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Full Length Research Paper

Impact of leukocytapheresis on the coagulation and lung injury in the endotoxemia dogs

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To explore the role of leucocyte in the coagulation and fibrinolysis system in endotoxin-induced lung injury, the impact of peripheral leukocytapheresis (LCAP) on the coagulation and fibrinolysis system, and the lung injury in the endotoxemia dogs were observed. Endotoxemia was introduced in dogs with lipopolysaccharidea (LPS, 2 mg/kg). A total of 30 dogs were randomly assigned into LPS group (dogs were treated with LPS alone), sham group (dogs underwent LPS treatment and then sham LCAP 12-14 h later) and LCAP group (dogs underwent LPS treatment and LCAP 12-14 h later) (n=10 per group). The levels of peripheral activator protein C (APC), soluble thrombomodulin (sTM) and plasminogen activator inhibitor-1 (PAI-1) were measured 0 h before LPS treatment and 2, 6, 12, 14, 16, 24 and 36 h after LPS treatment. Result showed that the serum level of APC at 14 and 16 h after LPS treatment in LCAP group was significantly higher than that in LPS group at corresponding time points (P<0.05). The serum levels of sTM and PAI-1 at 14 and 16 h after LPS treatment in LCAP group was significantly lower than those in LPS group at corresponding time points (P<0.05). The incidence of lung injury in LCAP group was lower than that in LPS group (P<0.05). At the early stage of endotoxemia, LCAP can improve the balance of coagulation and fibrinolysis which may partly contribute to the improvement of oxygenation index and attenuation of lung injury.

Key words: Endotoxemia, leukocytapheresis, coagulation/fibrinolysis.

INTRODUCTION

The increase of neutrophil count is one of the mechanisms involved in the endotoxemia induced lung injury (Hashimoto et al., 2008) and the dysfunction of coagulation/fibrinolysis is an important pathological feature in this disease (Ware et al., 2007; Ware et al., 2003). Studies have demonstrated that the activated neutrophils can secret elastase which may promote the release of endothelial cell thrombomodulin into the blood and impact the anticoagulation. However, whether neutrophils can affect lung injury through acting on the coagulation/fibrinolysis system remains still unclear (Boehme et al., 2002; Gando et al., 2004). Leukocytapheresis (LCAP) has been used in the treatment of high leukocyte acute leukemia (HLAL). Evidence shows that decrease of peripheral neutrophils can improve the lung injury secondary to the HLAL (Liu et al., 2005). In the present study, the changes in the coagulation/fibrinolysis system and acute lung injury were investigated in endotoxemia dogs receiving LCAP to explore the mechanisms underlying the effect of leukocytes on the coagulation/fibrinolysis in the

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endotoxemia induced lung injury.

MATERIALS AND METHODS

Reagents and instruments

Lipopolysaccharidea (LPS; 055:B5, Sigma, USA), leukocyte analyzer (Abbott CELL-DYN 1700, USA), blood gas analyzer (i-STAT, Abbott Laboratories, USA), leukocyte measure and polymorphonuclear cells (PMN) counts (SYSMEX, XE-2100, Japan), ELISA kits (Abcam, UK) for neutrophil elastase (NE), activated protein C (APC), thrombomodulin (TM) and plasminogen activator inhibitor type-1 (PAI-1), leukocyte separator (COM.TEC, Fresenius HemoCare GmbH, Germany) were used in the present study.

Animals and processing

A total of 30 healthy male dogs weighing 15.6±2.9 kg were purchased from the Animal Center of Research Institute of Field Surgery, Daping Hospital of the Third Military Medical University. This study was approved by the ethics committee of Daping Hospital. The animals were allowed to fast for 12 h and then intravenously anesthetized with sumianxin (0.04 ml/kg) followed by maintenance of anesthesia with intravenous 1.5% sodium pentobarbital (1 mg/kg/h) (Zeng et al., 2006). Oral intubation was performed with 7.5# tube which was connected to the ventilator. Controlled mechanical ventilation mode was used and the fraction of inspired oxygen (FiO_2) was 29%. The respiratory rate remained 18 beats/min, initial tidal volume 10 ml/kg, and inspiratory to expiratory time ratio 1:2. If necessary, the tidal volume was regulated to maintain the $PaCO_2$ at 40±5 mmHg. The bilateral femoral veins and the saphenous vein of left hindlimb were exposed and catheters connected to the tubes were inserted to these veins. Then the arterial catheter was inserted into the right femoral artery aiming to monitor the mean arterial pressure (MAP) and for blood gas analysis. Swan-Ganz catheter (5#) was inserted into the left common carotid vein to monitor the pulmonary artery wedge pressure (PAWP). The inspiratory oxygen concentration remained stable and the intravenous infusion was adjusted according to the MAP and PAWP. Supplement of lactated ringer solution (7 ml/kg/h) was done. When the MAP was <70 mmHg, the 10 ml/kg/h lactated ringer solution was administrated and 7 ml/kg/h lactated ringer solution was performed once the PAWP was >18 mmHg.

Grouping and modeling of endotoxemia

The endotoxemia was introduced accordingly as previously described (Tabor et al., 1987). The animals were randomly assigned into LPS group, sham group and LCAP group (n=10 per group). Through the preliminary test, we found that white blood cell count increased to the physiological levels 12 h after injection of endotoxin, while neutrophil count was significantly higher than baseline. So in the study, dogs in LPS group were intravenously treated with LPS (2 mg/kg). LPS was diluted with 100 ml normal saline and then infused in 30 min; dogs in sham group received LSP treatment followed by sham LCAP 12-14 h later; dogs in LCAP group received LPS treatment followed by LCAP 12-14 h later. The white blood cell count and blood gas were continuously monitored. The diagnosis of acute lung injury and acute respiratory distress syndrome (ARDS) was based on the criteria developed in the American–European consensus conference on ARDS (Bernard et al., 1994). LCAP was performed according to the procedures used for isolation of monocytes.

Detection of parameters

Measurements of neutrophil elastase, activator protein C, thrombomodulin and plasminogen activator inhibitor-1

The peripheral blood was obtained at 0, 2, 6, 12, 14, 16, 24 and 36 after LPS treatment and centrifuged at 3000 r/min for 10 min at 4°C. The supernatant was collected and stored at -80°C. The levels of NE, APC, TM and PAI-1 were determined with enzyme-linked immunosorbent assay (ELISA) methods according to manufacturer's instructions.

Measurement of oxygenation index (arterial partial pressure of oxygen/inspired oxygen)

The heparin anticoagulated peripheral blood was collected at 0, 2, 6, 12, 14, 16, 24 and 36 after LPS treatment and the arterial partial pressure of oxygen (PO_2) was measured by a portable blood gas analyzer followed by calculation of PO_2/FIO_2.

Wet-to-dry ratio of the lung

At 36 h after LPS treatment, the inferior lobe of right lung was obtained and weighed (wet weight; W). The lung tissues were then dried in an oven at 80°C for 72 h until the weight remain stable and weighed again (dry weight; D). The wet to dry ratio (W/D) of the lung was calculated.

Histopathology

At 36 h after LPS treatment, the inferior lobe of right lung was obtained and fixed in 4% paraformaldehyde followed by embedding in paraffin and sectioning. Then, Hematoxylin-Eosin Stain was performed and the changes in the histopathology were observed under microscope.

Statistical analysis

Statistical analysis was carried out with statistical package for the social sciences (SPSS) version 13.0 statistical software package. Qualitative data were expressed as mean ± standard deviation ($\bar{x}$±s) and analyzed with chi square test, while comparisons were done with repeated measures analysis of variance. Least significant difference (LSD) was used to analyze the difference in the data between two groups. A value of P<0.05 was considered statistically significant.

RESULTS

Changes in the count of leukocyte and neutrophils

At 2 h after LPS treatment, the leukocytes were decreased significantly (P<0.05) and then had a tread to increase. It returned to the baseline level at 12 h after LPS treatment. The levels of leukocyte and PMN in LCAP group at 14 and 16 h after LPS treatment were markedly lower that those in LPS group (P<0.01), however there were not remarkable differences between LPS group and sham group at any time point (P>0.05). Moreover, the levels of leukocyte and PMN in all groups at 36 h after LPS treatment were significantly higher than those at 0 h after LPS treatment (P<0.01) (Table 1).

Table 1. Levels of WBC and PMN at different time points (n=10, $\bar{x}$±s, ×10^9/L).

Parameter/group	0 h	2 h	6 h	12 h	14 h	16 h	24 h	36 h
				WBC				
LPS group	13.047±1.308	3.855±1.138	8.141±1.681	13.357±1.983	15.560±2.000	17.180±1.295	21.469±3.702	26.858±1.242**
Sham group	13.035±1.725	3.661±0.91	7.092±1.598	12.813±1.137	15.273±1.308	17.113±1.413	22.013±3.541	26.215±1.737**
LCAP group■	12.789±1.948	3.242±1.277	8.168±1.767	12.713±1.936	10.852±2.270▲▲	12.884±1.148▲▲	19.684±3.087	25.713±1.257**
				PMN				
LPS group	7.981±1.033	1.598±0.593	4.982±1.071	9.262±1.570	11.925±2.599	14.276±2.15	16.804±2.493	20.805±1.256**
Sham group	7.938±1.034	1.622±0.364	4.431±0.960	8.428±0.911	11.713±2.213	14.014±2.470	17.023±2.159	20.589±2.571**
LCAP group■	7.908±1.169	1.517±0.48	4.595±1.060	9.086±1.519	7.431±2.376▲▲	9.318±1.873▲▲	15.070±3.017	19.269±1.836**

Note: *P<0.05, **P<0.01 vs 0 h; ▲P<0.05,▲▲P<0.01 vs LPS group at corresponding time points; ■P<0.05 LCAP group vs LPS group or sham group.

Effects of leukocytapheresis on the serum levels of neutrophil elastase, soluble thrombomodulin, activator protein C and plasminogen activator inhibitor-1 in endotoxemia dogs

After LPS treatment, the NE, sTM and PAI-1 increased gradually and APC decreased progressively. In LCAP group, the serum levels of NE, sTM and PAI-1 at 14 and 16 h after LPS treatment were significantly lower than those in LPS group (P<0.05), and the APC level was higher than that in LPS group (P<0.05). But no significant differences in these parameters were observed between LPS group and sham group (P>0.05). Moreover, the serum levels of NE, sTM, APC and PAI-1 at 36 h after LPS treatment were markedly different from those at 0 h after LPS treatment (P<0.05) (Table 2).

Effect of LCAP on the PO_2/FIO_2 in endotoxemia dogs

The blood gas levels reduced over time after LPS treatment and reached a minimal level at 36 h after LPS treatment. At 24 and 36 h after LPS treatment, the blood gas levels in LCAP group were dramatically higher than those in LPS group (P<0.05), but there were no marked differences between LPS group and sham group at any time point (P>0.05) (Table 3).

Correlation between the total serum sTM and total NE

There was a positive correction between total serum sTM and total NE (R=0.707, P<0.05) (Figure 1).

Wet to dry ratio at 36 h after LPS treatment

When compared with LPS group, the W/D in LCAP group was markedly decreased (5.678±0.29 in LPS group and 5.416±0.177 in LCAP group; P<0.05). Furthermore, no marked difference was noted between LPS group and sham group (P>0.05).

Histopathological features 36 h after LPS treatment

In each group, a lot of neutrophils exuded and aggregated in the pulmonary capillaries, interstitium and alveolus accompanied by exudation of red blood cells, pulmonary interstitial edema, telangiectasia, hyperemia and blood cell exudation. However, the injury degree in LPS group and sham group were more serious than in LCAP group (Figure 2).

Incidence of acute lung injury

10 dogs were included in each group and 7, 8 and 2 dogs with acute lung injury were observed in the LPS group, sham group and LCAP group, respectively. The incidence of acute lung injury in LCAP group was markedly lower than that in LPS group and sham group (P<0.05), but no significant difference was noted between LPS group and sham group (P>0.05).

DISCUSSION

The involvement of neurophils in the lung injury has been confirmed but report on the role of neutrophils in the coagulation is less found in

Table 2. Levels of NE, sTM, APC and PAI-1 at different time points (n = 10, $\bar{x}$±s).

Parameter/group	0 h	2 h	6 h	12 h	14 h	16 h	24 h	36 h
NE(ug/L)								
LPS group	260.096±28.462	293.395±32.470	353.754±42.788	408.16±50.245	446.69±50.857	499.041±50.963	576.589±50.854	646.208±51.064 **
Sham group	258.167±19.737	281.334±22.424	339.646±25.615	392.414±41.724	441.707±41.018	495.205±40.766	582.375±40.585	656.697±41.124 **
LCAP group■	241.391±23.949	270.832±20.518	330.014±37.401	369.911±39.305	382.215±41.069▲▲	425.081±39.389▲▲	549.227±31.566	618.677±32.171 **
sTM(ug/l)								
LPS group	4.628±0.379	6.069±0.438	7.891±0.461	10.009±0.798	11.005±0.854	12.047±0.954	17.557±2.242	23.528±2.192 **
Sham group	4.404±0.314	5.803±0.390	7.428±0.685	9.505±0.798	10.681±0.914	11.443±0.918	17.917±2.364	24.398±1.975 **
LCAP group■	4.471±0.247	5.861±0.274	7.379±0.524	9.483±0.865	9.688±0.914▲	10.492±0.865▲	16.131±1.536	23.107±1.097**
APC(ug/l)								
LPS group	105.686±9.950	89.832±7.943	75.264±4.427	55.939±4.135	45.881±4.024	35.935±4.057	19.520±4.089	11.733±2.224**
Sham group	111.952±9.168	88.621±9.259	74.474±5.801	59.411±5.410	43.884±5.330	33.384±5.092	18.371±5.127	10.291±3.424**
LCAP group■	113.495±9.161	95.541±8.136	79.417±6.169	58.217±4.330	50.805±4.422▲▲	40.480±2.993▲▲	21.759±4.320	12.639±2.289**
PAI-1(ng/l)								
LPS group	77.925±1.918	90.380±3.881	105.881±3.792	127.516±7.275	138.348±7.363	147.702±7.509	190.807±10.063	265.668±9.680**
Sham group	77.200±2.055	88.680±2.898	104.155±5.122	126.444±8.348	137.592±8.301	144.754±7.860	192.164±10.092	266.053±9.686**
LCAP group■	76.551±1.723	87.726±4.189	101.427±5.983	121.948±7.760	128.023±7.756▲	135.249±7.683▲	183.325±10.372	260.240±9.675**

Note: *P<0.05, **P<0.01 vs 0 h; ▲P<0.05,▲▲P<0.01 vs LPS group at corresponding time points; ■P<0.05 LCAP group vs LPS group or sham group.

Table 3. PO_2/FIO_2 at different time points (n=10, $\bar{x}$±s)

Parameter/group	0 h	2 h	6 h	12 h	14 h	16 h	24 h	36 h
NE (ug/L)								
LPS group	393±35	378±30	364±31	341±34	333±25	329±24	287±30	274±29 **
Sham group	384±32	373±32	359±31	345±31	341±25	328±21	296±24	265±29 **
LCAP group■	410±32	392±34	376±31	368±34	343±33	337±22	319±23▲	311±25 **▲

Note: *P<0.05, **P<0.01 vs 0 h; ▲P<0.05,▲▲P<0.01 vs LPS group at corresponding time points; ■P<0.05 LCAP group vs LPS group or sham group.

LCAP, through decreasing the release of NE, not only directly improve the lung injury (Hashimoto et al., 2008), but also indirectly improve the histological features of the lung and decreases the incidence of lung injury through attenuating hypercoagulation (Boehme et al., 2002; Gando et al., 2004).

LCAP is a clinical method with which the circulating white blood cells are removed by a blood cell separator. Study shows that HLAL patients with ALI recovered after LCAP treatment (Liu et al., 2005). In the present study, the circulating white blood cells were partially removed and the low levels of white blood cells and PMN remained for several hours.

The circulating NE is mainly derived from PMN and NE is a critical enzyme causing lung injury (Dahlbäck and Villoutreix, 2005). In the blood

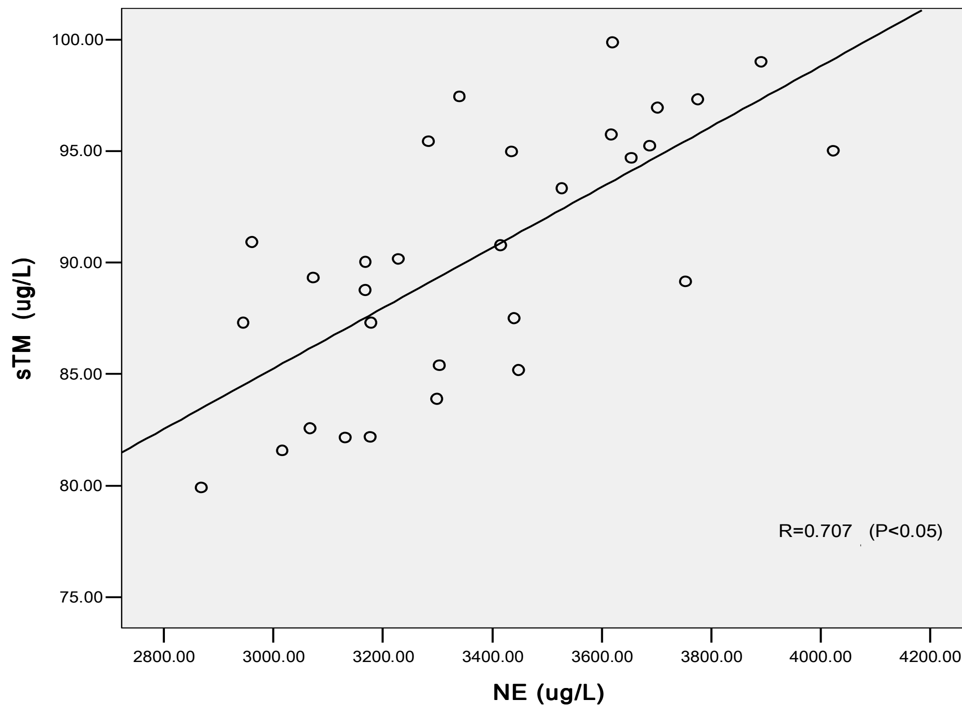

Figure 1. Correlation between the total serum soluble thrombomodulin and total neutrophil elastase.
There was a positive correction between total serum soluble thrombomodulin and total neutrophil elastase (R=0.707, P<0.05).

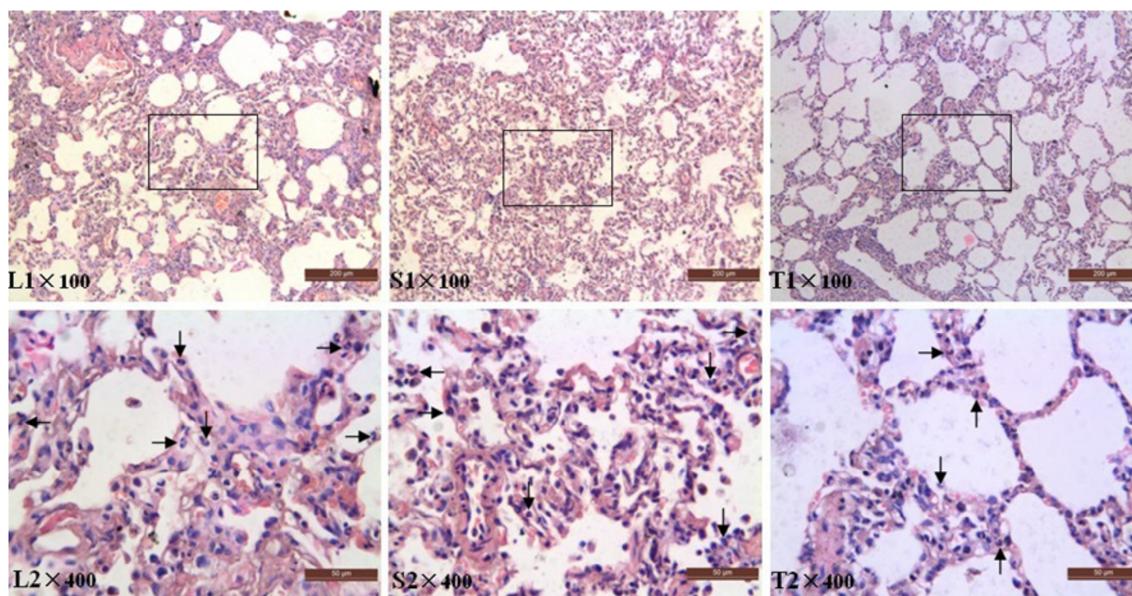

Figure 2. Hematoxylin-Eosin Staining and histopathological features of lung tissue.
L, LPS group; **S**, Sham group; **T**, LCAP group. L2, S2 and T2 represent the enlarged area of the frame area of L1, S1 and T1, respectively. The arrow represents neutrophils.

undergoing LCAP, the NE level is markedly reduced due to significant decrease of PMN count, which may lead to marked improvement of oxygenation index (P<0.05), one of the main causes resulting in reduced incidence of lung

injury (P<0.05).

In recent years, increasing attention has been paid to the role of APC in the anti-coagulation in endotoxemia because the decrease of serum APC is the key feature of coagulation in the lung injury. Study shows that APC can irreversibly hydrolyze coagulation factors (Va and VIIIa) resulting in their inactivation, which disrupts the coagulation cascade and interrupts the subsequent production of thrombin, finally leading to improvement of hypercoagulation (Dahlbäck and Villoutreix 2005). In addition, APC can inhibit another two important plasmin inhibitors (PAI-1 and thrombin activatable fibrinolysis inhibitor (TAFI)) which promotes the generation of plasmins and their activation (Espana et al., 2005). Therefore, in the ways abovementioned, APC exerts anti-coagulation and pro-fibrinolysis effects leading to a coagulation balance. Moreover, APC can activate the protease-activated receptor (PAR-1) eliciting corresponding intracellular signaling transduction, which may induce the reorganization and stability of cytoskeleton and intensify the intercellular conjunction. These effects lead to decrease of endothelial permeability, attenuation of lung edema and improvement of blood gas exchange finally resulting in mitigation of lung injury. Evidence shows that in endotoxemia, a lot of neutrophils are activated and then the TM is released into the blood. The released TM is unable to activate APC and therefore the functions of APC in coagulation are compromised. Our result revealed when compared with LPS group, the APC level was dramatically elevated following LCAP, which may be related to the attenuation of endothelial injury, decreased release of TM and increase of APC. Additionally, the serum NE level is decreased following LCAP, which then reduce the degradation of APC by NE. These effects finally lead to improvement of coagulation and marked decrease of pulmonary micro-thrombosis, which may be one of important mechanisms underlying the protective effects of LCAP on acute lung injury.

In the endotoxemia, the serum sTM increases which is relevant with the PMN activation and increase of PMN count and NE (Gando et al., 1995). PMN and NE alone or combination can lead to damage to micro-vascular endothelial cells which may cause the release of TM from the endothelial cells into the blood and decrease of TM on the endothelial cells leading to increase of serum sTM (MacGregor et al., 1997; Li et al., 2008). This response then decreases the APC and subsequently enhances the coagulation and inhibits the fibrinolysis finally resulting in lung injury secondary to endotoxemia. After LCAP, the serum sTM decreases but the exact mechanism is still unclear and may be associated with decreased release of NE due to reduced PMN count. In the present study, the correlation between sTM and NE was analyzed and result revealed positive association between sTM and NE (R=0.707, P<0.01), which is consistent with previously reported (Dahlbäck and Villoutreix, 2005). LCAP can decrease the serum NE level which directly attenuates the lung injury. In addition, LCAP can also improve the coagulation through reducing sTM which indirectly attenuates the lung injury. PAI-1 mainly acts on the fibrinolysis system and is a crucial anti-fibrinolysis factor. Study shows that PAI-1 level is closely related to the disease severity and the occurrence and development of diseases (Zeerleder et al., 2006). The increase of PAI-1 inhibits the fibrinolysis and promotes the pulmonary microvascular micro-thrombosis, one of pathological features in lung injury (Ware et al., 2007). In the study on the LPS induced liver injury, PMN activation leads to production of NE which then increases the serum PAI-1 but serum with anti-PMN activity can decrease the PAI-1 (Deng et al., 2007). In addition, APC can degrade the PAI-1 resulting in inactivation of PAI-1 (de Fouw et al., 1987). In our study, after LCAP, the NE released was reduced which leads to attenuation of endothelial cell damage, increase of APC and possible reduce of serum PAI-1. But the mechanism underlying the decrease of PAI-1 following LCAP is unknown.

The NE released by white blood cells, especially the neutrophils, can inhibit the decrease of APC and suppress the increase of sTM and PAI-1 in endotoxemia dogs, which improve the coagulation and fibrinolysis resulting in attenuation of inflammation because coagulation/fibrinolysis system and inflammation system form a complex network. Thus, the improvement of APC, sTM and PAI-1 following LCAP can increase the oxygenation and decrease the incidence of acute lung injury. We speculate LCAP may become a novel strategy in the prevention and treatment of acute lung injury.

Abbreviations: LCAP, leukocytapheresis; **LPS**, lipopolysaccharidea; **APC**, activator protein C; **sTM**, soluble thrombomodulin; **PAI-1**, plasminogen activator inhibitor-1; **HLAL**, high leukocyte acute leukemia; **NE**, neutrophil elastase; **TM**, thrombomodulin; **FiO_2**, inspired oxygen; **MAP**, mean arterial pressure; **PAWP**, pulmonary artery wedge pressure; **ARDS**, acute respiratory distress syndrome; **PO_2**, arterial partial pressure of oxygen; W, wet weght; D, dry weight; **W/D**, wet to dry ratio; **TAFI**, thrombin activatable fibrinolysis inhibitor; **PMN**, polymorphonuclear cells; **PAR-1**, protease-activated receptor; **ELISA**, enzyme-linked immunosorbent assay.

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