclonal antibody, and mice CD8-PerCP monoclonal antibody were purchased from Biolegend (100,305,100,407,100,731, San Diego, CA, USA).

2.2. Preparation and concentration determination of bacterial suspension

The *E. coli* CMCC (B) 44,102 standard strain was inoculated in 100 ml of sterile medium, cultivated on a shaker at 200 rpm for 12 h at 37 °C, and 1 ml of the medium was collected and diluted. The dilution gradient was 10 times, and diluted up to 10^9 times. At 10^5, 10^6, 10^7, 10^8 and 10^9 dilutions, each 500-μl medium was poured into a Petri dish containing sterile agar medium, and the Petri dish was turned clockwise to allow the liquid to be sufficiently tiled. Then, Petri dish was inverted and cultured in an incubator for 12 h at 37 °C. After incubation, the Petri dish was taken out, and the bacterial colonies on the Petri dish were counted. Then, the concentration of bacteria (CFU/ml) in the original culture solution was calculated [20].

2.3. Animals

Male BALB/c mice (body weight: 20 ± 2 g; 6–8 weeks) were obtained from the Experimental Animal center of the Military Medical Science Academy of the People's Liberation Army. (Beijing, China). Mice were housed in a pathogen-free environment and were given *ad libitum* access to food and water. Indoor temperature was maintained at 22 ± 2 °C with an artificial light/dark period of 12 h. All experiments were in line with national and institutional guidelines for the care and use of laboratory animals.

2.4. Immunosuppressed mice

The selected BALB/C mice were 6–8 weeks old. Mice were fasted and allowed to drink water *ad libitum* for 12 h before the operation. Mice in the immunocompromised model group (IM group) were injected with 25 $mg \cdot kg^{-1} \cdot d^{-1}$ [21,22] of cyclosporine A, once every other day, for a total of three sessions.

2.5. The immune system was detected by flow cytometry

Seven days after immunosuppression in mice, the whole blood of mice was analyzed by flow cytometry, and $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD4^+/CD8^+$ were detected. The peripheral anticoagulant blood of mice was added with 2 ml of erythrocyte lysate for 10 min to split the red blood cells. The clear and colorless peripheral blood was centrifuged at 1500 rpm for 5 min. Then, 45 μl of peripheral blood, anti-mouse CD3-FITC monoclonal antibodies, anti-mouse CD4-PE monoclonal antibodies and anti-mouse CD8-PerCP monoclonal antibodies were added, respectively. After severe shock, these were kept in a fridge for 30 min at 4 °C, and Hank's balanced salt solution was added for washing and centrifugation. Finally, cell volume was adjusted to 0.6 ml and detected by flow cytometry. In the front scatter light (FSC) and side scattered light (SSC) scatter plots, the lymphocyte door was set, and the lymphocytes were examined for PerCP, FITC and PE fl21cence intensity. All data were obtained using the BD FACSCanto™ IIflow cytometer, and FlowJ software was used to process the data [23].

2.6. Establishment of the model of sepsis with low immune system

The selected BALB/C mice were 6–8 weeks old. Mice were fasted and allowed to drink water *ad libitum* for 12 h before the operation. Mice were injected with 25 $mg \cdot kg^{-1} \cdot d^{-1}$ of cyclosporine A, once every other day, for a total of three sessions. Seven days after immunosuppression in mice, the *E. coli* CMCC (B) 44,102 standard strain was centrifuged, and the concentration of 1.0×10^9 CFU/ml was set. Mice were intraperitoneally injected with 300 μl of the *E. coli* CMCC (B) 44,102 standard strain at a concentration of 1.0×10^9 CFU/ml. The establishment of the immunosuppressive sepsis model was completed.

Mice were randomly divided into four groups ($n = 10$, each group): sham group, the immunocompromised model group (IM group), immunocompromised sepsis model group (ISM group), and routine sepsis model group (RSM group). Mice in the IM group were intraperitoneally injected with 300 μl of saline after immunosuppression. Mice in the ISM group were intraperitoneally injected with 1×10^9 CFU/ml of bacterial concentration of the *E. coli* CMCC (B) 44,102 standard strain after immunosuppression. Mice in the routine sepsis model group were intraperitoneally injected with 1×10^9 CFU/ml of bacterial concentration of the *E. coli* CMCC (B) 44,102 standard strain. Mice in the sham group were intraperitoneally injected with 300 μl of saline. After completion of the operation, mice in the four groups were tested for related indexes.

2.7. Detection of inflammatory factors

Blood samples were collected for tumor necrosis factor (TNF)-α and