Time- and dose-dependent severity of lung injury in a rat model of sepsis

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Abstract
Different animal models of experimental lung injury have been used to investigate mechanisms of lung injury. Lipopolysaccharide (LPS) administration is the most often used approach to model the consequences of bacterial sepsis. We created an endotoxemia rat model, simulating sepsis-related lung injury, in order to quantify the time and dose dependent severity lesions induced by the administration of lipopolysaccharide. Our study included 42 male Wistar rats, randomly divided into four groups: one control group (n=6) and three experimental groups (n=12/group) in whom we induced sepsis by intraperitoneal injection of progressively increasing doses of LPS (3, 5, 10 mg/kg). At six hours, the animals included in the groups with higher doses of LPS developed thrombocytopenia, elevated lactate levels, and liver and renal injury in a dose and time dependent manner. The severity of hypoxemia at six hours correlated with the increasing doses of LPS, with a slight improvement at 24 hours. Lung injury scores became more severe with increased dose and time of exposure to LPS without reaching the level of hyaline membranes formation. We also demonstrated translocation of a protein from the airspaces into plasma (RAGE – receptor for advanced glycation end products). Induction of sepsis using LPS is a known experimental model, but LPS treatment in rats does not cause the severe endothelial and epithelial injury that occurs in humans with acute respiratory distress syndrome (ARDS). In our study, the clinical, laboratory and histopathological findings confirmed sepsis and the damage of the alveolar-capillary membrane in a dose-dependent manner.

Keywords: lipopolysaccharide, ARDS, rat, experimental model of sepsis.

Introduction
Acute respiratory distress syndrome (ARDS) represents a devastating condition characterized by diffuse injury of the alveolar-capillary wall and alveolar and interstitial edema consecutive to increased pulmonary vascular permeability [1]. ARDS may result from heterogeneous etiologies, but is most often a common endpoint of sepsis, hemorrhagic shock, and trauma [2] and represents a significant source of morbidity and mortality in the intensive care units. In general, patients with sepsis-related ARDS had a higher disease severity and worse clinical outcomes than those with non-sepsis-related ARDS [3]. Currently, there are very few effective therapies for ARDS, other than supportive therapies and the use of lung protection strategies [4].

Sepsis and ARDS are evolving affections, with different stages, different environments, governed by inflammatory and anti-inflammatory, cellular and humoral responses.

The lung, like any parenchyma will react to aggression by an inflammatory syndrome. It is initially a protective physiological response, which subsequently by the interaction between leukocytes and endothelial cells will cause appearance of lesions at the level of the lung, contributing to the pathogenesis of ARDS.

The pathophysiology of ARDS consists of overlapping acute “inflammatory” and delayed “repair/fibrotic” phases [5]. Thus, the histopathological changes observed in ARDS can be divided into the overlapping phases of exudation, regeneration, and healing, which may be distinguished by either resolution or repair leading to fibrosis.

The pathogenic basis of ARDS and factors governing susceptibility are incompletely understood despite almost 45 years of intense investigation. ARDS is a heterogeneous syndrome associated with complex interactions among the predisposing conditions, comorbidities, and genetic determinants [3]. Different animal models of experimental lung injury have been used to investigate mechanisms of lung injury. Lipopolysaccharide (LPS) administration is the most often used approach to model the consequences of bacterial sepsis.

The objective of this paper was to quantify the dose- and time-dependent severity lesions in sepsis and ARDS, induced by the administration of lipopolysaccharide (LPS) in a rat model of sepsis.

Materials and Methods
Animal care
Our study included 42 male Wistar rats, provided by “Cantacuzino” National Institute of Research and Development for Microbiology and Immunology, Bucharest, Romania, aged 10–12 weeks and weighing 220–260 g.
randomly divided into four groups: control group (n=6) and three experimental groups (n=12/group).

All experimental procedures with animals followed the international recommendations for the use and care of animals and all experimental protocols were approved by Institutional Review Board of the University of Medicine and Pharmacy of Tîrgu Mureş, Romania. The animals were acclimatized to the usual laboratory conditions 14 days before the experiment. They were kept in cages with circadian rhythm of light at the stable temperature of 23°C. During the experiment, the rats were fed with standard laboratory rodent feed and water ad libitum.

**Experimental design and LPS-induced lung injury**

After four hours of stopping nutrition, we administered lipopolysaccharide (lipopolysaccharide from *Escherichia coli* 0111:B4, Quadratech Diagnostics Ltd.) intraperitoneally, in gradually increasing doses 3, 5, 10 mg/kg, to experimental subjects included in groups 1, 2 and 3, for induction of sepsis and ARDS. In the control group, the rats were treated the same as in LPS groups, except LPS is substituted with an equal volume of normal saline (NS).

Lung injury and systemic effects were quantified in terms of hypoxemic, laboratory and histopathological changes at 6 and 24 hours after LPS administration.

Thus, at six hours after administration of lipopolysaccharide, we anesthetized the rats in the control group and six animals in the experimental groups with a mixture of Ketamine–Xylazine (2:1; 0.3 mL/rat, intraperitoneal). After a thorough dissection, we cannulated abdominal aorta and arterial blood samples were collected in order to determine blood gasometry, laboratory analyzes and to obtain plasma samples by centrifugation (2000 rpm for 10 minutes). The obtained plasma samples were stocked in sterile tubes and kept at -70°C by the time of processing.

After blood collection, the rats were sacrificed right away and stamps of lung, renal, hepatic and myocardium tissues were collected. Blood gasometry was measured by using a Gem Premier 4000 blood gas analyzer.

At 24 hours after administration of lipopolysaccharide, the rest of the animals (six rats from each experimental group) were subjected to the same procedure.

**Lung histology**

Lung tissue fragments were processed by standard histological methods: fixing in formalin (24 hours), inclusion in paraffin, sectioning and staining with Hematoxylin–Eosin (HE) and trichromic Goldner–Szekely, and evaluating under optical microscopy (Nikon Eclipse E600).

Lung injury scores were quantified by a pathologist blinded to the treatment groups according to the guidelines provided by *American Thoracic Society* [6]. Lung injury was assessed on a scale of 0–2 for each of the following criteria: (1) neutrophils in the alveolar space; (2) neutrophils in the interstitial space; (3) number of hyaline membranes; (4) amount of proteinaceous debris; and (5) extent of alveolar septal thickening. The final injury score was derived from the following calculation:

\[
\text{Score} = \frac{20 \times (1) + 14 \times (2) + 7 \times (3) + 7 \times (4) + 2 \times (5)}{\text{number of fields} \times 100}
\]

The resulting injury score is a continuous value between zero and one (inclusive).

**Cytokine measurement in serum**

A variety of specific biomarkers for epithelial injury during acute lung injury (ALI) have been proposed, with receptor for advanced glycation end products (RAGE) the most recently advocated [7]. RAGE is expressed on the basal surface of type 1 epithelial cells, and has been shown to increase in bronchoalveolar lavage fluid (BALF) of murine models of lung injury [8, 9] and plasma of patients with ALI/ARDS [10].

In our study, RAGE serum levels of the rats included in the control group and the experimental groups with the lower (5 mg/kg) and the higher (10 mg/kg) LPS doses administered, were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Abcam).

**Statistical analysis**

For statistical analysis, we used SPSS ver. 20 (CA, USA). In order to characterize groups, we employed descriptive statistics (mean, standard deviation, minimum, maximum). The means between groups were compared for statistical significance using the paired *t*-test. The confidence intervals were set at a 95% with a significant *p*<0.05.

**Results**

**General observations**

At six hours after administration of LPS, all the animals receiving LPS showed physical signs of systemic illness including tachypnea, lethargy, piloerection, rejection of food and water with a severity in correlation with the increasing doses of LPS. Four animals included in groups with higher dose of LPS (5, 10 mg/kg, intraperitoneal) developed diarrhea. At 24 hours after administration of LPS, lethargy persisted only in the group with the highest dose of LPS. The animals included in the control group did not show any sign of systemic disease.

**Systemic inflammation associated with LPS**

Transient inflammatory reactions are used to protect the body against infection and toxin invasion. To determine the effect of LPS on multiple organ function, the following serum marker enzymes: SGPT (serum glutamic pyruvic transaminase), SGOT (serum glutamic-oxaloacetic transaminase), creatinine and blood urea nitrogen (BUN) levels were measured to see the effect on liver and kidney respectively. We measured the plasma lactate level at the two time points as a surrogate of inadequate tissue perfusion with concurrent shift toward increased anaerobic metabolism. Changes of circulatory parameters, including white blood cells number (WBC) and platelet number, in the earlier (six hours after LPS), and later (24 hours after LPS) stages were also examined.

An initial significant decrease in circulating WBC, which peaked at six hours after LPS challenge, was found only in groups with higher doses of LPS administered (5, respectively 10 mg/kg, intraperitoneal). The WBC counts slowly returned to the basal level at 24 hours after LPS challenge (Figure 1).
At six hours from administration, the animals included in the groups with higher doses of LPS (5 and 10 mg/kg) developed thrombocytopenia (Figure 2), elevated lactate levels (Figure 3), liver injury (Figures 4 and 5), and renal injury (Figures 6 and 7) in a dose-dependent manner. At 24 hours, the thrombocytopenia, elevated lactate levels, liver and kidney injuries persisted in case of groups with lower dose of LPS administered (3, 5 mg/kg) or even worsened in the group with the highest dose of LPS administered (10 mg/kg).

**Arterial blood gas analysis**

PaO2 or the partial pressure of oxygen dissolved in plasma is a measure of how much oxygen the lungs are delivering to the blood. We collected blood from abdominal aorta after NS or LPS treatment and analyzed PaO2.

We found that PaO2 in all the LPS groups, at six hours, was significantly reduced compared to that in the control group (Figure 8). In our study, LPS administration did not produce a substantial hypoxemia, not even in the group with the highest doses of LPS (10 mg/kg). In the early stage, the severity of hypoxemia correlated with the gradually increasing doses of LPS administered, with a PaO2 lower mean value of 70.67±6.31 mmHg.

At 24 hours, the hypoxemia improved, again in a dose-dependent manner. Comparing the control group to LPS groups, the statistical significance is however borderline ($p=0.053$), and only in case of group with highest dose of LPS administered (10 mg/kg) (Figure 8).

![Figure 1 – Mean value of WBC at six hours and 24 hours after LPS administration. At six hours, there is a statistical significant drop in mean WBC between a dose of 3 mg/kg and 5 mg/kg ($*p<0.0001$). The change in WBC from a dose of 5 mg/kg to 10 mg/kg is not significant ($**p=0.077$) nor is at 24 hours interval ($p=0.895$). Compared with the control group only the change in WBC count at six hours is significant ($***p<0.0001$).](image1)

![Figure 2 – Mean platelet count at six hours and 24 hours after LPS administration. We found no statistical significant change irrespective of dose or time after LPS administration between groups. Compared with the control group at six hours and 24 hours and a 10 mg/kg dose, the change was significant ($***p=0.001$, $###p<0.0001$).](image2)

![Figure 3 – Mean lactate at six hours and 24 hours. There is no statistical significance between groups at six hours post-LPS administration ($p=0.703$, $**p=0.124$). We found a significant difference between the control group and the 10 mg/kg group at six hours and 24 hours ($***p=0.032$, $###p=0.006$). We also found a borderline significance at 24 hours between the 3 mg/kg group and the 5 mg/kg group ($p=0.051$).](image3)

![Figure 4 – Mean GPT (glutamic pyruvic transaminase) at six hours and 24 hours after LPS administration. There is no significant change between groups at six hours ($p=0.413$, $**p=0.502$). There is a significant change between the 5 mg/kg and 10 mg/kg at 24 hours ($p=0.007$). There is a significant change between the control group and the 10 mg/kg at 24 hours ($***p<0.0001$).](image4)
Figure 5 – Mean GOT (glutamic-oxaloacetic transaminase) at six hours and 24 hours after LPS administration. There is a significant change between the 3 mg/kg and the 5 mg/kg group at six hours (**p=0.002). There is also a significant change between the 3 mg/kg, 5 mg/kg, 10 mg/kg groups at 24 hours (##p=0.036, ###p=0.023). Compared with the control group, there is a significant difference in both 10 mg/kg at six hours and 24 hours (***, ###p=0.001).

Figure 6 – Mean blood urea nitrogen (BUN) at six hours and 24 hours after LPS administration. There is no significant change between groups at six hours. We found a statistical significance in BUN change between the control group and the group with 10 mg/kg at six hours and 24 hours (***, ###p<0.0001). There is also a significant change in BUN at 24 hours between the 5 mg/kg and 10 mg/kg group (##p<0.0001).

Figure 7 – Mean creatinine value at six hours and 24 hours after LPS administration. There is a significant change between the 5 mg/kg and 10 mg/kg at six hours and 24 hours (**p=0.004, ###p<0.0001). There is also a significant change in creatinine between the control group and the 10 mg/kg at six hours and 24 hours (***p=0.006, ###p<0.0001).

Figure 8 – Mean PaO2 values at different LPS dosage, measured at six hours. Increasing the dosage from 5 mg/kg to 10 mg/kg is correlated with a statistical significance in PaO2 drop (*p=0.19, **p=0.032). There is significant difference between the control group and the 10 mg/kg group (***p<0.0001). Mean PaO2 values at different LPS dosage, measured at 24 hours. Increasing the dosage from 5 mg/kg to 10 mg/kg is correlated with a significant drop in PaO2 (##p=0.017). If we compare the control group with the 10 mg/kg group, there is a borderline significance (###p=0.053).

Histopathological observation of lung tissue

We examined the histology of lung tissues of all the rats in this study by HE staining (Figure 9). Control animals, treated with saline had no histological abnormalities in their lungs (Figure 9a).

Six hours after intraperitoneal injection of LPS, the rats developed congestion of pulmonary minute vessels and alveolar septum capillaries, even in the group with the lowest dose of LPS administered (3 mg/kg) (Figure 9b). With the increasing dose of LPS administered (5 mg/kg), rats showed alveolar septal thickening, interstitial and alveolar infiltration with erythrocytes, neutrophils and monocytes, collapse of air space and alveolar hemorrhage (Figure 9d). A similar array of progressive lung abnormalities was more predominant in group treated with the highest dose of LPS (10 mg/kg) (Figure 9f). The injury progressed with increased time of exposure to LPS (Figure 9, c, e and g). None of the LPS treated groups developed severe fibrin exudation into alveolar space, with the presence of typical hyaline membranes.
Figure 9 – (a) Normal microscopic aspects of the lung in the control animals group, treated with saline solution; (b) The congestion of pulmonary vessels and alveolar septum capillaries; (c) Inflammatory infiltrate with neutrophils in the alveolar septa; (d) Marked thickening of the alveolar septa, interstitial and alveolar infiltration with erythrocytes, neutrophils and monocytes; (e) Alveolar hemorrhage; (f) Alveolar hemorrhage, marked thickening of the alveolar septa and focal collapse of alveolar spaces; (g) Marked thickening of the alveolar septa and the focal collapse of air spaces. HE staining: (a) ×100; (b–d and f) ×200. Trichromic Goldner–Szekely staining: (e) ×400; (g) ×200.
Lung injury scores became more severe with increased dose and time of exposure to LPS (Figure 10).

**Disruption of epithelial barrier associated with LPS**

The exudative phase of ALI/ARDS is characterized by formation of lung edema due to diffuse alveolar damage. RAGE was identified on the basal surface of alveolar type I cells. In our study, the plasmatic levels of RAGE increased significantly with the increasing dose of LPS administered (Figure 11).

**Discussion**

Due to the difficulties of obtaining histological lung samples from humans, many studies providing valuable data for understanding the mechanisms of lung injury were conducted in animal models. Those models however, are limited in their ability to represent the complex clinical syndrome of ALI/ARDS because they cannot replicate several of the confounding factors, including the effects of age, chronic medical diseases such as liver and renal insufficiency, and the impact of genetic and environmental factors that may contribute to the clinical syndrome of ALI/ARDS [11]. Duration and severity of injury and especially the degree of subsequent resuscitation, not always match close enough clinical condition. Animal models have been based on either direct lung aggression such as bleomycin, endotoxins or suction acid, washing of the surfactant and oxygen toxicity; or indirect pulmonary aggression such as intravenous administration of endotoxins, complement or micro-emboli.

Nevertheless, all animal models in use have common features with human affection such as the influx of inflammatory cells and endothelial injury. Animal models of endotoxin-induced ALI have demonstrated an alteration of the alveolar–capillary barrier, with a more pronounced impairment of the endothelium, while in humans appears more pronounced epithelial injury [12].

Exposure of LPS is a well-known method of inducing acute lung inflammation and ARDS. In serum, LPS binds to a specific LPS binding protein (LBP) [13], forming a complex that activates the CD14/TLR4 receptor structure on monocytes and macrophages [14, 15]. TLR4 activation triggers the inflammatory reaction cascade by the means of production of pro-inflammatory cytokines, like TNF-α and IL-1β [16, 17]. Thus, the use of LPS provides information about the effects of host inflammatory responses, which occur in bacterial infections [18].

For this model of lung injury, species differences in LPS sensitivity are expected [11]. The mechanism for these differences is represented mainly by the species differences in the innate immune response [15]. For example, Toll-like receptor 4 (TLR4) from humans and mice recognize different lipopolysaccharide structures [19]. Also, animals whose lungs contain pulmonary intravascular macrophages (PIM), a resident population of mature macrophages that adhere to endothelial cells in pulmonary capillaries, develop manifestations of sepsis and lung injury with very small doses of LPS, in the μg/kg range. In contrast, animals that lack PIM, such as dog, rat, mouse, rabbit, and non-human primates require much higher doses, in the range of mg/kg [15]. In rats, the LD50 value of *E. coli* LPS is reported to be ~50 mg/kg [20]. In addition to species susceptibility, there are also differences in LPS responses between different strains of the same species [15]. In the literature, the most frequently used dose of
LPS for intraperitoneal injection in experimental studies on rats is around the value of 10 mg/kg.

We created an endotoxemia rat model, simulating sepsis-related lung injury, in order to quantify the time and dose-dependent severity lesions induced by the administration of lipopolysaccharide. Whereas LPS is easy to administer, and the results tend to be reproducible within experiments, we intend to use this model as a tool to test new biomarkers that might present a promise for use in the clinical practice.

LPS activates alveolar macrophages and causes neutrophils to infiltrate and damage the lungs. When activated, the neutrophils produce additional cytokines, playing a crucial role as signaling molecules that initiate, amplify, and perpetuate inflammatory response on the local and systemic levels [21]. Three stages of ARDS may be distinguished that overlap temporally and spatially. A hallmark of first exudative phase ARDS is diffuse alveolar damage consisting of widespread epithelial and endothelial injury and death [7], interstitial thickening; and the formation of microthrombi (evidence of endothelial injury and intraluminal activation of the coagulation cascade). This is followed by a proliferative phase characterized by alveolar epithelial cell hyperplasia and interstitial fibrosis [6]. In the absence of recovery, some patients may progress to a fibrotic stage characterized by diffuse fibrosis and other changes in the lung structure [22].

Our ARDS model was replicating a very acute phase, because blood and histology samples were extracted after six hours and 24 hours after LPS intraperitoneal injection. Lung tissue hyperemia, alveolar septal thickening, neutrophil accumulation, collapse of air space and alveolar hemorrhage are all pathological changes associated with exudative phase of ARDS. Experimental results showed that the rats in LPS-treated groups exhibited varying degrees of lung injury, which correlated with the increasing dose of LPS administered. The injury became more severe with increased time of exposure to LPS.

In humans, a hallmark of diffuse alveolar damage is represented by the deposition of hyaline membranes composed of fibrin and other proteinaceous debris as evidence of the disruption of the alveolo-capillary membrane [6]. In our study, the fibrin exudation into alveolar space was not severe and we did not demonstrate the presence of typical hyaline membranes. At the microscopic level, murine lungs differ from human lungs in that they have a larger number of Clara cells in the distal airways extensive bronchial-associated lymphoid tissue, and a virtual absence of submucosal glands beyond the proximal trachea [23]. In the setting of ALI, murine lungs rarely demonstrate typical hyaline membranes [6].

Highly susceptible to injury, alveolar type I cell covers the majority of the alveolus. The breakdown of the epithelial barrier promote fluid accumulation in the alveolar space and interstitium [21]. RAGE was identified on the basal surface of type I cells. RAGE belongs to the immunoglobulin superfamily and functions as a multi-ligand receptor that propagates the inflammatory response via nuclear factor-kappa B (NF-κB), thus increasing production of proinflammatory cytokines, reactive oxygen species (ROS) and proteases [9]. In patients with ALI/ARDS, plasma sRAGE levels peaked at day 1 and decreased over time, whereas higher levels were detected in patients with more severe lung dysfunction [24]. In another study, baseline plasma levels of sRAGE were significantly higher in patients with ALI/ARDS, with or without severe sepsis, than in patients with severe sepsis only and in mechanically ventilated controls [25]. In a separate retrospective nested case control study of 192 patients, RAGE was 1 of seven biomarkers out of 21 measured that had a high diagnostic accuracy in distinguishing ALI from non ALI in trauma patients [26]. We found that plasma RAGE levels correlated with increasing dose of LPS administered, which suggests that our model produces significant epithelial dysfunction.

In terms of hypoxic changes, in our study, LPS administration produced a statistically significant hypoxemia comparing to control group, but it did not produce a substantial hypoxemia defined as PaO$_2$≤60 mmHg, that is a prerequisite for disease diagnosis, not even in the group with the highest doses of LPS (10 mg/kg). Although the severity of hypoxemia correlated with gradually increasing doses of endotoxin administered, at 24 hours the hypoxemia is improving. Comparing the control group to LPS groups, the statistical significance is only borderline ($p=0.053$), and only in case of group with highest dose of LPS administered (10 mg/kg).

Our present results indicated that after LPS challenge, the circulating WBC were significantly decreased initially (at six hours) followed by a return toward normal range at the later stage of inflammation (at 24 hours). Our results are consistent with those reported by others [15, 27, 28]. In contrast, thrombocytes count, declined gradually in a time-dependent manner.

In LPS-treated groups with higher doses (5, 10 mg/kg, respectively), a significant increase in all the enzymes and creatinine and blood urea levels was observed in a time and dose-dependent manner, when compared with control rats, indicating multiple organ dysfunction. Again, these results are consistent with those reported by others [20].

A review article on animal models of acute lung injury [15] stated that the hemodynamic response to intravenous LPS is characterized by an initial phase of decreased cardiac output, and a fall in arterial pressure and that this initial phase is followed by slow improvement in the hemodynamic profile, over 4–6 hours. Lactic acid is the normal endpoint of the anaerobic breakdown of glucose in the tissues. In the setting of decreased tissue oxygenation, lactic acid is produced in the anaerobic cycle of energy production [29]. In our study, we used lactate as a surrogate of inadequate tissue perfusion with concurrent shift toward increased anaerobic metabolism. We found a significant increase in the lactate level at six hours after LPS challenge, even in the group receiving the lowest dose with a small improvement at 24 hours. Our results support the above statement regarding the hemodynamic response to intraperitoneal LPS administration.

A recent American Thoracic Society article provided guidelines for what investigators must consider measuring in all pre-clinical models of acute lung injury [6]. To determine if acute lung injury has occurred, they recommended that at least three of the four “main features” of acute lung injury be identified. Our endotoxemia rat model
managed to accomplish three of the four “main features”. We demonstrated the accumulation of neutrophils in the alveolar or the interstitial space, thickening of the alveolar wall and enhanced injury as measured by a standardized histology score. We also demonstrated translocation of a protein from the airspaces into plasma (RAGE) and a statistically significant hypoxemia comparing to control group.

We did not perform any of the recommended measurements of the inflammatory response in the lung, but we manage to prove the systemic inflammation associated with LPS administration.

Conclusions

Induction of sepsis using LPS is a known experimental model, but LPS treatment in rats does not cause the severe endothelial and epithelial injury that occurs in humans with ARDS. The main advantage of endotoxemia rat models is the use of LPS is that LPS is easy to administer, and the results tend to be reproducible within experiments. In our study on rats, the clinical, laboratory and histopathological findings confirmed sepsis and the damage of the alveolar–capillary membrane in a dose-dependent manner. The pulmonary microscopic lesions corresponded to the inflammatory or exudative phase of ARDS without reaching the level of hyaline membranes formation. This animal model of ARDS might be adequate in order to generate information about the pathophysiology of lung injury but not to test novel therapeutic interventions in complex biological systems.

Conflict of interests

The authors declare that they have no conflict of interests.

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