Int J Clin Exp Med 2017;10(8):11796-11803
www.ijcem.com /ISSN:1940-5901/IJCEM0046169

Original Article

Comparison of anticoagulation and thrombolysis therapies in lipopolysaccharide-induced disseminated intravascular coagulation

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Received December 8, 2016; Accepted April 8, 2017; Epub August 15, 2017; Published August 30, 2017

Abstract: Disseminated intravascular coagulation (DIC) is a significant health threat in numerous infectious and noninfectious conditions. Combinations of anticoagulants and thrombolics are the standard of care, however, the lack of a complete understanding of all underlying causes for creation of microthrombi and organ failure results in uncertainties for the best course of treatment. We have developed a rat model of sepsis using infusion of lipopolysaccharide (LPS) in order to test the efficacy of various drug treatments. In our model, LPS causes a reduction in white blood cells, platelets and fibrinogen and increases in prothrombin and activated partial thromboplastin time, D-dimer, plasminogen activator inhibitor, alanine aminotransferase and serum creatinine. Here we present results of treatments of the model with urokinase, plasmin and low molecular weight heparin. Both thrombolytics and anticoagulants provide varying degrees of protection against creation of microthrombi and reversal of changes in blood parameters due to the LPS infusion. Both types of treatments also protect against pathological lesions present in the model.

Keywords: Disseminated intravascular coagulation, microthrombosis, thrombolytic, anticoagulant, urokinase, plasmin, low molecular weight heparin

Introduction

Disseminated intravascular coagulation (DIC) is a dynamic pathologic process characterized by continuous and extensive activation of systemic coagulation, resulting in disseminated microthrombi within small- and medium-sized blood vessels, which ultimately leads to organ failure [1]. DIC can be associated with both suppressed microcirculation and (severe) bleeding simultaneously; the latter is likely caused by exhaustion or high consumption of platelets and coagulation factors [2]. DIC can be a factor in patients with a wide range of disorders including infectious disease, severe trauma, cancer, obstetrical disorders, giant hemangiomas, and microangiopathic hemolytic anemia [3]. Elevated plasma levels of plasminogen activator inhibitor-1 (PAI-1) potentially contribute to DIC manifestations caused by infections, such as significant inhibition of fibrinolysis, multiorgan dysfunction and shock [4]. Moreover, patients with multiple organ failure (MOF) displayed higher levels of PAI-1 and low levels of fibrin (original) degradation products (FDP), suggesting that elevated PAI-1 may suppress fibrinolysis, resulting in the occurrence of MOF in DIC [5]. Multi-center, randomized controlled trials have demonstrated that anticoagulant therapy using low molecular weight heparin (LMWH) did not reduce the mortality of patients with sepsis combined with DIC, likely due to the inability to remove the existing microcirculation thrombi [6-8]. Interestingly, Asakura et al. has shown in a rat model of DIC induced by the endotoxin lipopolysaccharide (LPS) that thrombolytic agents plus urokinase (UK) can improve organ function [9]. However, it is not clear whether there is any difference in the treatment efficacies between anticoagulation and thrombolytic therapies in this type of DIC. In this study, we generated a DIC model induced by LPS in Wistar rats [9-12]. The rats were subsequently treated with the thrombolytic agents UK

or plasmin (PL) and the anticoagulant LMWH. We analyzed the outcome of these two types of therapies, including measuring the level of platelets, white blood cells, coagulation ability, fibrinolysis, and liver and kidney function in these rats. Our study provides experimental data that should facilitate better clinical utilization of available therapies for DIC.

Materials and methods

Experimental animals and reagents

We used healthy, female, non-pregnant Wistar rats, weighing 160-200 g and aged 6-8 weeks, which were purchased from the Experimental Animal Center of Sichuan University. These rats were housed and maintained according to national laboratory animal standards and all procedures were approved by our institutional animal committee.

Endotoxin LPS (Escherichia coli 055: B5 L40-05, Sigma, USA); urokinase (Nanjing University Pharmaceutical Co., Ltd., Nanjin, China); plasmin (Science Sun, Beijing, China); low molecular weight heparin (Sanofi China); Martius-Sarlet-blue (MSB, Sigma); rat D-dimer, plasminogen activator inhibitor-1 (PAI-1) enzyme-linked immunosorbent assay kit (Beijing Chenglin Biotechnology, Beijing, China).

Criteria for rat model of DIC

The DIC model in Wistar rats was generated using criteria as described in previously published studies [11, 12]. In brief, when compared to the control group, at least three of the following five requirements were met: significant decrease of fibrinogen (FIB), significant decrease of platelets (PLT), significant increase in the fibrinogen degradation product D-dimer, significant extension of prothrombin time (PT) and activated partial thromboplastin time (APTT), and a reduction in microcirculation fibrin thrombosis.

Animal grouping and treatment

Forty eight Wistar rats were randomly divided into six groups according to the type of treatment: normal saline (NS), endotoxin 1 hour model (LPS1h), endotoxin 4 hour model (LPS4h), urokinase (UK), plasmin (PL) and low molecular weight heparin (LMWH). There were 8 rats in each group. After 12 hours of fasting, all rats received intraperitoneal anesthesia by using sodium pentobarbital (30 mg/kg) followed by sustained infusion of the abovementioned drugs or vehicle into the tail vein. Experimental DIC was induced as indicated below: NS group (NS 2.5 mL/h × 4 h), LPS1h group (LPS 4 mg/kg/h × 1 h + NS 2.5 mL/h × 1 h), and LPS4h group (LPS 4 mg/kg/h × 4 h + NS 2.5 mL/h × 4 h). All three treatment groups received a continuous infusion of LPS (4 mg/kg/h) for 4 hours. The drugs dissolved in 10 mL NS solvent were added 1 hour after the start of the continuing LPS infusion for the next 3 hours: UK group (LPS 4 mg/kg/h × 4 h + UK 4 IU/g/h × 3 h), PL group (LPS 4 mg/kg/h × 4 h + PL 2 IU/kg/h × 3 h), and LMWH group (LPS 4 mg/kg/h × 4 h + LMWH 25 IU/kg/h × 3 h).

After the 4-hour infusion, a total of 3.2 mL of abdominal aortic blood samples were collected and sent to a laboratory within an hour for the following tests. One mL of the blood collected in an EDTA anticoagulant tube was used for routine blood tests including platelet count (PLT) and white blood cell count (WBC). One mL was collected into a pro-coagulant tube for measuring the indices of organ damage including the level of serum alanine aminotransferase (ALT) and serum creatinine (Cr). One point two mL of blood collected in a 3.2% sodium citrate anticoagulant tube was used for measuring the indices of coagulation and fibrinolysis including APTT, PT and FIB. After the latter tests, 0.5 mL of the 3.2% sodium citrate anticoagulant blood were recycled and used to measure D-dimer and PAI-1 levels by an enzyme-linked immunosorbent assay (ELISA). Both kidneys were taken immediately after blood collection and preserved as formalin-fixed, paraffin-embedded (FFPE) tissues, which were cut at 4 μm and processed with hematoxylin-eosin (HE) and Martius-Sarlet-blue (MSB) staining. The renal histopathological damage and the formation of fibrin microthrombosis in glomeruli and microvessels were evaluated under an optical microscope.

Statistical analysis

All data are presented as means ± standard error (SEM). Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison tests. $P < 0.05$ was considered statistically significant.

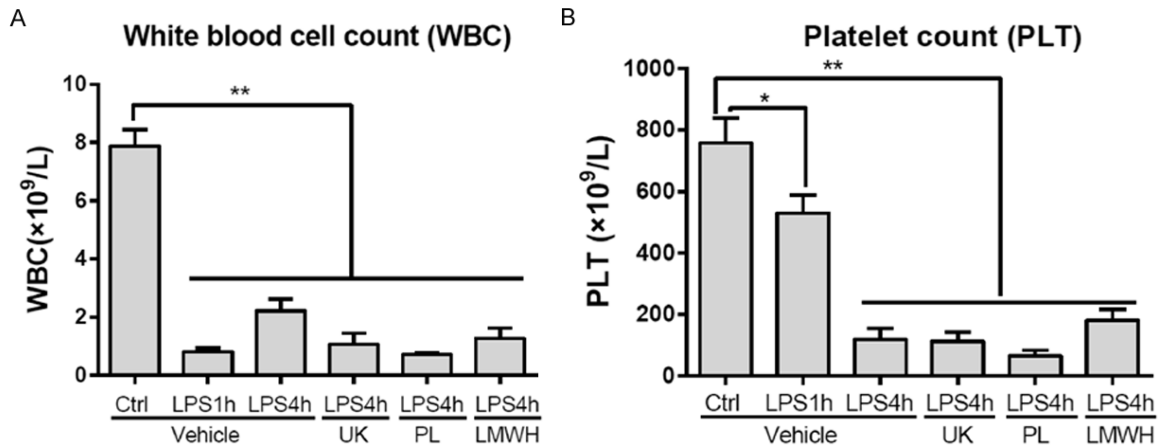

Figure 1. Drug treatment efficacy of Blood cell counts in an LPS-induced rat model of DIC. (A) White blood cell count (WBC) and (B) platelet count (PLT) were significantly reduced by LPS exposure for 1 and 4 h, which was not inhibited by drug treatment with coagulation and fibrinolysis reagents in rats. Data are mean ± SEM. n = 8 each. *$P < 0.05$, **$P < 0.01$ determined by one-way ANOVA with Tukey's test for multiple comparisons.

Results

Induction of DIC model

Although LPS application for 1 hour (LPS1h) showed a significant effect on the WBC count, PLT count, FIB, D-dimer and PAL-1, it did not show a significant effect on PT, ALT or Cr (**Figures 1-3**). However, the LPS4hour application (LPS4h) showed a significant effect on all measured parameters (**Figures 1-3**). Therefore, an LPS4hour application is more effective than LPS for 1 hour for the induction of DIC in Wistar rats.

The effect of drug treatment on blood cell count

To determine whether UK, PL, or LMWH have any effect on routine blood counts in LPS-induced DIC rats, we counted both white blood cells (WBC) and platelets (PLT). Although LPS application for both 1 h and 4 h caused a significant reduction in the levels of both WBC and PLT, we did not observe any significant effect by any of these drugs comparing the two application times (**Figure 1**).

The effect of drug treatment on LPS-induced coagulation and fibrinolysis

To determine the anticoagulant effect of UK, PL, and LMWH, we examined the plasma level of fibrinogen (FIB), prothrombin time (PT), and activated partial thromboplastin time (APTT). The level of FIB was significantly reduced upon LPS application for 1 h ($P < 0.05$) or 4 h ($P < 0.01$), which was not inhibited by treatment with UK, PL, or LMWH (**Figure 2A**). For APTT and PT, LPS4h application, but not LPS1h, showed significant enhancement in the respective values compared to the NS group (**Figure 2B**, **2C**). Remarkably, thrombolysis therapy with UK or PL did not show any rescuing effect for these two blood tests (**Figure 2B**, **2C**). Anticoagulation therapy with LMWH exhibited significant inhibition ($P < 0.01$) of increased prothrombin time by LPS4h (**Figure 2B**, **2C**). Interestingly, although LMWH did not show a significant inhibition of the LPS4h-induced increase in APTT, the APTT was significantly ($P < 0.01$) lower in the LMWH group than in the UK and PL groups (**Figure 2C**).

We further analyzed the level of D-dimer, a small protein fragment generated by fibrinolysis after a blood clot is degraded, which is often associated with elevated fibrinolysis [13]. As expected, the plasma level of D-dimer in both LPS1h and LPS4h groups was significantly higher than that in the NS group (**Figure 2D**). Treatment with all three drugs significantly reduced the LPS-induced elevation in D-dimer levels. We also found that the plasma level of PAI-1, which is associated with coagulation, was significantly elevated by LPS application, while UK, PL, or LMWH treatment inhibited this upregulation (**Figure 2E**).

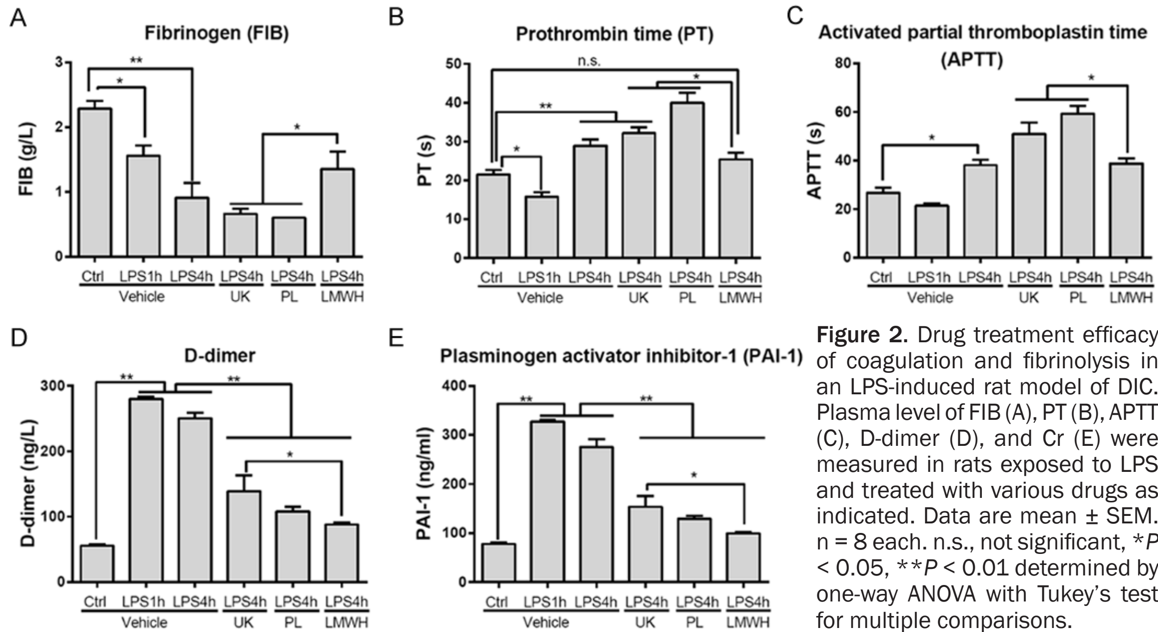

Figure 2. Drug treatment efficacy of coagulation and fibrinolysis in an LPS-induced rat model of DIC. Plasma level of FIB (A), PT (B), APTT (C), D-dimer (D), and Cr (E) were measured in rats exposed to LPS and treated with various drugs as indicated. Data are mean ± SEM. n = 8 each. n.s., not significant, *P < 0.05, **P < 0.01 determined by one-way ANOVA with Tukey's test for multiple comparisons.

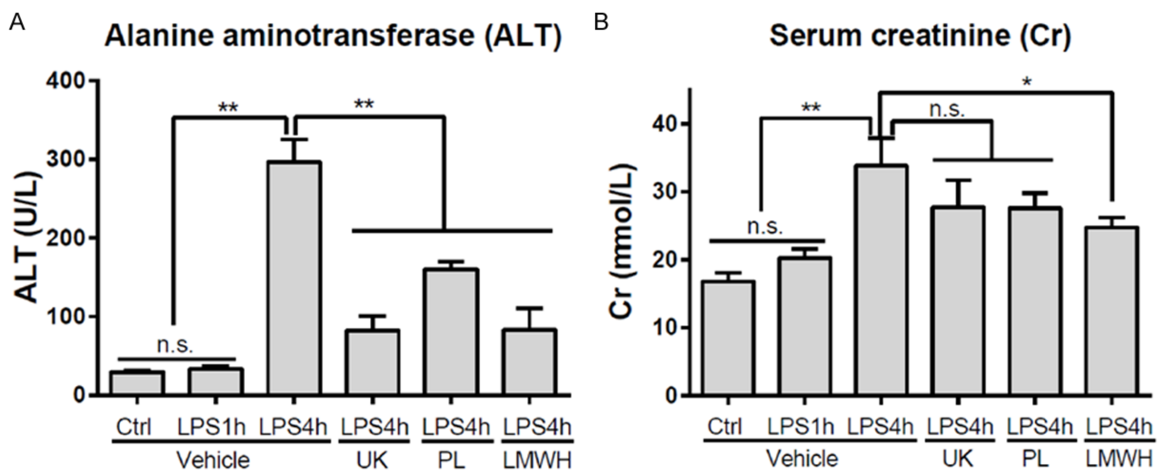

Figure 3. Drug treatment efficacy of organ injury index in an LPS-induced rat model of DIC. Plasma levels of ALT (A) and Cr (B) were measured in rats exposed to LPS and treated with various drugs as indicated. Data are mean ± SEM. n = 8 each. n.s., not significant, *P < 0.05, **P < 0.01 determined by one-way ANOVA with Tukey's test for multiple comparisons.

The effect of drug treatment on LPS-induced organ damage

We evaluated LPS-induced organ damage by measuring the serum level of alanine aminotransferase (ALT) and creatinine (Cr). While LPS1h failed to induce a significant change in the level of either ALT or Cr, LPS4h significantly elevated the serum level of both proteins (**Figure 3**). In the case of ALT, all three drugs showed significant rescuing effects, although UK and LMWH treatments exhibited a greater effect than PL (**Figure 3A**). However, in the case of Cr, only LMWH showed a significant effect, while neither UK nor PL produced a statistically significant result compared to the LPS4h vehicle treatment (**Figure 3B**).

Histopathological evaluation of fibrin thrombus

We also evaluated LPS-induced organ damage through examination of tissue integrity by immunohistochemistry. Compared to the NS group, minor injuries, including mild edema in renal tubular epithelial cells and mild dilatation and congestion in renal interstitial capillaries

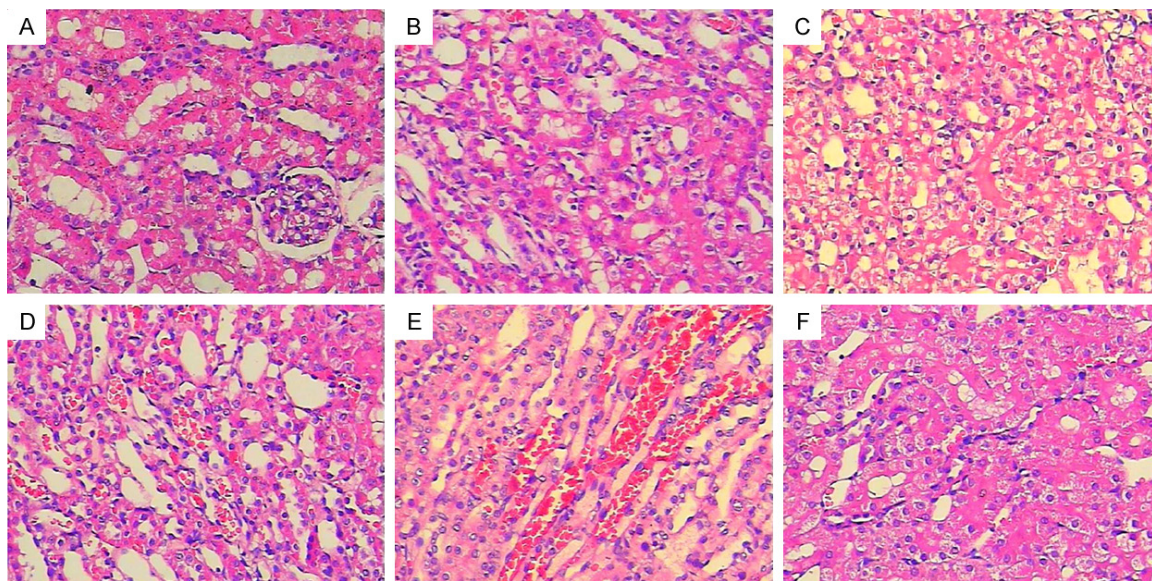

Figure 4. Histopathological evaluation of fibrin thrombus by HE staining in an LPS-induced rat model of DIC. Images (200×) of renal tissues from rats exposed to control (A), LPS1h (B), LPS4h (C-F) and treated with vehicle (A-C), UK (D), PL (E), or LMWH (F).

were observed in LPS1h group tissues (**Figure 4A**, **4B**). In contrast, we observed much more severe damage in LPS4h group tissues, including more extensive edema in renal tubular epithelial cells and more obvious dilatation and congestion in renal interstitial capillaries (**Figure 4C**). We did not observe necrosis in either LPS1h or LPS4h groups. While the PL group only showed congestion in the renal interstitium, the UK group showed both congestion and hemorrhage (**Figure 4D**, **4E**). The LMWH group also exhibited reduced congestion and hemorrhage in the interstitium, and edema remission in renal tubular epithelial cells (**Figure 4F**).

We next used MSB reagent to stain glomerular fibrin depositions (GAD) red, chronic fibrins purple, red blood cells yellow, and nuclei blue (**Figure 5**). We detected no microthrombi in the NS group (**Figure 5A**). Our results show that LPS application resulted in the generation of microthrombi with a greater number detected in the LPS4h group than in the LPS1h group (**Figure 5B**, **5C**). After treatment with UK, LP, and LMWH, we detected fewer microthrombi in each of the three treatment groups than in the LPS4h group (**Figure 5C-F**).

Discussion

In this study, we have successfully established a rat model of DIC by LPS application for 4 hours, as indicated by changes in the plasma levels of coagulation and fibrinolysis factors and renal tissue damage quantifiable by immunohistochemistry. We further demonstrated that treatment with thrombolytic and anticoagulant agents UK and plasmin, and LMWH, respectively, alleviated LPS-induced DIC with variable efficacy. Thus, this study provides useful insights for the clinical application of these drugs in DIC.

In our rat model of DIC, the WBC count declines significantly, indicating a poor prognosis in human sepsis, which may be caused by LPS-induced activation of leukocytes with subsequent apoptosis [14] and sequestration of neutrophils at sites of epithelial cell damage, leading to a decrease in the pool of circulating leukocytes and a severe inflammation reaction in DIC [15]. Severe inflammation reactions may aggravate the damage caused by endothelial anticoagulant systems, especially those mediated by protein C [16]. This may cause the insufficiency of anticoagulant treatments that are unable to balance hyperactive coagulation, leading to wide formation of fibrin thrombi. It has also been found that D-dimer, the product of fibrinolysis, is significantly increased initially but then decreased, indicating that total fibrinolytic ability may decline after a brief rise, resulting in an inability to dissolve a large num-

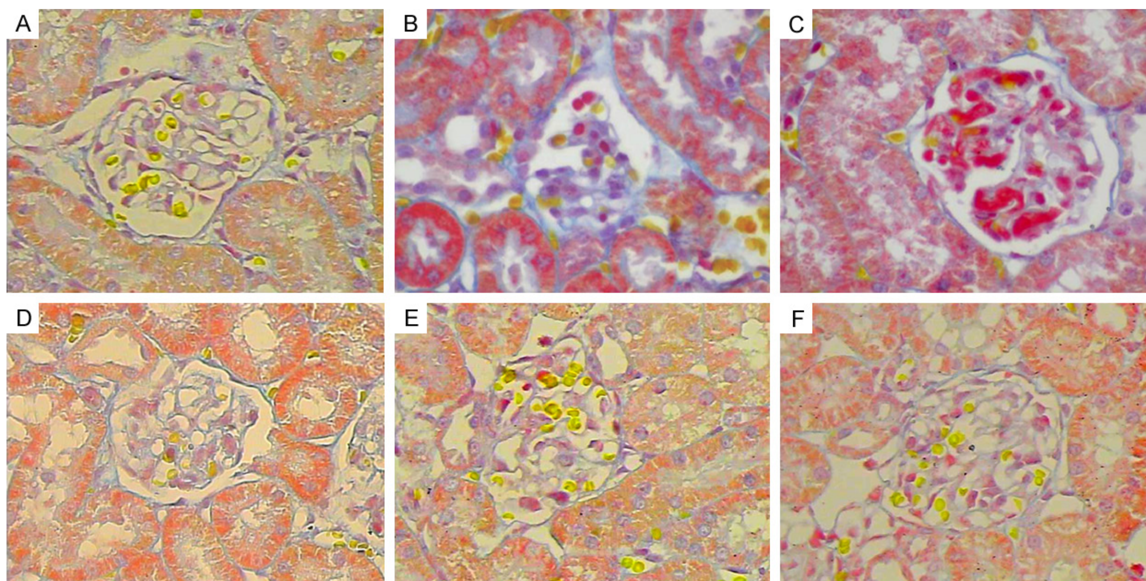

Figure 5. Histopathological evaluation of fibrin thrombus by MSB staining in an LPS-induced rat model of DIC. Images (200×) of renal tissues from rats exposed to control (A), LPS1h (B), LPS4h (C-F) and treated with vehicle (A-C), UK (D), PL (E), or LMWH (F).

ber of microthrombi and leading to microcirculation thrombosis and organ dysfunction [16].

We demonstrated that the plasma level of fibrinogen was significantly decreased in LPS-induced DIC in rats. This event may be associated with continuous activation of the coagulation system induced by LPS, which can exhaust the availability of fibrinogen, resulting in insufficient anticoagulation that causes a decline in fibrinolytic ability [4, 14]. It has been suggested that this pathological process involves the elevation of PAI-1 caused by endothelial dysfunction, the generation of thrombin activated fibrinolysis inhibitor (TAFI), and the enhancement of fibrinolytic inhibition due to platelet-activated a2-antiplasmins [15, 16]. Interestingly, we have found that prothrombin time, evaluating the extrinsic pathway of coagulation, was initially decreased after one hour of LPS insult, implying that the activation of coagulation, was then upregulated after LPS application for four hours, suggesting an eventual loss of coagulation ability [17]. Similarly, activated partial thromboplastin time (APTT), an assay evaluating the intrinsic and common pathway of coagulation, was also decreased and then increased after LPS application for 1 h and 4 h, respectively. In line with increased coagulation in LPS4h animals, only anticoagulation agent LMWH, but not thrombolytic agents UK and plasmin, showed visible effects in inhibiting LPS-induced upregulation of coagulation. These results suggest that the molecular mechanisms underlining the therapeutic action in DIC is different between these types of drugs [7, 8, 18, 19].

Meanwhile, we have shown that the level of D-dimer, a fibrin degradation product (FDP), was significantly elevated by LPS application at either 1 h or 4 h, which was inhibited by all three drugs. This result suggests that both anticoagulative and thrombolytic therapies were effective in inhibiting thrombosis leading to the overall reduction of D-dimer, an index for thrombosis [20]. Interestingly the efficacy of these drugs was different, as the level of D-dimer varied among three different drug-treated groups, indicating a possible difference in the molecular mechanisms underlining therapeutic action among these groups. Similarly, LPS-induced elevation in PAI-1 level was significantly reduced to varying degrees by these drugs. Our results support the idea that anticoagulative and thrombolytic drugs alleviate thrombosis through different mechanisms [7, 8, 18, 19].

Moreover, we demonstrated that all three drugs can alleviate LPS-induced organ damage at comparable levels, as measured by the

blood level of alanine aminotransferase (ALT), an indicator of liver injury [21], and creatinine (Cr), an indicator of renal dysfunction [22]. Our immunohistochemistry results further demonstrate that the effects varied among these drugs. LPS-induced extensive edema in renal tubular epithelial cells and dilatation and congestion in renal interstitial capillaries were alleviated differentially by the three different drugs. While both PL and UK showed less congestion and/or hemorrhage in renal interstitium, anticoagulation reagent LMWH exhibited reduced symptoms of congestion, hemorrhage, and edema. These results are consistent with the notion that DIC is a coagulation-dominant event [9, 10, 23, 24]. All three drugs showed effects on reducing microthrombi.

In conclusion, both anticoagulation (LMWH) and thrombolysis (PL and UK) therapies have shown variable abilities to reduce thrombi and protect organs. While both types of reagents showed an ability to prevent pathological lesions related to thrombolysis, the anticoagulation reagent LMWH showed an additional ability in the tests related to anticoagulation, including FIB, PT and APTT. This study should be of great value informing clinical studies related to anticoagulation and thrombolysis therapies.

Disclosure of conflict of interest

None.

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