Video Article

Use of Animal Model of Sepsis to Evaluate Novel Herbal Therapies

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Abstract

Sepsis refers to a systemic inflammatory response syndrome resulting from a microbial infection. It has been routinely simulated in animals by several techniques, including infusion of exogenous bacterial toxin (endotoxemia) or bacteria (bacteremia), as well as surgical perforation of the cecum by cecal ligation and puncture (CLP)¹⁻³. CLP allows bacteria spillage and fecal contamination of the peritoneal cavity, mimicking the human clinical disease of perforated appendicitis or diverticulitis. The severity of sepsis, as reflected by the eventual mortality rates, can be controlled surgically by varying the size of the needle used for cecal puncture². In animals, CLP induces similar, biphasic hemodynamic cardiovascular, metabolic, and immunological responses as observed during the clinical course of human sepsis³. Thus, the CLP model is considered as one of the most clinically relevant models for experimental sepsis¹⁻³.

Various animal models have been used to elucidate the intricate mechanisms underlying the pathogenesis of experimental sepsis. The lethal consequence of sepsis is attributable partly to an excessive accumulation of early cytokines (such as TNF, IL-1 and IFN- γ)⁴⁻⁶ and late proinflammatory mediators (e.g., HMGB1)⁷. Compared with early proinflammatory cytokines, late-acting mediators have a wider therapeutic window for clinical applications. For instance, delayed administration of HMGB1-neutralizing antibodies beginning 24 hours *after* CLP, still rescued mice from lethality^{8,9}, establishing HMGB1 as a late mediator of lethal sepsis. The discovery of HMGB1 as a late-acting mediator has initiated a new field of investigation for the development of sepsis therapies using Traditional Chinese Herbal Medicine. In this paper, we describe a procedure of CLP-induced sepsis, and its usage in screening herbal medicine for HMGB1-targeting therapies.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3926/>

Protocol

1. Establishment of Animal Model of Sepsis

1. Mice are anesthetized with ketamine (75 mg/kg, intramuscular, i.m.) and xylazine (10 mg/kg, i.m.), and placed in supine position.
2. Fix the feet of the mouse with tape to ensure a stable position.
3. Clean the abdomen with 3 alternating scrubs of betadine or other skin disinfectant and alcohol. Then, make a 15 mm midline incision to expose the cecum.
4. Ligate the cecum with a 4-0 silk suture at approximately 5.0 mm from the cecal tip, and then puncture the ligated cecal stump once with a 22-gauge needle to allow extrusion of stool.
5. The cecum is immediately replaced back to its normal intra-abdominal position.
6. Close the incision site in two layers, by first closing the musculature of the abdomen with absorbable sutures, and then closing the skin with wound clips or nonabsorbable sutures.
7. Resuscitate mouse with 0.5 ml normal saline solution and a single dose of imipenem (0.5 mg/mouse, subcutaneously) immediately after the completion of the surgical procedure.
8. Return the mouse back to a clean cage with free access to food and water.
9. At various time points post CLP, herbal extract or component is administered intraperitoneally.
10. Animal survival is monitored for more than two weeks. Moribund animals exhibiting difficulties to stand, agonal breathing, severe muscular atrophy and uncontrolled bleeding should be euthanized by overdose carbon dioxide inhalation.
11. It is important to note that circulating cytokine levels are important parameters in these studies. Various analgesics have been shown to affect cytokine release and activities, and therefore have been intentionally avoided in postoperative care.

2. Preparation of Herbal Extract

1. Extract herbs in hot water (85 °C) for 1–4 hr (1 hr for leaves, and 4 hr for roots).
2. Centrifuge the water-soluble fraction (3300 g, 20 min, 4 °C) to remove insoluble particles.
3. Filtrate the supernatant fraction through a 0.2 µm filter.
4. The clear water-soluble filtrate fraction is then fractionated by ultrafiltration using Centriprep YM-10 Centrifugal Filter (Catalog no. 4305, Millipore).
5. The resulted low (<10 kDa) and high (> 10 kDa) molecular weight fractions (MWF) are screened for HMGB1-inhibiting activities using macrophage cultures.
6. Intraperitoneal administration of HMGB1-inhibiting herbal extract or components at 24 hr post CLP to evaluate their therapeutic efficacy.

3. Peritoneal Macrophage Isolation

1. Thioglycollate broth (4%, 2.0 ml) is administered intraperitoneally into normal mice.
2. Primary peritoneal macrophages are harvested at 3 days as previously described¹⁰.
3. Macrophages are pre-cultured in DMEM medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine, and 1% penicillin.
4. Adherent macrophages are gently washed with, and cultured in, serum-free OPTI-MEM I medium two hours before stimulation with bacterial endotoxin (lipopolysaccharide, LPS, *E. coli* 0111:B4, Sigma-Aldrich).
5. At 16 hours after LPS stimulation, levels of HMGB1 in the culture medium are determined by Western blotting analysis¹¹.
6. The relative band intensity was quantified by using the NIH image 1.59 software to determine HMGB1 levels with reference to standard curves generated with purified HMGB1.

4. Representative Results

1. CLP induces systemic and local inflammation.

Within a few hours of CLP surgery, animals exhibit clinical signs of sepsis that include piloerection, lethargy, huddling, and decrease in food and water intake. Animals developing a severe peritonitis with consecutive systemic infection normally die within 48 - 96 hr. However, even age-, sex-, and genetic background-matched animals can respond to CLP surgery in a distinguishable manner in the course of experimental sepsis. For instance, at 48 hr post CLP, while some animals may have approached the moribund state (as defined in **Figure 1**), others may remain at a non-moribund state.

Comprehensive surveys of circulating cytokines revealed dramatic differences in levels of several cytokines (e.g., IL-6, KC, MIP-2, and sTNFR1) between mice in the moribund and non-moribund states (**Figure 1**)¹². Notably, these inflammatory mediators have been classified as surrogate markers of sepsis because their circulating levels are reliable predictors of lethal outcome in experimental¹²⁻¹⁴ or clinical sepsis¹⁵. In addition, CLP also induced local release of various pro- and anti-inflammatory cytokines and chemokines. For instance, at 48 hr post CLP, significant amounts of cytokines (e.g., IL-6) and chemokines (KC and MIP-2) can still be measured not only systemically in the blood, but also locally in the peritoneal lavage fluid (**Figure 2**).

2. Screening for HMGB1-inhibiting herbal extract or components.

Using macrophage cultures, we were able to evaluate the capacities of various herbal extract or components in inhibiting endotoxin-induced HMGB1 release (**Figure 3**). In light of their capacity in inhibiting HMGB1 release, we further explored their efficacy in animal models sepsis. Given the late and prolonged kinetics of HMGB1 accumulation in experimental sepsis⁸, the first dose of HMGB1 inhibitors was given 24 hr after the onset of sepsis - a time point when mice developed clear signs of sepsis including lethargy, diarrhea, and piloerection. As shown in **Figure 4**, delayed and repeated administration of a major green tea component, epigallocatechin-3- gallate (EGCG), beginning at 24 h after onset of sepsis, significantly rescued mice from lethal sepsis¹². Even when given orally, EGCG still rescued mice from lethal sepsis, significantly increasing animal survival rates from 16% to 44%¹⁶. It would be important to determine whether combinational therapies with several herbal components could achieve a significant better protection in animal models of sepsis. Taken together, these experimental data validated our approach to screen novel therapeutic agents using an animal model of CLP-induced sepsis.

5. Representative Results

Figure 1. Circulating levels of surrogate markers are dramatically higher in septic animals approaching moribund state. Balb/C mice were subjected to sepsis by CLP and monitored for the development of signs of sickness. At a late stage of sepsis (52 hr post CLP), blood was collected from 3 normal mice (-CLP), 3 septic mice approaching the moribund state, and 3 septic mice in the non-moribund state. Serum was pooled from each group, and assayed for the cytokine profile by antibody arrays. Note the dramatic difference in the relative levels of several surrogate markers between different groups. Adapted from doi:10.1371/journal.pone.0001153.g006 with granted permission from the publisher.

Figure 2. Detection of local and systemic cytokines and chemokines at 48 hr post CLP. Balb/C mice were subjected to sepsis by CLP, and blood or peritoneal fluid were harvested at 48 h post CLP. Relative levels of several cytokines and chemokines in pooled serum or peritoneal lavage fluid were measured by cytokine antibody arrays, and expressed as arbitrary units (AU). As controls, blood and peritoneal fluid samples were obtained from normal healthy animals (-CLP) without laparotomy.

Figure 3. Herbal component dose-dependently inhibited endotoxin-induced HMGB1 release in primary macrophage cultures. Primary murine peritoneal macrophages were stimulated with LPS in the absence, or presence of herbal component (e.g., EGCG, 15 µM). At 16 hours after LPS stimulation, levels of HMGB1 in the culture medium were determined by Western blotting analysis. Adapted from doi:10.1371/journal.pone.0001153.g006 with granted permission from the publisher.

Figure 4. Herbal component rescued mice from lethal sepsis. Balb/C mice were subjected to lethal sepsis by CLP, and herbal component (EGCG) was intraperitoneally administered at +24, +48, +72 hours post the onset of sepsis. Animal survival was monitored for up to two weeks. The Kaplan-Meier method was used to compare the differences in mortality rates between groups. *, $p < 0.05$ versus saline. Adapted from doi:10.1371/journal.pone.0001153.g006 with granted permission from the publisher.

Discussion

In the laboratory, several animal models of sepsis have been employed to understand the pathogenesis of sepsis in order to develop potential novel therapies. Their clinical relevance remains a subject of debate before the successful translation of animal studies into clinical applications for sepsis. Although neutralizing antibodies against early cytokines (e.g., TNF) were protective in animal models of bacteremia/endotoxemia^{17,18}, they actually worsen survival in animal model of sepsis¹⁹. Similarly, most anti-TNF agents failed to show efficacy in clinical trials of sepsis²⁰⁻²². This failure reflects partly on the complexity of the underlying pathogenic mechanisms of sepsis^{23,24}. In addition, it may also be attributable to pitfalls in the selection of: 1) feasible therapeutic targets or drugs; 2) optimal doses and timing of drugs; and 3) non-realistic clinical outcome measures (such as mortality rates)²⁵.

The recent discovery of HMGB1-targeting herbal extract and/or components, including Danggui²⁶, Green tea^{12,16}, and Danshen²⁷ has provided successful examples of preclinical investigation employing animal models of sepsis. Further investigation in this area will shed more light on the molecular cascades underlying regulation of the innate immune response, and provide clues for the development of therapeutics for various inflammatory diseases. When first establishing CLP in your laboratory, effort should be made to perform the surgery procedure as quickly and exactly as possible to ensure reproducibility, particularly when using a large number (30-40) of mice to compare the survival rates between several experimental groups of an experiment. The use of long-acting anesthetics (such as ketamine and xylazine) allows us to complete CLP surgical procedure on a large number of mice in a relatively short time frame, and meanwhile help to remove potential dose variance often occurred when using volatile anesthetics. Survival rates and systemic cytokine accumulation can be taken as signs of successful performance of CLP procedure.

CLP model has been widely used in rodents because of obvious advantages in low cost, simplicity of surgical procedure, and extensive pathological, immunological, physiological characterizations. However, there are a number of limitations of the mouse CLP model¹⁻³. For instance, like all animal models, a species disparity is highlighted by the fact that cecal ligation without puncture can be fatal in human but not in mice. Moreover, because of small size of mouse and dehydration subsequent to CLP, it is often difficult to obtain serial blood samples for cytokine measurement. These disadvantages can be partially overcome by establishing CLP models in larger animals^{2,3,27,28}. In addition, it is important to point out that the mortality rates and the progress of peritonitis in rodents are largely determined by the amount of stool extrusion, which are affected by the gauge of the needle used to puncture the cecum, the number of punctures, the total volume of ligated cecum and the viscosity of the stool^{2,3}. In addition, the dose and frequency of administration of antibiotics at early stage of CLP can also affect the mortality rates. Finally, animal sources and housing environment can also contribute to the variance of mortality rates.

Disclosures

A.E.S. and H.W. are co-inventors of patent applications related to HMGB1 inhibitors (tanshinones) as potential therapeutic agents for sepsis.

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