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Efficacy of a novel endotoxin adsorber polyvinylidene fluoride fiber immobilized with L-serine ligand on septic pigs*

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Abstract: A novel adsorber, polyvinylidene fluoride matrix immobilized with L-serine ligand (PVDF-Ser), was developed in the present study to evaluate its safety and therapeutic efficacy in septic pigs by extracorporeal hemoperfusion. Endotoxin adsorption efficiency (EAE) of the adsorber was firstly measured in vitro. The biocompatibility and hemodynamic changes during extracorporeal circulation were then evaluated. One half of 16 pigs receiving lipopolysaccharide (*Escherichia coli* O111:B4, 5 μg/kg) intravenously in 1 h were consecutively treated by hemoperfusion with the new adsorber for 2 h. The changes of circulating endotoxin and certain cytokines and respiratory function were analyzed. The 72 h-survival rate was assessed eventually. EAE reached 46.3% (100 EU/ml in 80 ml calf serum) after 2 h-circulation. No deleterious effect was observed within the process. The plasma endotoxin, interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) levels were decreased during the hemoperfusion. Arterial oxygenation was also improved during and after the process. Furthermore, the survival time was significantly extended (>72 h vs. 47.5 h for median survival time). The novel product PVDF-Ser could adsorb endotoxin with high safety and efficacy. Early use of extracorporeal hemoperfusion with the new adsorber could reduce the levels of circulating endotoxin, IL-6, and TNF-α, besides improve respiratory function and consequent 72 h-survival rate of the septic pigs. Endotoxin removal strategy with blood purification using the new adsorber renders a potential promising future in sepsis therapy.

Key words: Sepsis, Endotoxin, Adsorption, Polyvinylidene fluoride matrix immobilized with L-serine ligand (PVDF-Ser), Hemoperfusion, Pig

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1 Introduction

Sepsis or severe sepsis is a complex entity, with high morbidity and mortality in intensive care unit. Some progressions in the syndrome treatment have been achieved or validated, including early goal-directed therapy (EGDT), use of activated protein C (drotrecogin α), corticosteroids, and anti-cytokines, or endotoxin treatment (Bernard *et al.*, 2001; Rivers *et al.*, 2001; van der Poll, 2001). However, the general mortality still ranges from 28% to 47% in USA and Europe (Martin *et al.*, 2003; Vincent *et al.*, 2006), while up to 48% in China (Cheng *et al.*, 2007).

Extracorporeal apheresis for sepsis has been under great consideration, especially the technique of endotoxin (lipopolysaccharide, LPS) adsorption, on which promising outcomes have been achieved in preclinical (animal) and clinical trials (Stegmayr, 2008). As an adjunctive therapy for sepsis, direct hemoperfusion with polymyxin B bound and immobilized to polystyrene fibers (DHP-PMX) has been widely employed in clinical setting since 1994 in Japan (Shimizu *et al.*, 2006; Cruz *et al.*, 2007; 2009).

Accompanied with advances made in chromatography and industrial separation applications, sorbent technologies have made tremendous progress in the last two to three decades (Jaber and Pereira, 1997).

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Given the possible nephrotoxicity and neurotoxicity of PMX and its costly procedure (Cruz *et al.*, 2007), other biocompatible materials have been explored extensively in order to remove endotoxin or other mediators among inflammatory cascade. The materials include specific membranes as Lixelle® (Shimizu *et al.*, 2006), an adsorbent for cytokine removal (CTR) (Taniguchi *et al.*, 2007), diethylaminoethyl cellulose (Bengsch *et al.*, 2005), and polymethylmethacrylate (Nakamura *et al.*, 2010), particular adsorbent as resin (Tetta *et al.*, 2002; Amoureux *et al.*, 2004) and activated charcoal (Howell *et al.*, 2006), and some ligands as human serum albumin (Bracht *et al.*, 2009) and certain amino acids (Umgelter *et al.*, 2008) covalently immobilized onto macroporous polymeric beads. These adsorber cartridges selectively or non-selectively remove humoral endotoxin, overwhelming cytokines, and other bacterial components extracorporeally, for amelioration of the patient's condition by sepsis immunomodulation.

Polyvinylidene fluoride (PVDF) is a kind of material with non-toxicity, high biocompatibility, and chemical stability, being applied in much medical manipulation (Klinge *et al.*, 2002; Neuss *et al.*, 2008). We have reported that PVDF hollow fiber with chitosan or certain amino-acid ligands was employed to remove endotoxin in medical reagents (Sun *et al.*, 2005; 2006). Different amino-acid ligands have different endotoxin adsorption efficiency (EAE) and interaction mechanisms. L-Serine (Ser) ligand has been reported to possess favorable efficiency of endotoxin removal from serum in endotoxemic rabbits. Hydroxyl group of the ligand interacts with phosphoric residue of endotoxin by three couples of hydrogen bonds to form a stable cage-like structure (Wei *et al.*, 2007).

The present study was conducted to evaluate the safety and efficacy of hemoperfusion using the novel adsorber, PVDF-Ser, on endotoxin-challenged severe sepsis in pigs.

2 Materials and methods

2.1 Adsorber preparation

PVDF-Ser adsorber (Department of Chemical & Biochemical Engineering, Zhejiang University, China) had 6400 hollow fibers, the effective contact area of 13 m^2, the priming volume of 80 ml, and the gross length of 18 cm. The outer diameter of the PVDF hollow fiber is 1100 μm, while the inner diameter is 800 μm. As a new patent, it was protected by State Intellectual Property Office of China. The PVDF fiber cartridge without Ser ligand was also obtained as a control for the following in vitro study.

2.2 In vitro EAE study of PVDF-Ser adsorber

To evaluate the EAE at a dynamic state, 80 ml of calf serum solution with LPS (100 EU/ml; *Escherichia coli* O111:B4, Sigma, USA) was circulated within PVDF-Ser adsorber. The closed circuit was connected with polyethylene tubes and a peristaltic pump (BT01-100 mode, Baoding Longer Precision Pump Co., Ltd., China) at a flow rate of 80 ml/min. The endotoxin level was detected at every 30 min during a 2 h-circulation period.

2.3 Animal preparation

Twenty-four male pigs weighing (21±3.5) kg were supplied by a local farm, and then adapted to laboratory environment with water ad libitum for 24 h before brought into the experimental procedure. The study was approved by the Institutional Animal Care and Use Committee of Zhejiang University, China. The experimental animals were treated according to the animal ethics guidelines of the Chinese National Health and Medical Research Council.

2.4 Animal anesthesia and surgical preparation

Intramuscular injections of ketamine 5 mg/kg, atropine 10 μg/kg, and midazolam 0.5 mg/kg were administrated for premedication. Anesthesia was initiated with ketamine 5 mg/kg and midazolam 0.5 mg/kg intravenously, followed by midazolam 1–2 mg/(kg·h) and fentanyl 20–40 μg/(kg·h). The level of anesthetic state was assessed through pain stimuli to the fore hoof by a forceps without muscle relaxants. Additional doses of fentanyl or midazolam were given when analgesia or sedative was needed. The animals were orally intubated after given a bolus of pancuronium bromide 0.5 mg/kg for muscle paralysis. Thereafter, mechanical ventilation was implemented with oxygen in air (FiO_2 40%) and a positive end expiratory pressure level of 3–4 cmH_2O (Servo300 ventilator, Siemens, Sweden). A volume-control mode was introduced with a tidal volume of

10 ml/kg and a respiratory rate of 20 breaths/min. The body temperature was maintained constant around 37–38 °C by use of a heating blanket.

A 9-F single-lumen catheter was inserted into the left cervical artery for measurements of mean arterial pressure (MAP) and heart rate, sampling of arterial blood gas, hematology, and blood chemistry. A 10.5-F double-lumen catheter was introduced into the right internal jugular vein for infusion of fluid, endotoxin and anesthetics. A 14-F double-lumen catheter was cannulated into the right femoral vein and served as hemoperfusion access. A cystostomy catheter for urine drainage was positioned suprapubically into the bladder after a minor vesicotomy performed. All catheters were placed by direct cutdown under sterile conditions and then the wounds closed surgically. Every swine recovered from the effects of surgical operation after 2 h-stabilization period.

2.5 Experimental protocol

After baseline measurements, the swines were allocated to one of the following four groups: Group A without endotoxin challenge and extracorporeal hemoperfusion, n=4; Group B with extracorporeal hemoperfusion, but no endotoxin challenge, n=4; Group C with endotoxin challenge, n=8; Group D with endotoxin challenge and extracorporeal hemoperfusion using PVDF-Ser adsorber, n=8.

The hemoperfusion circulation was impelled by the above peristaltic pump with a blood flow rate of 80 ml/min. A bolus dose of 150 IU/kg heparin sulfate was infused intravenously for systemic anticoagulation, followed by a continuous infusion of 40 IU/(kg·h) in all animals, including those not receiving extracorporeal hemoperfusion. Groups A and B were designed for comparable study of the biocompatibility and hemodynamic changes during hemoperfusion with the adsorber. Hemodynamics, hematology, and blood chemistry were measured at baseline and every half-hour during the 2 h-circulation in Groups A and B. The animals in Groups C and D were infused continuously with 5 μg/kg endotoxin by a syringe pump over 1 h. Group D swines were consecutively treated with extracorporeal hemoperfusion using the novel adsorber for 2 h. The concentrations of plasma endotoxin, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), and arterial blood gases were detected at baseline and every half-hour during the above 3 h. Ringer's lactate was started intravenously at a rate of 8 ml/(kg·h), regulated to 12 ml/(kg·h) when MAP was less than 70 mmHg, and 18 ml/(kg·h) once MAP was less than 50 mmHg, for adequate fluid resuscitation, and vice versa. No colloids or vasoactive reagents were used. After hemoperfusion, mechanical ventilation, bypass devices, and fluid resuscitation were withdrawn from the experiment. The septic pigs were subsequently monitored until spontaneous death or for 72 h. Animals surviving to 72 h were then sacrificed with a bolus injection of 15 ml KCl under deep anesthesia.

2.6 Measurements

The endotoxin levels within calf serum and swine plasma were measured by the tachypleus amebocyte lysate assay (Chinese Horseshoe Crab Reagent Manufactory, Xiamen, China) through quantitative chromogenic technique using ultraviolet (UV) spectrophotometer (Zhejiang Institute of Metrology Sciences, China). The lowest level for detection was 0.01 EU/ml (The term EU described the biological activity of endotoxin and 120 pg of endotoxin from *E. coli* O111:B4 corresponded to 1 EU). Hemoglobin, white blood cells, and platelets were detected on Automatic Hematology Analyzer (ADVIA 120, Bayer HealthCare, Germany). Plasma creatinine, albumin, alanine aminotransferase, aspartate aminotransferase, total bilirubin, K, Na, and Cl were measured by use of an Automatic Biochemistry Analyzer (ADVIA 2400, Bayer HealthCare). Arterial blood gas was performed on Blood Gas Analyzer (Rapidlab 1265, Bayer HealthCare), while PT and APTT were tested on Coagulation Analyzer (Coatron M2, TECO, Germany). TNF-α and IL-6 concentrations were detected by the enzyme-linked immunosorbent assay kits (ELISA, Shanghai Bluegene Biotech, China). The survival state was observed for the following survival analysis.

2.7 Statistical analysis

Measurement data were expressed as mean± standard deviation (SD). Comparison analyses between and within groups were implemented using analysis of variance (ANOVA) for repeated measures followed by the least significant difference (LSD) test (post hoc). The survival rates were analyzed by the Kaplan-Meier method and log-rank test. The analyses

were performed with a statistical package for social science for windows (SPSS, Chicago, IL, USA), and a *P*-value less than 0.05 was considered statistically significant.

3 Results

3.1 In vitro EAE

Endotoxin level decreased evidently through PVDF-Ser adsorber during the 2 h-circulation time, particularly within the first half-hour period, whilst almost no changes were found through PVDF carrier cartridge (Fig. 1). There was 32.5% endotoxin removal through PVDF-Ser adsorber after the first half-hour circulation, meanwhile a moderate descent was achieved during the following 1.5 h. The EAE reached 46.3% after the 2 h-dynamic circulation, which meant 0.029 EU/cm^2.

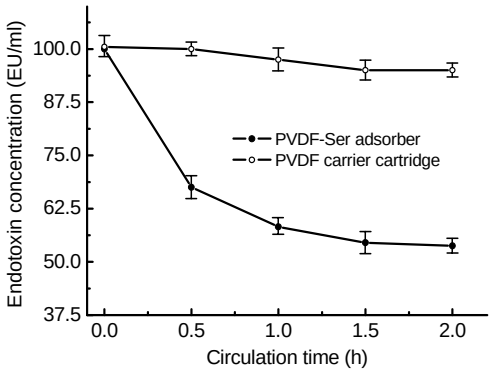

Fig. 1 Endotoxin adsorption efficiency in a dynamic way
Endotoxin level was significantly decreased through PVDF-Ser adsorber compared to that only through PVDF carrier cartridge during the 2 h-circulation period (P<0.01). 32.5% of total endotoxin amount (100 EU/ml in 80 ml calf serum) was removed during the first half-hour, while the adsorption efficiency reached 46.3% in the end; in other words, 0.029 EU/cm^2

3.2 In vivo biocompatibility and hemodynamic effect of the adsorber device

In Groups A and B, heart rate and MAP were not significantly changed during the 2 h-hemoperfusion (data not shown). The plasma creatinine, albumin, alanine aminotransferase, aspartate aminotransferase, total bilirubin, and electrolytes were relatively stable during the blood apheresis process (data not shown). The partial pressure of arterial oxygen (PaO_2), partial pressure of arterial carbon dioxide ($PaCO_2$), pH, and base excess were also not apparently influenced by direct hemoperfusion (data not shown). No swine in both groups died during the observation time. These attractive results demonstrated that the novel adsorber had no deleterious effect on hemodynamics, renal, hepatic, or respiratory function.

Hemoglobin and leukocyte levels were not affected yet. However, platelet count was decreased by about 30% at the end of 2 h-blood apheresis process. Reduction of platelet count involved in the hemoperfusion process was not thought to be a specific phenomenon derived from the novel material, but a generally observed phenomenon during extracorporeal circulation (Gritters-van den Oever *et al.*, 2009), which could be evaluated in future preclinical and clinical trials.

3.3 Effect on plasma endotoxin

In Groups C and D, the plasma endotoxin level ascended quickly during the 1 h-LPS infusion. The level reached approximately ten-fold at the end of infusion when compared to the baseline (0.3 EU/ml vs. 0.03 EU/ml, Fig. 2). The decrease of endotoxin level was more striking during the 2 h-extracorporeal

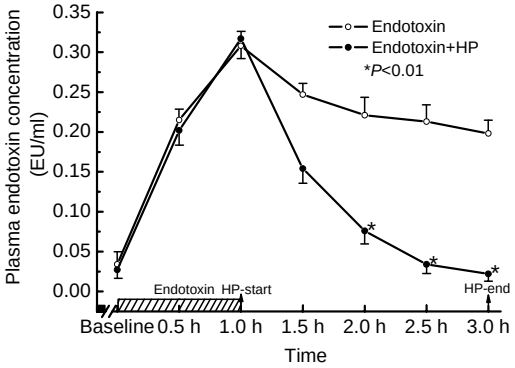

Fig. 2 Changes of plasma endotoxin level during 1 h-lipopolysaccharide (LPS) infusion and then hemoperfusion (HP) therapy
The graph presented the changes of circulating endotoxin concentration in septic pigs undergoing extracorporeal HP using the PVDF-Ser adsorber. The endotoxin level was elevated after 1 h-LPS infusion (5 μg/kg) to approximately ten-fold compared to the baseline. The level decreased more rapidly during HP therapy than that without HP treatment ($^*P$<0.01). The difference could be observed as early as 1 h after HP initiation. Note that the endotoxin level descended to a nearly normal stage at the end of 2 h-HP process

hemoperfusion using the adsorber compared to that without hemoperfusion therapy (P<0.01). The reduction could be observed as early as 1 h-circulation. The level almost descended to the baseline at the end of treatment.

3.4 Effect on inflammatory mediators

IL-6 and TNF-α in septic swines were released excessively during and after LPS infusion (Fig. 3). The IL-6 level reached the peak at about 2 h after LPS infusion start, while the peak of TNF-α level shifted forward by about 0.5 h. The process of the mediators' accumulation was broken after the consecutive hemoperfusion intervention. Both mediators' levels during the treatment with the adsorber decreased more obviously than that without the therapy, early in the first half-hour of treatment (P<0.01).

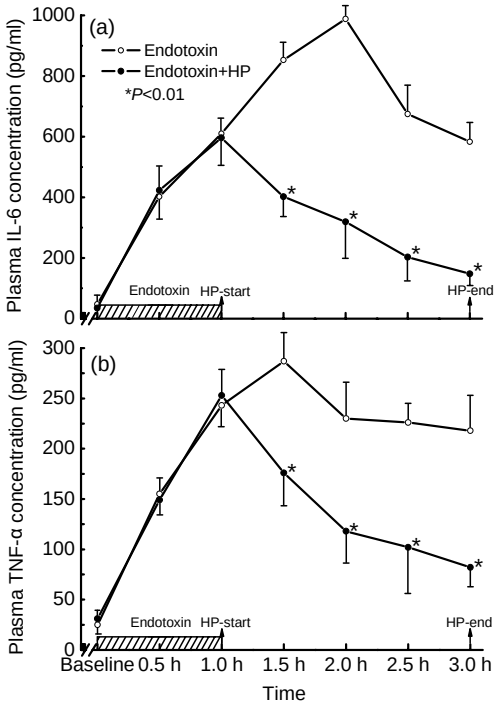

Fig. 3 Changes of circulating IL-6 and TNF-α levels during endotoxin infusion and hemoperfusion (HP) therapy

The graphs exhibited the changes of plasma IL-6 (a) and TNF-α (b) concentrations in septic swines treated by HP using the PVDF-Ser adsorber. The IL-6 level without HP intervention ascended to its peak after approximately 2 h after the start of endotoxin infusion, while TNF-α level did for about 1.5 h. However, the rising course was blocked when HP initiation. The levels decreased significantly during HP process compared to that without HP therapy, even as early as 0.5 h after the therapy ($^{*}P$<0.01)

3.5 Effect on PaO_2

Acute arterial hypoxemia in septic pigs was apparently observed after 1 h-endotoxin challenge (about 200 mmHg vs. 90 mmHg). Hopefully, PaO_2 ascended gradually during the hemoperfusion therapy using the adsorber, early after 0.5 h-treatment (Fig. 4). Moreover, arterial oxygenation was improved significantly after 1.5–2 h hemoperfusion compared to that without the therapy (P<0.05). This meant that the impairment of lung as an endotoxin-sensitive organ could be attenuated by the timely therapy.

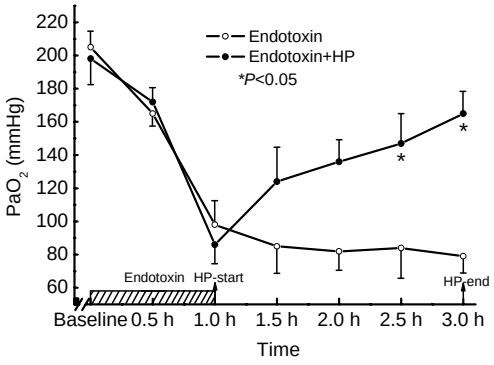

Fig. 4 PaO_2 changes during endotoxin infusion and hemoperfusion (HP) therapy

The graph displayed the changes of partial pressure of arterial oxygen (PaO_2) in septic pigs by HP therapy with the PVDF-Ser adsorber. During the stage of endotoxin challenge and HP treatment, the pigs were mechanically ventilated with fraction of inspired O_2 (FiO_2) 40%. PaO_2 decreased after endotoxin induction, to approximately 90 mmHg. Hopefully, an upward trend was observed as early as 0.5 h after HP initiation. Arterial oxygenation was improved significantly after the 2 h-process, even after 1.5 h-treatment, compared correspondingly to that without the therapy ($^{*}P$<0.05)

3.6 Effect on survival

Five septic pigs (5/8, 62.5%) survived for 72 h after 2 h-hemoperfusion with the adsorber, whilst only two (2/8, 25.0%) survived for 72 h after severe sepsis insult without the hemoperfusion treatment (Fig. 5). The survival time was extended correspondingly (>72 h vs. 47.5 h for median survival time, P<0.05).

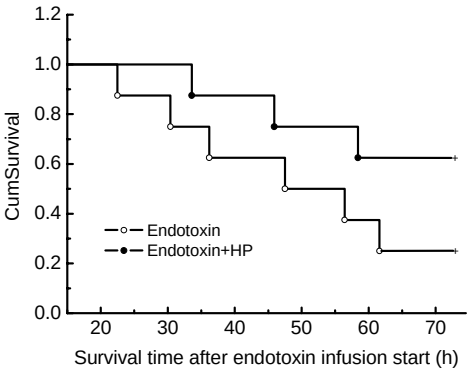

Fig. 5 Survival analysis for septic swines with hemoperfusion (HP) therapy

The graph showed the comparable analysis of survival time for septic pigs undergoing HP therapy using the PVDF-Ser adsorber. Five septic pigs (5/8, 62.5%) survived for 72 h after extracorporeal HP treatment; however, only two (2/8, 25.0%) survived for 72 h once without HP therapy. The survival time was significantly prolonged after the treatment (>72 h vs. 47.5 h for median survival time; $P<0.05$, by the Kaplan-Meier method and log-rank test)

4 Discussion

In the present study, a novel endotoxin adsorption column with PVDF as carrier matrix and L-serine as ligand has been developed for endotoxin removal. The design purpose of the new adsorber is for future potential use in an extracorporeal circulation system in treatment of septic patients. The results have revealed that hemoperfusion using the new adsorber could significantly reduce circulating endotoxin, IL-6 and TNF-α, and meanwhile improve respiratory function and 72 h-survival rate of the septic pigs.

Septic patients induced by Gram-negative bacterial infection are usually accompanied with elevation of endotoxin concentration. Higher levels portend a poor outcome in severe sepsis and septic shock (Marshall *et al.*, 2004). Even if there is no identified infection, endotoxemia could occur from bacteria or endotoxin translocation in gastrointestinal tract due to the epithelial barrier dysfunction. Furthermore, it has been regarded as a key trigger of severe sepsis and septic shock (Opal, 2002). So, endotoxin has long been under consideration for sepsis therapy. Some anti-endotoxin therapies have been tested in preclinical and clinical studies, such as certain endotoxin-neutralizing peptides and/or anti-endotoxin antibodies like E5 and HA-1A. However, none of these in clinical trials have been convinced to be effective in treatment of sepsis or septic shock (McCloskey *et al.*, 1994; Angus *et al.*, 2000; Wong and Luk, 2009). Therefore, extracorporeal endotoxin removal as a promising approach is arising in view of poor overall outcomes associated with severe sepsis.

The present in vitro study revealed that the novel adsorber PVDF-Ser had an efficient affinity to endotoxin, with about 50 EU endotoxin removal after 2 h-dynamic interaction, which was equivalent to PMX (Shoji, 2003). The efficiency could be amplified by the hollow fibers and the contact area multiplying along with technology advance. Another way could be two or more sessions of hemoperfusion process applied into sepsis treatment. Some clinicians and researchers might still suspect the efficacy of endotoxin removal strategy (Carlsson *et al.*, 2009), by comparing the exact quantity of endotoxin removal to the total amount in septic creature. However, we should pay attention to the level reduction of detectable circulating endotoxin and consequent alleviation of impaired organs. Early or super-early intervention of the hemoperfusion to block the hyper-inflammatory cascade might be another key factor in the strategy, since delay application at the peak or later of inflammatory reaction could discount the effect of endotoxin removal.

IL-6 represents activation status of inflammatory storm and reflects biological potency of several other cytokines, as it maintains much long in blood circulation once triggered release by TNF-α and IL-1. Its persistent elevation, compared to initial or peak level, should be valued more in sepsis development (Song and Kellum, 2005). Moderate increase of TNF-α level is essential in host defense and immunomodulation for sepsis, while excessive release always implies severe local or systemic immune response and unfavorable prognosis (Rigato *et al.*, 1996). Thus, IL-6 and TNF-α appear to be promising candidate cytokines for severity prediction and prognosis of severe sepsis.

Our present study demonstrated that circulating endotoxin, IL-6, and TNF-α could be reduced from blood compartment in septic pigs undergoing hemoperfusion with the novel adsorber. The mechanism how the cytokine levels were decreased remained unclear. One reason may be that the stimuli endotoxin

diminished timely and then the tissue injury mitigated to some extent. In other words, the cytokines' synthesis and release were slowed or restrained somewhat. The novel adsorber per se possessing cytokines avidity could not be completely excluded, though the adsorbent was designed to remove endotoxin. The outcome was generally in accordance with the results of previous research, when sepsis or septic shock was treated with DHP-PMX (Zagli *et al.*, 2010). However, cytokine exchanges between blood compartment and interstitial tissue had still been unclear, so tissue levels could be measured in the future if possible.

Arterial oxygenation, one indicative factor of respiratory function, was impaired after endotoxin insult, but improved through 2 h-hemoperfusion therapy with the adsorber. The 72 h-survival time was also prolonged after the treatment for severe sepsis. The encouraging results were consistent with the discoveries using DHP-PMX (Tsushima *et al.*, 2002; Cruz *et al.*, 2009). However, the outcomes should be viewed cautiously. Firstly, the hemoperfusion process ought to be implemented as early as possible, preferably before the peak of inflammatory reaction. Thus, the preliminary hours were the most efficient during the development and evolution of severe sepsis. In the present experiment, super-early intervention was carried out without any delay. Secondly, the endotoxin removal strategy was currently advocated only as adjuvant treatment for severe sepsis or septic shock. Whatever, there was no definite infectious focus and no antibiotic or corticosteroid used in the experiment, which did not soundly mimic a clinical condition. Thirdly, different LPS dosages (lethal or sublethal), intravenous modes (bolus or continuous), and administrative routes (intravenous or intraperitoneal) in LPS infusion models of sepsis had a different effect on hemodynamics, injury target, and severity extent of organ involved (Zanotti-Cavazzoni and Goldfarb, 2009). Therefore, relative experiment should be validated in other animal models and in clinical trials future. Lastly, the strategy of endotoxin elimination might be more favorable for hyperendotoxemia state of severe sepsis, which could be evaluated in future study.

In conclusion, the novel product PVDF-Ser could adsorb endotoxin with high safety and efficacy. Early use of extracorporeal hemoperfusion with the adsorber could decrease the levels of circulating endotoxin, IL-6, and TNF-α, as well as improve respiratory function and consequent 72 h-survival rate of the septic pig. Endotoxin removal strategy with blood purification using the new adsorber has a potentially promising role in therapy for septic patients.

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