

## Original Article

# Effects of Rhubarb combined with ulinastatin on T-cell subsets in sepsis rats

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**Abstract:** Objective: The pathogenesis of sepsis, a systemic inflammatory response syndrome, is very complicated and not well understood. However, the importance of lymphocyte percentage and ratio is implicated. Rhubarb is a traditional Chinese medication and plays a role in protecting gastrointestinal mucous and controlling the SIRS damage. Ulinastatin is a protease inhibitor that prevents overproduction of inflammatory cytokines. Currently, despite numerous sepsis clinical researches, the study on the effects of combined drug therapy on sepsis is lacking. In this study, we studied Rhubarb and Ulinastatin combination treatment on T lymphocyte subsets in sepsis induced by the cecal ligation and perforation (CLP). Immunosuppression happened at the early stage of severe sepsis in the CLP rat models, as CD3<sup>+</sup>, CD4<sup>+</sup>, CD4<sup>+</sup>/CD8<sup>+</sup> began to decline, dropped rapidly after 24 h and continuously decreased at 36 h. CD8<sup>+</sup> T lymphocyte showed no significant change in all groups after CLP. The morality of CLP rats was increased with Rhubarb treatment in test dose (1.2 g/100 g). The immunosuppression state of CLP rats ameliorated with UTI treatment at early stage. The immunomodulatory properties were improved along with drug treatment, and immunities were obviously increased after 24 h, moreover, continuously increased at 36 h. The relief effect of immunosuppression after CLP showed much better in Rhubarb combined with UTI treatment than UTI monotherapy. In conclusion, the combination drug treatment facilitates the improvement of sepsis by modifying the lymphocyte percentage.

**Keywords:** Sepsis rats, rhubarb, ulinastatin, inflammatory response

## Introduction

Sepsis is a systemic inflammatory response syndrome (SIRS) caused by infection or by serious complications from different incidents such as severe trauma, burn, shock, and surgery. There are 1.8 million new cases of sepsis worldwide every year, and the overall fatality rate increased to 30-50%, which is higher than the fatality rate of acute myocardial infarction [1]. The pathogenesis of sepsis is very complicated as there are numerous fundamental problems such as infection, diminished immunity, inflammation, blood coagulation, tissue injury, and pathophysiological changes of multiple organ systems. During the early stages of sepsis, there is an enormous release of inflammation mediators, known as a "cytokine storm", which then results in SIRS. As the disease progresses, the synthesis and release of anti-inflammatory cytokines, which are dominant in the late stage of SIRS, occurs. Besides inhibit-

ing the inflammation responses, these anti-inflammatory cytokines also show inhibitory effects on the body's immune system and cause apoptosis of T cells, B cells, NK cells, DC cells and monocytes. As a result, compensatory anti-inflammatory response syndrome (CARS) eventually develops [2]. This immunosuppressed state is one of the main causes of body injury and is the end result of sepsis [3-5]. The excessive inflammatory response during the early stage is correlated to the suppression of the immune system in the late stage: the stronger the early inflammation, the more suppressed the immune system becomes in the latter stage [6-8].

New research over recent years suggests that CARS and immunosuppression actually happens at the beginning of infection or damage instead of being compensatory responses to SIRS. Thus, excessive inflammatory response and immunosuppression might exist during the

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early stage of sepsis (especially severe sepsis and septic shock). Therefore, we should not focus on suppressing inflammation during treatment instead emphasize balancing the immune system of patients [9-11].

T lymphocyte counts and subsets distribution reflect the state of cellular immunity to some extent. T lymphocytes are divided into four subsets according to different functions and surface markers: inducer/helper T cells (Ti/Th), cytotoxic T cells (Tc), suppressor T cells (Ts) and delayed type hypersensitivity T cells. CD3<sup>+</sup> is the common surface marker of all kinds of T lymphocytes; thus, it can be used to represent the total number of T lymphocytes. CD4<sup>+</sup> is the surface marker for Ti/Th, which help B lymphocytes to produce antibodies and induce T lymphocytes transformation to effector cells. CD8<sup>+</sup> is protein in Ts, which mediate host cell destruction and dissolution after pathogen infection. The ratio of CD4<sup>+</sup>/CD8<sup>+</sup> is about 2:1 in mammals, depending on species, and it generally reflects the body's immunologic homeostasis state. CD4<sup>+</sup> and CD8<sup>+</sup> are in dynamic equilibrium with each other and feedback regulation determines the total effect of the immune responses together. The immune function is considered imbalanced if the ratio deviates from the normal value [12-14].

A herb that belongs to the polygonaceae family, Rhubarb is a traditional Chinese medicine with the following components: anthraquinones, Rhubarb polysaccharides, Rhubarb tannin, etc. Rhubarb plays a role in protecting gastrointestinal mucous by preventing toxins and bacteria dislocation, promoting gastrointestinal wriggle, accelerating endotoxin excretion and ameliorating visceral organs and peripheral blood circulation, which also helps control the SIRS damage by decreasing endotoxin activity. Ulinastatin (UTI) is a protease inhibitor that is synthesized and secreted by liver [15]. UTI is critical in inhibiting multiple protease activity, stabilizing lysosomal membrane, inhibiting lysosomal enzyme release, reducing inflammatory factor release, decreasing permeability of capillary, improving tissue edema, and helping neutralize peroxide [16, 17]. Numerous studies have indicated that UTI, similar to glucocorticoid, inhibits overproduction of inflammatory cytokines, but have no side effects unlike glucocorticoid. UTI improves the immunity by increasing the CD4<sup>+</sup> T lymphocyte counts, thus, the Ti/Ts ratio [18, 19]. Therefore, UTI shows

positive effects on both T cell function and quantity, not only alleviating cell immunosuppression but also strengthening the cytotoxicity of natural killer cell (NK cell).

Currently, there are numerous clinical research projects on sepsis, but few of them focus on the effects of combined drug therapy, especially Chinese and Western medicine combined treatment, on T lymphocyte subsets. In this study, we investigate the effects of Rhubarb and UTI on T lymphocyte subsets in sepsis rat models. Taking T lymphocyte subsets or ratio (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>+</sup>/CD8<sup>+</sup>), as the main indicators of an organism's immunological homeostasis will help to illustrate the pathogenesis of sepsis in the immune systems and potentially offer new ideas for more effective clinical treatment of sepsis.

### Materials and methods

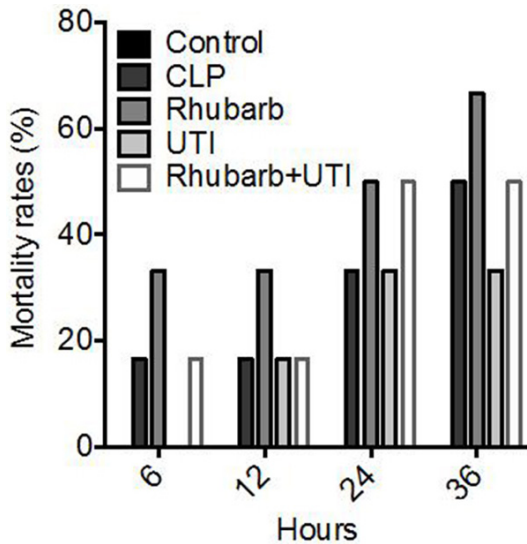
#### *Main reagents*

UTI for powder injection was purchased from Techpool (Techpool Bio-pharma Co., Ltd, Guangdong, China). Rhubarb powder was offered by Shenwei Big Pharmacy (Shijiazhuang, China). CD3<sup>+</sup>, APC, CD4<sup>+</sup> PE, and CD8<sup>+</sup> FITC antibodies were purchased from BD (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

#### *Preparation of the cecal ligation and perforation (CLP) rat model*

This model was prepared with a constant temperature of 25°C. Animals were not given any food for 12 hours before any surgical procedure, but they had accessed to water during this time. They were anaesthetized with an intraperitoneal injection of 10% chloral hydrate (0.4 ml/100 g). A 1.5 cm incision was made at the midline. Then, the abdominal layers were opened one at a time until the cecum was carefully isolated in a sterile environment. The cecum was then ligated by #4 sutures at the vascular bow, just below the ileocecal valve. After carefully avoiding any damages while ligating the vascular bow, we checked the unobstructed intestine. Next, we punctured ceca three times (triangular in shape) with a 12-gauge needle and formed a six-cecum fistula and applied sufficient pressure to extrude a single droplet of fecal material from the puncture site. Subsequently, the cecum was placed back into the abdominal cavity and the abdo-

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**Figure 1.** Mortality rates of rats after various times following treatment.

men layers were closed. The animals were resuscitated with a subcutaneous injection of normal saline (2-3 ml/100 g) to combat the fluid loss post operation, and they were kept under warm light for 1.5-2 h to recover. The rats showed abdominal distension, anorexia, increase in eye secretions, and temperature drop. The rectal temperature usually dropped below 34°C if the surgery was successful.

### Animal grouping

120 healthy Sprague-Dawley (SD) rats of either gender, 8-12 weeks of age, and weighing 204-251 grams were purchased from the experimental animal center of Hebei Medical University (Certificate No. 1303144). They were divided into five random groups with 24 rats in each group. The individual groups were labeled as following: control group (I), CLP group (II), CLP and Rhubarb treatment group (III), CLP and UTI treatment group (IV) and CLP and Rhubarb combined with UTI treatment group (V). After a placebo operation in group I and CLP operation in groups II, III, IV, V, each group was further divided randomly into four phases (6 h, 12 h, 24 h and 36 h) with 6 rats in each phase.

Group I (control group): Midline laparotomy was performed, and the cecum was carefully isolated. Then, the abdominal cavity was closed, and a subcutaneous injection of normal saline (2-3 ml/100 g) was given. Group II: (CLP model). Group III (experimental group 1): The same treatment as group II was given. Gavage was administered once every 12 h with Rhubarb

(1.2 g/100 g) immediately after CLP. The dose used in rats was 60 times that used in humans (0.02 g/100 g). The Rhubarb was soaked 3-4 h in normal saline and filtered to 2-3 ml before used. Group IV (experimental group 2): The same treatment as group II was given. Intraperitoneal injection of UTI (1000 U/100 g) was given immediately after CLP. The UTI was dissolved in 0.9% normal saline and administered every 12 h. Group V (experimental group 3): The same treatment as group II was given. Gavage was administered with Rhubarb and an intraperitoneal injection of UTI synchronously once every 12 h after CLP.

### Samples collection and preparation

Two ml blood samples were collected directly from the hearts of rats in each phase of each group, and the samples were kept in the EDTA anticoagulant vacuum tubes. Each sample was divided into an experiment tube and control tube, marked as Tube 1 and Tube 2. 2.5  $\mu$ L of CD3<sup>+</sup> APC, 2.5  $\mu$ L of CD4<sup>+</sup> PE, and 0.5  $\mu$ L of CD8<sup>+</sup> FITC were added into Tube 1 and 6  $\mu$ L of homotype control solutions were added into Tube 2. They were oscillated well, and 100  $\mu$ L of anticoagulant whole blood was added. Then, the tubes were incubated, protecting them from light at room temperature for 20 min after. Next, 1 mL of FACS Lysing Solution was added (hemolysin: deionized water = 1:9), and the tubes were protected from light for 6-8 min. They were centrifuged at 1500 rpm for 5 min, and the supernatant was poured. Then, the solutions were washed twice with PBS and re-suspended in cells. CD4<sup>+</sup>/CD8<sup>+</sup> ratio was calculated, and the percentages of T lymphocyte subsets (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>) in every 20,000 cells of each sample were analyzed via flow cytometry.

### Statistical methods

The data analysis was performed using SPSS 13.0. The data are presented as the mean  $\pm$  SEM. Variance analysis and student's t test were used for statistical analysis.  $P < 0.05$  and  $P < 0.01$  were used to measure statistical significance.

## Results

### General behavior

The rats in group I typically ate, drank, and moved around freely with no abnormal behaviors after recovery. Group II, III, IV and V rats

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**Table 1.** Percentage of lymphocytes among all experiment groups

Items	CD3 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>	CD4 <sup>+</sup> /CD8 <sup>+</sup>
Control group				
6 h	67.43±3.27	42.87±1.88	24.56±1.50	1.75±0.06
12 h	68.40±3.28	43.73±2.06	24.67±1.41	1.78±0.06
24 h	68.75±3.42	43.96±2.02	24.79±1.50	1.78±0.05
36 h	68.66±2.71	43.44±1.91	25.22±1.40	1.73±0.10
CLP group				
6 h	66.31±3.92	40.78±2.58 <sup>☆</sup>	25.53±1.60	1.60±0.07 <sup>☆</sup>
12 h	65.36±4.43	39.33±2.85 <sup>★</sup>	26.02±1.79	1.51±0.07 <sup>★</sup>
24 h	60.67±2.52 <sup>★</sup>	34.10±2.11 <sup>★</sup>	26.58±1.63 <sup>☆</sup>	1.29±0.12 <sup>★</sup>
36 h	58.70±2.47 <sup>★</sup>	31.79±2.44 <sup>★</sup>	26.91±1.48 <sup>☆</sup>	1.19±0.13 <sup>★</sup>
Rhubarb group				
6 h	67.86±4.18	42.01±2.76	25.85±1.56	1.63±0.05
12 h	65.04±5.09	39.05±3.78 <sup>★</sup>	25.99±1.62	1.50±0.10 <sup>★</sup>
24 h	62.78±3.76 <sup>★</sup>	37.10±2.40 <sup>▲,★</sup>	25.69±1.62	1.44±0.07 <sup>▲,★</sup>
36 h	65.03±3.86 <sup>▲</sup>	40.00±2.09 <sup>▲,☆</sup>	25.03±1.82 <sup>▲</sup>	1.60±0.05 <sup>▲,★</sup>
UTI group				
6 h	67.55±2.08	42.17±2.54	25.38±1.65	1.67±0.19
12 h	69.04±4.82	44.05±2.94 <sup>▲</sup>	24.99±1.99	1.77±0.06 <sup>▲</sup>
24 h	73.78±3.64 <sup>▲</sup>	46.91±2.76 <sup>▲,☆</sup>	24.87±0.93	1.89±0.05 <sup>▲,☆</sup>
36 h	75.04±3.57 <sup>▲,★</sup>	50.57±2.09 <sup>▲,★</sup>	24.47±1.66 <sup>▲</sup>	2.07±0.09 <sup>▲,★</sup>
Rhubarb + UTI group				
6 h	69.51±3.70	43.91±2.68 <sup>▲</sup>	25.59±1.55	1.72±0.10 <sup>▲</sup>
12 h	71.06±3.14 <sup>▲</sup>	46.52±1.96 <sup>▲,☆</sup>	24.53±1.71	1.90±0.12 <sup>▲,☆</sup>
24 h	77.41±3.05 <sup>▲,★</sup>	52.92±2.59 <sup>▲,★</sup>	24.50±1.35 <sup>▲</sup>	2.17±0.14 <sup>▲,★</sup>
36 h	78.64±4.39 <sup>▲,★</sup>	54.36±2.83 <sup>▲,★</sup>	24.28±1.79 <sup>▲</sup>	2.24±0.10 <sup>▲,★</sup>

Note: VS the CLP and Rhubarb treatment group, UTI treatment group and Rhubarb combined with UTI treatment group at the same time, <sup>▲</sup>*P* < 0.05, <sup>★</sup>*P* < 0.01; VS the control group and CLP group, Rhubarb treatment group, UTI treatment group and Rhubarb combined with UTI treatment group at the same time, <sup>☆</sup>*P* < 0.05, <sup>★</sup>*P* < 0.01 (*x* ± *s* %).

started to drink and feed 2-3 h after CLP, and they were characterized by depression, piloerection, drowsiness, seldom movements, chill, abdominal distension, increase in eye secretions, rectal temperature below 34°C, and eventual death. The mortality in groups III and IV was the highest, 50% to 60% at 24 h and 36 h, respectively (**Figure 1**). The mortalities in groups I, II, and V were lower relative to groups III and IV (**Figure 1**).

There was no significant difference in the percentage of lymphocyte subsets in control group at various time (*P* < 0.05) (**Table 1**).

*Comparison of CD3<sup>+</sup> T cell count between experiment groups, CLP group, and control group in the same phase*

The CD3<sup>+</sup> T cell count in the CLP group was significantly lower than the count in the control

group and the UTI treatment group at 24 h and 36 h phases (*P* < 0.01). The count in Rhubarb treatment group was significantly higher than that of the CLP group at 36 h (*P* < 0.01) and significantly lower than that of the control group at 24 h (*P* < 0.01). Compared to control group, the count was significantly higher in UTI treatment group (*P* < 0.01). The CD3<sup>+</sup> T cell count in the Rhubarb combined with UTI treatment group was significantly higher than the CLP group at 12 h, 24 h, and 36 h as well as higher than the control group at 24 h and 36 h phases (*P* < 0.01).

*Comparison of CD4<sup>+</sup> T cell count between experiment groups, CLP group, and control group in the same phase*

The CD4<sup>+</sup> T cell count in CLP group was significantly lower than in the control group and in the

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Rhubarb combined with UTI treatment group in all phases (both  $P < 0.05$  at 6 h and  $P < 0.01$  at 12 h, 24 h, 36 h). The count in the Rhubarb treatment group was significantly lower than in the control group ( $P < 0.01$  at 12 h, 24 h;  $P < 0.05$  at 36 h). The count in UTI treatment group was significantly higher compared to the count in the CLP group ( $P < 0.01$  at 12 h, 24 h, 36 h), and the count in the Rhubarb combined with UTI treatment group was significantly higher than the count in the control group ( $P < 0.05$  at 12 h;  $P < 0.01$  at 24 h, 36 h) at 12 h, 24 h, and 36 h phases. At 24 h and 36 h, the count in Rhubarb treatment group was significantly larger compared to the CLP group. Similarly, the count in UTI treatment group was significantly higher than in the control group (both  $P < 0.05$  at 24 h and  $P < 0.01$  at 36 h).

### *Comparison of CD8<sup>+</sup> T cell count between experiment groups, CLP group, and control group in the same phase*

The CD8<sup>+</sup> T cell count in CLP group were significantly higher compared to that in the control group at 24 h and 36 h (both  $P < 0.05$ ), while there was no statistical significance in the other phases ( $P > 0.05$ ). The count in Rhubarb treatment group and UTI treatment group both showed lower levels compared to that in the CLP group ( $P < 0.05$ ;  $P < 0.01$ , respectively). At 24 h and 36 h, the count in the Rhubarb combined with UTI treatment group was significantly lower than in the CLP group ( $P < 0.05$  at 24 h;  $P < 0.01$  at 36 h).

### *Comparison of CD4<sup>+</sup>/CD8<sup>+</sup> ratio between experiment groups, CLP group, and control group in the same phase*

The CD4<sup>+</sup>/CD8<sup>+</sup> ratio in the CLP group was significantly lower than in the control group and Rhubarb combined with UTI treatment group at all phases (both  $P < 0.05$  at 6 h;  $P < 0.01$  at 12 h, 24 h, 36 h). At 12 h, 24 h and 36 h, the ratio in the Rhubarb treatment group was significantly lower compared to that in the control group ( $P < 0.01$ ). Similarly, the ratio in UTI treatment group was also significantly decreased compared to that in the CLP group ( $P < 0.01$ ). Compared to the CLP group, the ratio in the Rhubarb treatment group was significantly lower at 24 h and 36 h ( $P < 0.01$ ). At the same time points the ratio in the UTI treatment group showed a significant increase compared to that

in control group ( $P < 0.05$  at 24 h and  $P < 0.01$  at 36 h).

## Discussions

We recorded and analyzed the number of each T lymphocyte subsets while referring to literature. Our analyses revealed no significant difference between the percentages of T lymphocytes subsets we calculated with the literature values. However, the T lymphocyte counts would fluctuate with strains, housing and approaches etc. To ensure the credibility of our experiment, we chose the T lymphocyte count in control rats as the T lymphocyte normal value range.

CD3<sup>+</sup> is expressed on the surface of all kinds of T lymphocytes; thus, it could be used as a marker to represent the total number of T lymphocytes. The reduction of CD3<sup>+</sup> T cells count in CLP group at 6 h, although with no statistical significance, indicates the existence of immunosuppression in the early phase or co-existence of immunosuppression and immune hyperfunction. The lack of significant change in CD3<sup>+</sup> T cells count conforms to the pathophysiology of early severe sepsis. Similar to CLP group, CD3<sup>+</sup> T cells count in the Rhubarb group came down until the 36 h mark, which might be explained by the threshold of effective blood concentration of Rhubarb not being reached, resulting in a failure to exert their pharmacological effects. Rhubarb is absorbed slowly by the body in traditional Chinese medicine. Rhubarb treatment accelerated the excretion of endotoxins in the intestine as time progresses. It plays a role in gastrointestinal mucosa protection, which, to some extent, reduced the absorption of endotoxins into blood and alleviated inflammation. The T lymphocytes count was fewer in the Rhubarb treatment group than control group, which indicates an immunosuppressed state in Rhubarb treatment rats. The CD3<sup>+</sup> T cells count was increased in UTI group and rose even more after 12 h, which was much higher than the count in the CLP group and in the control group. UTI has a role in enhancing immunity. UTI could reach effective blood concentration in a short time after intraperitoneal injection due to the powerful absorption effect of omentum majus. Related research has confirmed that intraperitoneal injection extended the effects with intravenous administration. Rhubarb combined with UTI treatment

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group showed similar effect as UTI group. The CD3<sup>+</sup> T cells count increased more quickly during 12-24 h compared to the UTI group. This was because of the protection effect in gastrointestinal mucosa and increased excretion of endotoxins by Rhubarb, which alleviated immunosuppression together with UTI and improved the recovery and improvement of immunity.

CD4<sup>+</sup> T cells are a subpopulation of CD3<sup>+</sup> T cells and represent a majority of the CD3<sup>+</sup> T cell population; therefore, the trends for CD4<sup>+</sup> T cells were similar to those of CD3<sup>+</sup> T cells. CD4<sup>+</sup> T cells are critical for helping B lymphocytes produce antibodies and inducing T lymphocytes to become effector cells. Some of CD4<sup>+</sup> T cells were exhausted by the numerous inflammatory mediators being released during the early stage of sepsis. While the inflammation responses were partly controlled by increasing anti-inflammatory mediators, there was also a suppression effect on body immunity. The changes of CD4<sup>+</sup> T cells, relative to CD3<sup>+</sup> T cells, reflected the existence of immunosuppression in the early stage of sepsis since they excluded the interference of CD8<sup>+</sup> T cells.

CD8<sup>+</sup> T cells are a type of T lymphocytes characterized as mediators of own cells killing and dissolution after pathogen infection. Since there was no involvement of virus and other pathogens in rat cell infection, the CD8<sup>+</sup> T cell count showed little change in all groups, except a slight increase in the CLP group which showed no statistical significance. The increase of CD8<sup>+</sup> T cells might strengthen the killing effects on host cells and improve immunosuppression as CD8<sup>+</sup> T cells played a role in cleaning death cells. UTI treatment reduced CD8<sup>+</sup> T cells in sepsis rats, which could alleviate killing effects on self-cells and ameliorate immunosuppression.

CD4<sup>+</sup>/CD8<sup>+</sup> ratio represents the body's immune activity of rats. The changed trend of CD4<sup>+</sup>/CD8<sup>+</sup> ratio is similar to CD4<sup>+</sup>, as the CD8<sup>+</sup> value had little change in these experiments.

Additional experiments need to be performed because of the high mortality rate of rates especially at 24 h and 36 h time points, which could have resulted in artificial error and incorrect data. The low survival of rats at 24 h and 36 h could be due to the following factors: (1), the survival rates increased since the rats were

far from severe sepsis after CLP, which delayed the failure of multiple systems organs; (2), individual difference, the rat survived which with stronger immunity and the higher survival rates in female rats than male rats; (3), the difference quality and quantity of fecal material during CLP would infect the abdominal cavity infection and sepsis severity. We aim to decrease the errors by utilizing same sex rats in the next experiments, as we cannot improve the condition through human interference.

The rats presented weight loss, dehydration, and anus mucosa redness after Rhubarb (60 times dose in human) gavage administration for 3 days in the pre-experiments. After gavage administration of Rhubarb in the formal experiments, the rat did not appear purgation, but lots of Rhubarb was deposited in the gastrointestinal tract, which caused high mortality. We will adjust the dose of Rhubarb and observe the results in a later study.

The rats exhibited reduced appetite and water intake after CLP. This behavior was correlated to treatment time, and it might cause a shortage of energy and water, which could result in serious circulatory failure at a later stage. We will perform abdominal cavity fluid replenishment, since we failed to find suitable treatment measures referring to the previous work.

In conclusion, immunosuppression happened at the early stage of severe sepsis in the CLP rat models, as CD3<sup>+</sup>, CD4<sup>+</sup>, CD4<sup>+</sup>/CD8<sup>+</sup> began to decline, dropped rapidly after 24 h and continuously decreased at 36 h. CD8<sup>+</sup> T lymphocyte showed no significant change in all groups after CLP. The morality of CLP rats was increased with Rhubarb treatment in test dose (1.2 g/100 g). The immunosuppression state of CLP rats ameliorated with UTI treatment at early stage. The immunomodulatory properties were improved along with drug treatment, and immunities were obviously increased after 24 h, moreover, continuously increased at 36 h. The relief effect of immunosuppression after CLP showed much better in Rhubarb combined with UTI treatment than UTI monotherapy.

### Disclosure of conflict of interest

None.

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