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Development of venous-venous extracorporeal blood purification circuits in rodents for sepsis

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Abstract

BACKGROUND—Unlike pharmacological interventions in sepsis, extracorporeal blood purification which is widely used in septic patients is not typically studied in experimental rodents. Most of the previous studies have performed extracorporeal blood purification in larger animals and typically use artery-venous (AV) vascular access. We developed a venous-venous (VV) purification model in the rat as a treatment for sepsis.

METHODS—Using adult male Sprague Dawley rats we cannulated the femoral artery or vein and the jugular vein with P50 tubing and created a VV or AV circuit. Blood flow was determined by arterial pressure in the AV circuit while in the VV circuit the blood flow was regulated using a rotary pump. The safety of this circuit was evaluated using the changes of blood interleukin-6 (IL-6), rectal temperature and seven-day survival with sham extracorporeal circulation (circuit connection without treatment) to the control (without circuit). The main side effects of this V-V circuit with A-V circuit were compared.

RESULTS—The difference of IL-6, body temperature and cumulative survival were no statistically significant after extracorporeal circulation. The main side effects of extracorporeal circulation occurred less often with VV compared to AV therapy: massive bleeding (2.5% vs. 15%, $p=0.04$), clot formation (2.5% vs. 15%, $p=0.04$). This VV circuit has been successfully used in different septic rodent models with different techniques (hemoadsorption and hemofiltration).

CONCLUSIONS—VV blood purification in a rodent model appears to be effective and is safer than AV circuit.

Keywords

apheresis; sepsis; animal model; rat; hemofiltration

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CONFLICT OF INTEREST STATEMENT

None declared.

1. Introduction

Sepsis is one of the main causes of death in critically ill patients. The pathophysiology of sepsis is complex and not completely understood. The pro-inflammatory and anti-inflammatory response can lead to cell and organ dysfunction and, in many cases, death [1]. Thus, the goal of the intervention is to restore the homeostasis of circulating mediators rather than to specifically inhibit pro- or anti-inflammatory mediators.

Extracorporeal blood purification therapy is a special treatment, in which blood circulates through a device where waste products and water are removed, and replacement fluid is added and purified blood is return to the patient. The blood purification has also been reported to remove a wide array of inflammatory mediators. The effects are broad-spectrum and auto-regulating. It is also demonstrated to restore immune function through improving antigen-presenting capability, adjusting leukocyte recruitment, oxidative burst and phagocytosis, and improving leukocyte responsiveness [2]. Extracorporeal blood purification might become an alternative therapy, particularly given recent high-profile failed trials in sepsis.

A great deal of work has been doing in order to find and optimize the best extracorporeal blood purification therapy for sepsis. Most of the animal work has been done in big animals, such as sheep [3], pigs [4,5] and dogs [6,7]. Due to blood volume limitation, it appears difficult to carry out extracorporeal blood purification in rodents. Although some recent studies were performed in rodents, this rodent model was artery-venous circulation in which flow rates were affected by the artery blood pressure [8, 9, 10, 11].

The purpose of our work was to develop a venous-venous blood purification model for sepsis.

2. Material and Methods

2.1. Materials & Reagents

Animals—Adult, male Sprague-Dawley (SD) rats (500–600g) (Charles River Laboratories, Wilmington, MA).

Anesthesia/Analgesia—Pentobarbital sodium injection (Hospira Inc., Lake Forest, IL) for general anesthesia. Buprenorphin (0.3 mg per kg body weight) (Ben Venue Laboratories, Inc., Bedford, OH) for post-operative analgesia.

Sterile alcohol prep pads (Fisher Scientific, Pittsburgh, PA).

Sterile saline solution (0.9% wt/vol) saline (Hospira Inc., Lake Forest, IL).

Sterile heparin sodium injection (Hospira Inc., Lake Forest, IL).

Isopropyl alcohol 70% (Fisher Scientific, Pittsburgh, PA).

Escherichia coli endotoxin (O111:B4, Sigma Chemical, St. Louis, MO).

Escherichia coli bacteria strain E25922 (American Type Culture Collection, Rockville, MD).

Polyethylene-50 catheters (Becton Dickinson, Franklin Lakes, NJ).

22 gauge bendable tips (Inter-Med Inc., Racine, WI).

18 gauge & 20 gauge needles (Becton Dickinson, Franklin Lakes, NJ).

Equipment

Surgical equipments: dissection scissors, micro-dissection scissors, curved surgical forceps, straight anatomical forceps and needle holder (Roboz Surgical Instrument Co., Gaithersburg, MD).

Rotary mini- pump (Fisher Scientific, Pittsburgh, PA)

Sterilized hemoadsorption cartridge (1ml, CytoSorb, RenalTech International, New York, NY).

Sterilized hemofiltration cartridge (5ml, Oxiris®, Gambro, Meyzieu cedex, France)

Heating pad (Gaymar Industries, Orchard Park, NY)

Clinical thermometers (Jorgensen Laboratories, Inc., Loveland, CO).

2.2.Procedures

2.2.1.Preoperative preparation—Experiments were complied with national and institutional regulations concerning the use of animals for research purposes. The weight of the animals was determined since anesthetics administration is based on it. We also recommend using sterilized latex gloves, face mask and surgical gown as a protective equipment for the operator keeping at the same time the surgical field reasonably aseptic.

Animals were anesthetized with pentobarbital sodium (50 mg per kg body weight i.p.). The intensity of anesthesia was monitored by toe pinch using tweezers. Adequate anesthesia should result in no response of extremity (e.g., no flexion of extremity).

Potential surgical skin area was shaved using an electric trimmer and disinfected with alcohol prep pads. Animals were placed onto Styrofoam pads on their backs, with heads oriented away from the operator.

2.2.2. Set up of sepsis

A. Endotoxin injection induced sepsis: Before endotoxin administration, the animals were maintained in a steady state at least for 30 min. *Escherichia coli* endotoxin was administered intravenously (over 1 min) at a dose of 20 mg/kg (diluted in 1 ml of 0.9% saline). This dose and strain of endotoxin were selected because they result in physiologic conditions that closely resemble human septic shock [8]. These conditions include hemodynamic instability, diffuse capillary leak syndrome, and metabolic acidosis. In sham controls, the same amount of saline was injected. Each animal was resuscitated with injecting pre-warmed normal saline (37 °C; 5 ml per 100 g body weight) subcutaneously.

B. Bacterial clot implantation induced sepsis: A calculated dose of live *E. coli* bacteria ($2 \times 10^8 - 2 \times 10^9$ CFU/ml) was inoculated in the peritoneal cavity. The bacteria was prepared in a fibrin clot and counted as described by Ahrenholz [12]. Briefly, the clot was prepared in the following manner. A 2% solution of bovine fibrinogen in sterile phosphate buffered saline (PBS) was prepared. For rats receiving the *E. coli* implant, the target bacterial load was suspended in this solution. The fibrinogen solution was adjusted to the rat's weight (10mL of solution/Kg); in a 500-g rat, the volume of 2% fibrinogen solution was approximately 5 mL. Next, 5U of thrombin/mL of 2% fibrinogen solution was added, and these reagents were mixed well in a tube – the mixture would clot in approximately 20–30 seconds. A clot of 1 inch in length and 1/2 inch wide was formed. Once the rat was

anesthetized, an abdominal incision of approximately 1.5 inch was made. The clot was placed into the left flank, just above the cecum. The clot did not immediately attach the abdominal wall. It was allowed to float freely until an inflammatory response was induced. Once inflammation was induced, the clot began to adhere to the gastric intestinal tract. Usually, it remained localized into the left abdominal flank, among loops of small intestine (based on autopsy of the animals in past experiments). In sham controls, the same size of clots without live bacteria was planted in the peritoneal cavity. Animals were resuscitated by injecting pre-warmed normal saline (37 °C; 5 ml per 100 g body weight) subcutaneously.

C. Cecal ligation and puncture (CLP) induced sepsis: After the peritoneal cavity was open, cecum was located and isolated by using blunt anatomical forceps. The mesentery of the cecum was carefully dissected. The cecum was ligated at the designated position for the desired severity grade. The ileocecal valve was avoided to be ligated so that intestinal continuity was maintained. The cecum was perforated by single through-and-through puncture midway between the ligation and the tip of the cecum in a mesenteric-to-antimesenteric direction. The tied segment was gently pressed to ensure that a small amount of feces was extruded on to the surface of the bowel. The cecum was returned to the peritoneal cavity. The wound was closed using 4-0 braided absorbable suture for the muscle layer and the skin with surgical staples. In sham controls, the cecum was exposed but not ligated or punctured, then returned to the abdominal cavity. Animals were resuscitated by injecting pre-warmed normal saline (37 °C; 5 ml per 100 g body weight) subcutaneously.

2.2.3. Set up of extracorporeal blood purification therapy circuits—We started the blood purification in different time points based on different septic models, as different models induced sepsis in different time points. For the endotoxin induced sepsis, we started extracorporeal blood purification immediately after endotoxin injection. For live bacterial clot implantation induced sepsis, we started the therapy 20–24 hr after implantation. For CLP induced sepsis, we started the therapy 18–20 hr after CLP.

A. Hemoadsorption: Before the device was set up, the polyethylene-50 catheters were connected with 22 gauge bendable tips. All the tubing was sterilized with Isopropyl alcohol 70%. Then the tubing was flushed with heparinized saline (heparin 30 IU/ml). During general anesthesia, the femoral vein and jugular vein were carefully dissected and isolated (Figure 1 A & B). The vein was cut with microdissection scissors. The catheter introducer was inserted inside the vein (Figure 1 C) and the polyethylene-50 catheter was put beneath the introducer (Figure 1 D). The catheter was advanced slowly and gently until the catheter reached venae cavae (Figure 1 E). When the catheter was in venae cavae, it was easy to draw blood (Figure 1 E & F). The extracorporeal circulation was set up as femoral vein, hemoadsorbent column, mini pump and jugular vein. The flow rate was controlled at 0.8–1.0 ml/min (similar to 12–15ml/min in a 70kg of patient) with the pump (Figure 2A).

B. Hemofiltration: As the total volume of the hemofiltration circuit (filter + tubing) is about 5 ml, the circuit was pre-filled with 5 ml of septic blood from another animal (donor). Prepared the tubing system, cannulated the veins, and set up the extracorporeal circulation as hemoadsorption. The ultrafiltration outlet and replacement inlet tubing system were connected. The replace fluid was provided to the replacement system. The ultrafiltration fluid was collected from the ultrafiltration outlet (Figure 2B). Both inlet and outlet flow rates were controlled exactly the same rates with the mini-pump (approximately one third of rate as the whole the circulation rate).

C. Sham treatment: The extracorporeal circulation was set up as hemoabsorption without any treatment columns. The circulation was pre-filled with saline or septic blood as the treatment circulation (Figure 2C).

During the period of blood purification, the anesthesia was maintained with pentobarbital sodium (10–20mg per kg body weight i.p. every 1–2hrs). All animals were maintained temperature at about 36–37°C with heating pad.

2.3. Measurements and statistical analysis

To explore the safety of this device, we compared the changes of blood interleukin-6 (IL-6), rectal temperature (T), and seven-days survival with 4 hr's sham extracorporeal circulation (n=14) compared to the same CLP animals (n=14) without extracorporeal circulation (CLP control). Data (IL-6 and T) were expressed as mean $\pm$ standard error (SEM). We also compared the incidence of the main side effects (massive bleeding, clots formation, and air embolism) between the AA circuit and the VV circuit. The differences of numeric data (IL-6 and temperature) between the two groups were compared with paired T-test. The Chi-Squared test was used to compare the differences of categorical data. The survival time was assessed by Kaplan-Meier and overall survival in each group was compared using Fisher's exact (SPSS11, Chicago, IL). $P < 0.05$ was considered to be significant differences.

3. Results

Since 2008, we have done almost 300 cases of blood purification therapies using this venous-venous rodent model of extracorporeal circulation, which has been successfully used in different septic rodent models with different techniques (hemoabsorption and hemofiltration) [13,14,15,16]. Ten to fifteen minutes were required to perform the bacterial clot peritoneal implantation or CLP procedure and about one hour was required to set up the entire blood purification circuit.

In order to estimate the potential harmful side effects of such a circuit, we compared the survival rates of the rats that underwent sham extracorporeal circulation with control rats (only fluid resuscitation) after the same CLP procedure in all animals. Although the cumulative survival of the sham treatment was worse than that of CLP control, the difference was not statistically significant (35.71% vs 42.86%, $P > 0.05$, Figure 3A). The concentrations of IL-6 in sham group were mild higher than those in CLP control. However, the differences were not statistically significant (308.33, vs 283.75 pg/ml, $P > 0.05$, Figure 3B). There was also a tendency to decrease body temperature with the sham extracorporeal circulation (36.7 vs 36.95°C, $P > 0.05$, Figure 3C). The main side effects of extracorporeal circulation were massive bleeding (defined as more than 20% of the effective blood volume loss during the procedure day), clots, air embolism and hypothermia. There were fewer side effects with VV compared to AV therapy: massive bleeding (2.5% vs. 15%, $p = 0.04$), clot formation (2.5% vs. 15%, $p = 0.04$, Table 1). These side effects could be minimized with enough heparinization, careful observation and heating pad.

4. Discussion

We chose this kind of rat, as its effective circulative blood volume is about 15–20 ml. It may be the smallest animal used in the study of extracorporeal blood purification. Its age is similar to that of the middle-to old human. The SD rats have a consistent reproducible inflammatory response in different septic model, which resembles clinical sepsis.

Many of the problems with the use of animal data in sepsis stem, not only from the animal models per se, but from how those results have been adapted to clinical trial designs [17].

Animal models provide insights about specific components of the septic process but cannot truly mimic the full clinical complexity and intrinsic heterogeneity of patients with sepsis. Endotoxin or lipopolysaccharides (LPS), as the principal component of the gram negative bacterial cell wall, stimulates the release of inflammatory mediators from various types of cells, responsible for the initiation of the sepsis process. This has formed the basis for the simplest sepsis model. This model produces a rapid onset of hypodynamic circulatory collapse and a more rapid resolution or decline to mortality. It is suitable for the study of acute changes, such as the mediator changes after the blood purification therapies. However, this model reflects a primarily systemic challenge without an infectious focus. There is general agreement that LPS injection may serve as a model for endotoxic shock but not for sepsis [18]. Thus, caution must be exercised whenever assessing clinical efficacy of blood purification in animal models of endotoxemia. CLP is widely used and known to closely mimic the pathophysiology of septic human patients. It is polymicrobial sepsis, characterized with an early hyperdynamic and a late hypodynamic phase if fluid resuscitation is enough. The overall lethality is closely related to the conditions under which CLP is used, such as the length of the ligation, the number and size of puncture [19]. The lethal short-term survival and sub-lethal long-term survival septic models can be produced based on requirements. However, the main problem of the CLP model is the variability as this model delivers a variable microbiological dose. Another problem for sub-lethal long-term survival model produces only limited cytokines, which is carefully considered in the blood purification study [9]. In order to control the microbiological dose, pure bacterial culture peritonitis models have been developed [12,14]. When the same number of bacteria is implanted intraperitoneally in a fibrin clot, highly reproducible sepsis is induced with compromised myocardial performance [12], and a high mortality rate [20]. However, this model cannot accurately mimic real clinical sepsis as CLP model, as it is not a polymicrobial sepsis and fails to reproduce the synergy between aerobic and anaerobic organisms seen in human peritonitis. Until now, CLP induced sepsis is still the gold standard for animal model. The problem of variability is easily overcome by increasing the sample size. The severity of sepsis can be titrated based on the design.

Our research team had previously worked with the arterial-venous blood purification circuit in rats [8,9]. This technique cannot mimic clinically venous-venous blood purification. Moreover, it is affected by the blood pressure and cardiac output. We therefore tried to set up the venous-venous blood purification.

The main difficulty during vascular access was that it was difficult to draw blood from the venous line, as it was easily collapsed. We tried different tubing and adjusted the catheter in different sites. The critical point was to put the catheter in venae cavae and draw blood slowly. Finally we set up this venous -venous blood purification circuit in rats.

The overall safety of this system was first evaluated between septic rats treated with the sham venous-venous blood purification and septic rats treated with control (only saline). The mortality of the extracorporeal circuit group was a little bit higher than that of CLP control, although the difference was no statistical significance. It may be related to the activation of inflammatory response induced by the extracorporeal circulation [21]. The tubing we used in the circuit is made of common polyethylene, which may activate the inflammatory mediators. To decrease the activation, the tubing should be optimized.

The main side effects during extracorporeal circulation were bleeding, clots and air embolism in the system, which were compared between the VV circuit and AV circuit. There were actually fewer massive bleeding and clots formation in this VV circuit compared to AV circuit (table1). Because of no arterial cannulation, the risk of bleeding from puncture site in artery is decreased. The flow rate in AV circuit was controlled by the blood pressure

while the flow rate of this venous-venous blood purification could be easily controlled with the mini-pump. It is likely to induce the clots in the AV circuit if the flow rates decrease due to lower blood pressure. We used 20u/ml of heparin (diluted in saline, 0.2–0.3ml/hr) to flush the tubing in order to prevent the potential clot formation. Another advantage of this VV system over AV system is that it offers a more stable blood flow rate, assures a more precise removal of solutes and produce a better efficacy. A strong evidence of the efficacy is that this system has been successfully coupled with the hemoabsorption or hemofiltration. The incidence of air in the circuit may be higher than that in AV system. However, we didn't find there were more air embolisms in the VV circuit compared to AV circuit, as we also adjusted the location of the inlet tubing to prevent the potential air into the vein.

There are some limitations in this device. First of all, there is no attachment in this device which can adjust the temperature during the process of blood purification. If the treatment of blood purification persists for a longer time, there might be a significant decrease in animal's body temperature. In our experiments, the heating pad was used and levels were adjusted to maintain the animal's body temperature. Secondly, there are not attachments to monitor the hemolysis or thrombus in the circuit. We had to use some other ways to monitor and prevent hemolysis and thrombus during the circulation. Fortunately, we did not find much hemolysis or thrombus during our experiments. However, it still needs to be improved in our future study.

In conclusion, this venous-venous blood purification rodent model appears to be effective and safe. We recommend its use for studies which aim will be to assess extracorporeal therapies in rats.

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Figure 1. Procedures for vascular cannulation

- A. Isolate the vessels from the surrounding tissue.
- B. Separate the vein from the artery.
- C. Cut the vein and insert the catheter introducer.
- D. Put the catheter beneath the introducer
- E. Adjust the position of the catheter until it is easy to aspirate the blood.
- F. Vascular cannulation is finished.

Figure 2. Set up the extracorporeal blood purification
A. Hemoadsorption.
B. Hemofiltration.
C. Sham treatment.

A

B

C

Figure 3. Effects of sham extracorporeal blood purification (EBP) device on vital signs
 A. Changes of seven days survival.
 B. Changes of IL-6 after 4hr's sham extracorporeal blood purification (mean $\pm$ SEM, pg/ml).
 C. Changes of rectal temperature after 4hr's sham extracorporeal blood purification (mean $\pm$ SEM, °C).

Table 1

The comparison of main side effects between artery-venous circuit and venous-venous circuit (incidence)

Side effects	Artery-venous circuit	Venous-venous circuit	P values
Massive bleeding	15%	2.5%	0.04
Air embolism	5%	7.5%	>0.05
Clot formation	15%	2.5%	0.04