Pathologic and hematologic studies in experimental leptospirosis in guinea pigs: thrombocytopenia, but not disseminated intravascular coagulation

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Abstract

Background: Thrombocytopenia is a common finding in leptospirosis, but its exact mechanism is unknown. Results of studies on coagulation alterations during leptospirosis were inconsistent, some findings showed that the prominent levels of thrombocytopenia seen in severe leptospirosis do not reflect the occurrence of disseminated intravascular coagulation (DIC) syndrome, but others reached the conclusion that the hemorrhages observed in leptospirosis are due to DIC. The aim of this study is to elucidate whether DIC is an important feature of leptospirosis.

Methods: The leptospirosis model of guinea pigs was established by intraabdominal inoculation of *L. interrogans* strain *Lai*. The pathologic changes and leptospires were detected by hematoxylin and eosin (HE) staining, immunohistochemistry staining and electron microscopy. Platelet or fibrin thrombus was detected by HE staining, Martius Scarlet Blue (MSB) staining and electron microscopy. Hematologic tests including hemostatic molecular markers such as 11-dehydrogenate thromboxane B2 (11-DH-TXB2), thrombomodulin (TM), thrombin-antithrombin III complex (TAT), D-Dimer and fibrin(ogen) degradation products (FDPs) were examined in guinea pigs to evaluate the coagulative alterations in leptospirosis by quantitative enzyme-linked immunosorbent assay (ELISA).
Results: Pulmonary hemorrhage appeared 24 hours after leptospires intraabdominal inoculation, progressing to a peak at 96 hours after infection. Leptospires were detected 24 hours post-inoculation in the liver, 48 hours in the lung and 72 hours in the kidney by immunohistochemistry staining. Spiral form suggestive of intact leptospires was initially found, but starting from 48 hours after infection, leptospires were seen in granular form resulting from gradual degradation. Platelet aggregation. Phagocytosis of erythrocytes and plateletes by hepatic Kupffer cells were also found. No platelet or fibrin thrombi were found in morphological observation. Thrombocytopenia was seen in experimental leptospirosis in all infectied guinea pigs in our study. Analysis of hematologic molecular markers showed that 11-DH-TXB2 and TM in plasma of guinea pigs were elevated significantly; TAT which reflects the thrombin generation had a decline trend after infection; D-dimer and FDPs increased statistically, nonetheless the increasing did not have clinical significance; D-dimer and FDPs elevated slightly; plasma fibrinogen level was increased.

Conclusions: Pathologic, immunohistochemical and hematological studies in experimental leptospirosis in guinea pigs indicate that the thrombocytopenia found in guinea pigs does not correlate with the occurrence of DIC. The platelet aggregation and hepatic Kupffer cells phagocytosis may among the potential causes of thrombocytopenia.
**Background**

Leptospirosis is a globally important zoonotic disease caused by the pathogenic *Leptospira spp* including *L. interrogans*, *L. kirschneri*, *L. noguchii*, *L. borgpetersenii*, *L. santarosai*, and *L. weilii* etc[1]. Pathogenic leptospires excreted in urine of infected animals may penetrate human body through skin or mucous membranes after the contact of the host with contaminated water or soil. Leptospirosis is characterized by a broad spectrum of clinical manifestations, which goes from a subclinical infection and self-limited anicteric febrile illness to Weil's disease, a severe and potentially fatal disease characterized by hemorrhage, acute renal failure and jaundice[2]. Haematological manifestations are relatively common in leptospirosis, the most common being thrombocytopenia[3], but the mechanism of thrombocytopenia is unknown. Vasculitis, decreased thrombocyte production, and increased peripheral destruction and consumption of thrombocytes have been considered as potential causes of thrombocytopenia[3].

Studies on coagulation alterations during leptospirosis are scanty and mostly retrospective. Interestingly, The results of these studies were inconsistent: some findings showed that although thrombocytopenia occurs in up to 50% of Weil's disease patients[4], the prominent levels of thrombocytopenia seen in severe leptospirosis often do not reflect the
occurrence of DIC syndrome[5-8]; but others reached the conclusion that
the hemorrhages observed in leptospirosis are due to DIC[9-14]. The
purpose of this study was to elucidate whether DIC is an important
feature of experimental leptospirosis in guinea pigs.

**Methods**

**Bacteria**

Isolates of *L.interrogans* strain *Lai* were obtained from the Institute for
Infectious Disease Control and Prevention (IIDC), Beijing, China.
Leptospires were maintained by serial passages in guinea pigs for
preservation of virulence and counted with a Petroff Hauser counting
chamber for experimental infection. Strain was grown in liquid
Ellinghausen-McCullough-Johnson-Harris (EMJH) medium at 28°C
under aerobic conditions and collected at a density of about $10^8$ bacteria
per ml.

**Animals**

Forty guinea pigs (Laboratory animal center of Shanghai Second Medical
University), weighing 150 to 200 grams, were housed in individual cages
and fed with standard guinea pig chow and water *ad libitum*. Five groups
including a negative control group were defined, each group has eight
guinea pigs. Guinea pigs were injected intraperitoneally with 1ml of the
culture ($5\times10^8$ leptospires). Negative control animals were injected with
EMJH media only. Guinea pigs were euthanized on the time of 24h, 48h, 72h or 96h after infection. The Animal Research Committee of the Shanghai Second Medical University approved all animal studies.

**Light Microscopic Studies**

Viscera of the normal and infected guinea pigs were fixed in neutral-buffered 4% formaldehyde, processed routinely, embedded in paraffin, cut into 4-µm serial sections and stained with HE.

**Immunohistochemistry**

Paraffin-embedded tissue sections were incubated at 60°C for 30 minutes. Two xylene washes were applied for 5 minutes, followed by 2 × 100% ethanol for 1 minute, 2 × 95% ethanol for 1 minute, 1× 70% ethanol for 1 minute and incubation in 3% H₂O₂ in methanol for 10 minutes. After 2× 5-minute washes in phosphate-buffered saline (PBS), sections were incubated in 0.2mg/ml trypsin at 37°C for 15 minutes. Sections were again washed ×2 in PBS for 5 minutes, primary rabbit antibody specific for *L.interrogans* strain *Lai* was then added at a dilution of 1:6000 in 0.05M Tris-HCl PBS for 90 minutes. Rabbit antiserum to *L.interrogans* strain *Lai* was prepared using a modified procedure as previously described[15]. After again washing tissue sections, EnVision™ (EnVision system, DAKO) was added for 40 minutes. After washing, staining was visualized using 3, 3’-diaminobenzidine (EnVision system, DAKO) for 10 minutes and the reaction was stopped using H₂O. Before mounting,
slides were washed in H₂O, dehydrated by dipping in 95% ethanol 40 times followed by 100% ethanol for 40 times and 3×5-minute washes in xylene. Sections were counterstained in weak hematoxylin.

**Martius Scarlet Blue (MSB) staining**

Tissues from the livers, kidneys and lungs of the normal and infected guinea pigs were fixed in neutral-buffered 4% formaldehyde, processed routinely, embedded in paraffin, cut into 4-µm serial sections and stained with MSB according to the routing procedures.

**Electron Microscopy**

Tissues were minced into 1- to 2-mm pieces and immersed for more than 2 hours in 2% glutaraldehyde in PBS (pH 7.0). Tissue was then washed in PBS (20 minutes × 3) and post-fixed in 1% osmium tetroxide. Tissues were washed again and dehydrated in graded ethanol, and embedded in Epon. One-µm sections were cut and mounted and stained with toluidine blue. From these sections, regions of interest were selected for ultrastructural study. Seventy-nm sections were cut and stained with uranyl acetate and lead citrate and examined with a PHILIP CM-120 electron microscope.

**Hematology**

At 24, 48, 72 and 96 hours after infection, immediately before the anesthesia, blood was taken by cardiac puncture for analysis of blood parameters and anticoagulated with EDTA (ethylene diaminetetraacetate).
acetic acid; 10 mM) or citrate (final concentration, 3.2%). Each time point consisted of 8 guinea pigs from each experimental group. Platelets were measured using an automated cell counter (Sysmex K-4500, Sysmex, Japan). Fibrinogen was assayed according to Clauss and plasma levels of coagulation factor activity by a 1-stage clotting assay on an automated clotting analyzer SYMEX CA-1500 (Sysmex CA-1500, Sysmex, Japan). Hematologic molecular markers were measured using quantitative ELISA: 11-DH-TXB2 kit was provided by Cayman Chemical Company, TM kit was provided by American Diagnostica Inc, TAT kit was provided by DADE BEHING, D-dimer and FDPs kit was provided by Sun Bioengineering Co.Ltd, Shanghai, China.

Statistics

Hematologic results are presented as mean values±SD. Statistical analysis was performed using analysis of variance (ANOVA). $P <0.05$ was considered statistically significant.

Results

Gross Findings

Gross autopsy revealed distinct, well-circumscribed, multi-focal areas of hemorrhage visible on surfaces of the lungs, and extensive hemorrhage on peritoneal surfaces 24 hours after infection with $L. interferans$ strain $Lai$. Hemorrhage on the surfaces of the lungs became more severe,
progressing to a peak at 96 hours; diffuse pulmonary hemorrhage was taken place in 3 of 8 guinea pigs (Fig 1). Generalized petechiae or ecchymoses in the peritoneum and other internal organs like heart, stomach, intestine and perinephros were also seen.

**Histologic Findings (HE staining, Immunohistochemistry, MSB staining) and Electron Microscopy Study**

**Liver**

HE staining of infected liver tissue demonstrated that the pathologic alterations were first seen at 48 hours after infection, progressing to the peak at 96 hours. Hepatocyte necrosis was observed as groups or as scattered individual hypereosinophilic cells with pyknotic nuclei. Cellular discohesion of hepatocytes ranged from focal to diffuse was also observed. Hypertrophy and hyperplasia of Kupffer cells were seen in the sinusoids. Mild to moderate increases in numbers of monocytes and neutrophils were observed in portal tracts (Fig 2A).

Spiral forms of leptospires similar in shape to whole intact leptospires was initially found 24 hours post-inoculation in the livers by immunohistochemical staining, starting from 48 hours after infection, leptospires were seen in granules form resulting from gradual degradation (Fig 3A).

Electron microscopy studies of liver at 72-96 hours after intraabdominal
inoculation showed that there were deformed erythrocytes in the hepatic sinusoid, breaches were found in the membrane of erythrocytes. Ultrastructural observation revealed there were platelet aggregation, phagocytosis of erythrocyte and platelet by hepatic Kupffer cells were also found (Fig 4).

**Kidney**

In the kidneys, interstitial nephritis was the major finding, accompanied by an intense cellular infiltration composed of neutrophils and monocytes. Occasionally, acute inflammatory cells were present in and around the renal tubules. However, glomeruli appeared normal. Erythrocytes could be seen in the urinary space and renal tubules (Fig 2B). Immunohistochemical staining revealed large numbers of intact leptospires 72 hours after infection. Leptospires can be seen within the renal tubules and in the interstitium, in some cases, a few were present within glomeruli (Fig 3B). Degenerated leptospires were found 96 hours after infection in the interstitium and in tubules.

**Lung**

The histological study in the lungs confirmed the gross findings, infected guinea pigs had significant alveolar hemorrhage. Early hemorrhage appeared randomly as small foci, as severity increased, coalesced areas of hemorrhage developed. Generally, there was a mild to moderate increase of interstitial cellularity of the alveolar septa due to increased numbers of
mononuclear cells and occasional neutrophils. Increased numbers of neutrophils in capillaries were also observed (Fig 2C).

*Leptospires* were detected in the lumen of septal capillaries and in the interstitium at 48 hours after infection by immunohistochemistry staining. Leptospires in spiral forms were initially found, leptospires were seen in granules form starting from 48 hours after infection. There were less leptospires in the lungs compare to the livers and kidneys at the same period (Fig 3C).

HE, MSB staining and ultrastructural studies showed that though there were conspicuous pathogenic changes in the organs, no microthrombi were observed in the liver, kidneys and lungs in guinea pigs during leptospirosis.

**Hematologic results**

The results of the hematologic investigation are shown in Table 1. BPC of infected guinea pigs decreased remarkably compare to normal control (564±82 ×10⁹/L) after infection with *L. interrogans*, the mean value was 97×10⁹/L, 30×10⁹/L at 72 and 96 hours, respectively. Mean platelet volume (MPV) level was increased to 8.09±0.33 fL 96 hours after infection compare to the normal control (6.31±0.19 fL) in experimental leptospirosis in guinea pigs.
Plasma 11-DH-TXB2 level in control group was 3.51±0.55µg/L, the value was increased after infection, progressing to a peak of 7.46±1.67µg/L at 96 hours.

Plasma TM level in control group was 2.91±0.30µg/L, after infection with *L. interrogans*, the value was increased steadily, progressing to a peak of 6.87±0.62µg/L at 96 hours.

The result of Plasma TAT level showed there was a decline trend after infection, the value at 96 hours was 2.04±0.25µg/L compare to the 3.66±0.66µg/L in control group.

Plasma D-dimer and PDFs levels in experimental leptospirosis in guinea pigs had similar increase. D-dimer level in control group was 0.13±0.01 mg/L, the value was increased, progressing to a peak of 0.27±0.05 mg/L 72 hours after infection with *L. interrogans*. PDFs level in control group was 2.01±0.08 mg/L, the value was increased after infection, progressing to a peak of 3.20±0.43 mg/L at 72 hours.

Plasma fibrinogen level in control group was 3.19±0.61g/L, the value was increased after infection with *L. interrogans*, reached 5.92±0.83g/L at 96 hours.

### Discussion

DIC is a disorder that is characterized by systemic intravascular activation of the coagulation system, simultaneously leading to
intravascular thrombi, compromising an adequate blood supply to various organs, and to bleeding as a consequence of the exhaustion of platelets and coagulation factors[16]. Bleeding is the commonest manifestation of DIC, but microvascular thrombosis is a more important cause of morbidity and mortality. DIC is not a disease on itself but is always secondary to an underlying disorder, sepsis or severe infection induced by any microorganism is one of the most frequently occurring diseases that are known to be associated with DIC[17].

The clinical presentation of DIC varies widely, which may complicate the clinical and laboratory diagnosis. Most of routinely available laboratory tests are capable of documenting a deficit in platelets and coagulation factors, but are not able to directly detect activation of coagulation. Since low levels of plasma coagulation factors may be caused by other mechanisms than DIC, many of the underlying conditions that are associated with DIC may cause a low platelet count in the absence of DIC, such as the thrombocytopenia that may occur during severe infection.

Detection of microthrombi was initially considered as necessary for the definition of DIC followed by laboratory assessment of hemostatic abnormalities, molecular markers for activation of coagulation and fibrinogen to fibrin conversion are highly sensitive for the diagnosis of DIC. It is currently considered that hemostatic markers such as TAT, D-dimer et al should be useful for early diagnosis of DIC[18].
Plasma 11-DH-TXB2 is an useful parameter of TXA2 formation which implies the activation of platelet[19]. Plasma TM level is regarded as a molecular marker reflecting injury of endothelial cells. It is often increased in case of diffuse endothelial damage such as in DIC, diabetic microangiopathy, plasmodium falciparum and rickettsial infections. In several systemic inflammatory diseases, Plasma TM levels are correlated to the activity of the disease[20]. Elevated plasma concentration of TAT may well reflect the increased generation of thrombin, the high sensitivity may be helpful in detecting even low-grade activation of coagulation[21]. D-dimer is a specific fibrin degradation product and also serves as a marker for plasmin activation. FDPs serves as a marker of plasmin activation are elevated in 85%-100% patients with DIC, but the specificity of high levels of FDPs is limited and many other conditions, such as trauma, recent surgery, inflammation or venous thrombo-embolism, are associated with elevated FDPs. D-dimer levels are high in patients with DIC, but also poorly distinguish patients with DIC from patients with venous thromboembolism, recent surgery or inflammatory conditions[22].

In the current study, pathologic and hematologic laboratory tests including routine and hematologic molecular markers were used to elucidate whether the DIC is a feature of experimental leptospirosis in guinea pigs. Our results showed that thrombocytopenia was seen in
experimental leptospirosis in guinea pigs. Reduced BPC with enlarged MPV suggesting that the thrombocytopenia was caused by accelerated platelet clearance rather than diminished production during *L. interrogans* infection. The increased plasma 11-DH-TXB2 level indicated that there was platelet activation, followed by platelet aggregation and hepatic Kupffer cells phagocytosis. Increased TM level in guinea pig’s plasma reflect injury of endothelial cells in leptospirosis. D-dimer and FDPs DIC increased statistically, nonetheless the increasing did not have clinical significance. When DIC was induced in control mice by lipopolysaccharide (LPS) injection, D-dimer was elevated 6-fold over background levels[23]. TAT that reflects the thrombin generation had a decline trend after infection with *L. interrogans* other than increase. Plasma fibrinogen level was increased in our study, the reason may be fibrinogen act as a acute phase reaction protein in inflammation. Levi M et al[17] figured that fibrinogen may be unsuitable for the diagnosis of DIC, because it increases in the inflammatory reaction. The results of hematologic laboratory tests indicate that the thrombocytopenia found in experimental leptospirosis in guinea pigs in our study does not correlate with the occurrence of DIC.

More importantly, intravascular fibrin thrombi were not found in the kidneys, liver and lungs in guinea pigs by HE, MSB staining and electron microscopy throughout the study, this also proves that the
thrombocytopenia in our study does not correlate with the occurrence of DIC.

Nally JE and colleagues revealed that there was no chemical or microscopic evidence for DIC, in that the D-dimer was not elevated, intravascular fibrin was not found, and fibrinogen levels were elevated, not diminished[24]. Though thrombocytopenia occurred in 86.6% of 30 patients, it did not seem to be due to DIC consumption. Nicodemo AC et al concluded that thrombocytopenia, uremia and coagulation disorders, individually or as a group, should be included among the contributing factors that lead to and worsen bleeding episodes[25]. DIC is not believed to be a feature in leptospirosis.

Thrombocytopenia can be a complication of many viral, bacterial, fungal, and protozoan infections. In some instances, infection-induced thrombocytopenia is severe enough to cause bleeding. Many, but not all, severe bacterial infections induce thrombocytopenia as a result of DIC, a condition precipitated by the generalized activation of the coagulation cascade in response to bacterial components such as LPS and peptidoglycan[26, 27], DIC in rats can be induced by a 4-h infusion of LPS (30 mg/kg) via the tail vein[28]. Leptospirosis virulence has been attributed in part to the effect of the leptospiral LPS[29], the LPS of *L. interrogans* has a chemical structure and biologic effects similar to that of gram-negative bacteria, however, the former is approximately 12 to 20
times less toxic, and its role in the pathogenesis of the disease appears rather secondary[4]. The structurally unique molecule and relatively low toxicity of *L. interrogans* LPS may be the reason of thrombocytopenia in our study does not correlate with the DIC, in addition, the platelet aggregation and hepatic Kupffer cells phagocytosis may among the potential causes of thrombocytopenia.

**Conclusions**

In conclusion, pathologic, immunohistochemical and hematological studies in experimental leptospirosis in guinea pigs indicate that the thrombocytopenia found in guinea pigs does not correlate with the occurrence of DIC. The platelet aggregation and hepatic Kupffer cells phagocytosis may among the potential causes of thrombocytopenia.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

HLY, XCJ & XKG designed the research project. XCJ and WJL are the pathologists who examined tissue samples. XYZ and BYH coordinated the leptospira culture and participated in the pathology experiments. HLY carried out the hematology experiments. HLY,XKG and XCJ drafted the manuscript, GPZ participated in the design of the study and helped to draft the manuscript. All authors contributed in the writing and preparation of the manuscript. All authors read and approved manuscript.
Acknowledgments

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References

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**Figure Legends**

**Figure 1**
Hemorrhage of the lungs of the guinea pigs injected with *L.interrogans*. A: 24 hours after infection; B: 48 hours after infection; C: 72 hours after infection; D: 96 hours after infection

**Figure 2**
Pathologic changes in the liver (A), kidney (B) and lung (C) of guinea pigs infected with *L.interrogans*.(HE staining, magnification × 200)

**Figure 3** Positive immunohistochemistry staining demonstrating spirochetes in the liver (A), kidney (B) and lung (C) of guinea pigs infected with *L.interrogans*. (EnVision, magnification, × 200)

**Figure 4**
Electron photomicrographs of hepatic sinusoid from infected guinea pigs 72 hours after *L.interrogans* strain *Lai* intra-abdominal inoculation. A: deformed erythrocytes (×4500); B: the breach of erythrocyte membrane (×12000); C: phagocytosis of platelet and deformed erythrocyte induced by hepatic Kupffer cells (×4500); D: platelet aggregation (×3000).
### Table 1

Evolution of hematological detection in experimental leptospirosis in guinea pigs (n=8 in each group), data are presented as mean±SD. (*P<0.05, compare with control)

<table>
<thead>
<tr>
<th>Index of detection</th>
<th>Control</th>
<th>24h after infection</th>
<th>48h after infection</th>
<th>72h after infection</th>
<th>96h after infection</th>
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<tbody>
<tr>
<td>BPC(10⁹/L)</td>
<td>564±82</td>
<td>352±40*</td>
<td>307±50*</td>
<td>97±35*</td>
<td>30±14*</td>
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<tr>
<td>MPV(fL)</td>
<td>6.31±0.19</td>
<td>6.53±0.51</td>
<td>6.51±0.18</td>
<td>7.04±0.51*</td>
<td>8.09±0.33*</td>
</tr>
<tr>
<td>11-DH-TXB2(µg/L)</td>
<td>3.51±0.55</td>
<td>5.12±0.73*</td>
<td>4.85±0.86*</td>
<td>5.31±1.31*</td>
<td>7.46±1.67*</td>
</tr>
<tr>
<td>TM(µg/L)</td>
<td>2.91±0.30</td>
<td>3.40±0.41*</td>
<td>5.63±0.57*</td>
<td>6.62±0.49*</td>
<td>6.87±0.62*</td>
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<tr>
<td>TAT(µg/L)</td>
<td>3.66±0.66</td>
<td>2.87±1.01</td>
<td>3.27±1.56</td>
<td>2.32±0.77*</td>
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<tr>
<td>D-dimer(mg/L)</td>
<td>0.13±0.01</td>
<td>0.19±0.06*</td>
<td>0.24±0.02*</td>
<td>0.27±0.05*</td>
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<tr>
<td>FDPs(mg/L)</td>
<td>2.01±0.08</td>
<td>1.99±0.07</td>
<td>2.70±0.53*</td>
<td>3.20±0.43*</td>
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<tr>
<td>fibrinogen (g/L)</td>
<td>3.19±0.61</td>
<td>3.62±0.62*</td>
<td>4.99±0.63*</td>
<td>5.87±0.71*</td>
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