Induction of acute respiratory distress syndrome in rats by lipopolysaccharide and its effect on oxidative stress and antioxidant status in lung

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Acute lung injury (ALI) or its severe form, acute respiratory distress syndrome (ARDS) is an important cause of mortality in the human population. Despite significant advances made, the mortality associated with ALI remains unchanged. The objective of the present study was to evaluate the role of oxidative stress, alveolar antioxidant status and multiple organ injury in ARDS induced by lipopolysaccharide (LPS) in rats. Rats were divided into 4 groups, group I control rats were given saline intraperitoneally, whereas groups II, III and IV (LPS-treated) rats received an intraperitoneal injection of LPS (10 mg/kg body weight) and sacrificed after various time intervals. In LPS-treated rats, we observed increased levels of oxidative products, decreased levels of antioxidants in lung tissues and increased levels of serum marker enzymes, suggesting multiple organ injury. Bronchoalveolar lavage fluid (BALF) neutrophil content and protein concentration in LPS-treated rats were significantly elevated in a time-dependent manner. Histological studies revealed neutrophil influx and diffused alveolar damage in LPS-administered rats. These results clearly suggested that increased oxidant levels led to oxidative stress, antioxidant deficiency attenuating lung inflammation and tissue damage. LPS administration resulted in multiple organ failure, leading to increased mortality.

Keywords: Acute respiratory distress syndrome, Lipopolysaccharide, Oxidative stress, Alveolar antioxidants, Multiple organ failure.

Every cell in the body requires a constant supply of oxygen to produce energy, grow, repair or replace itself and maintain the vital functions. Lungs play an important role in delivering the purified oxygen to the cells. The vascular endothelium serves as the key barrier between the intravascular compartment and extra-vascular tissues and plays a critical role in a large number of physiological and pathological processes. Endothelial cells (ECs) are integrally involved in regulating blood flow, coagulation, leukocyte trafficking, edema formation, wound healing and angiogenesis. Because of their location at the blood-extravascular tissue interface, ECs are constantly exposed to circulating mediators that may perturb the above-mentioned endothelial barrier functions.

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is characterized by an extensive neutrophil influx into the lung, the expression of proinflammatory mediators and damage of the lung epithelium and endothelium. Reactive oxygen species (ROS) causes ARDS, plays an important role in pulmonary vascular endothelial damage and is hypothesized to be responsible for clinical manifestation of ARDS. An imbalance between exposed oxidants and endogenous antioxidants results in oxidative stress which is involved in the pathogenesis of a number of growing diseases, including lung pathologies, such as ARDS, chronic obstructive pulmonary disease (COPD) and asthma. Over a period of 25 years, globally the annual incidence of ALI/ARDS is 3,35,000 with 1,47,000 deaths per year. The mortality rate of ALI/ARDS has not significantly changed over the last decade. Hence, elucidating the pathogenic mechanism mediated by oxidant stress is the need of the hour.

The endotoxin lipopolysaccharide (LPS) is a well known inducer compound for ALI/ARDS and induces ALI by mainly dysfunctioning the pulmonary surfactants. Recognition of LPS by a host receptor(s) is the first step in a multi-step
sequence, leading to activation of a number of signal transduction cascades in lung cells. The downstream effectors of these pathways result in production of a variety of inflammatory mediators, including proinflammatory cytokines and chemokines, adhesion molecules, ROS and nitric oxide by various cell types in the lung. In the present study, level of oxidative stress and antioxidant status have been investigated in LPS-treated ARDS rats. In addition, serum marker enzymes for organ injury, bronchoalveolar lavage fluid (BALF) cell analysis, vascular leakage and lung histopathological abnormalities have also been studied to determine the effect of LPS on various organs and tissue damage.

**Materials and Methods**

**Materials**

LPS (*Escherichia coli*, serotype 055:B5), o-anisidine, hexadecyltrimethyl ammonium bromide (HTAB), bovine serum albumin (BSA), 2-thiobarbituric acid (TBA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 2,4-dinitrophenyl hydrazine (DNPH), 3,3'-diaminobenzidine, reduced glutathione (GSH) were obtained from Sigma Chemical Co. (St. Louis, USA).

**Animals and experimental design**

Male albino rats of Wistar strain, 200-250 g of body weight were obtained from Central Animal Facility, Indian Institute of Science, Bangalore, India and kept under conventional housing conditions (22°C, 55% humidity and 12 h day/night cycle) in our University animal house. The rats were fed with standard pellet diet and water *ad libitum*. All procedures were approved and complied with the standards for the care and use of animal subjects as stated in the guidelines laid by Institutional Animal Ethical Committee (IAEC), Bharathidasan University, Tiruchirappalli.

Rats selected from colonies were randomized into 4 groups, comprising of six rats each and were fasted overnight. Group I: control animals (24 h saline treated); Groups II, III, and IV were given LPS (10 mg/kg body weight) intraperitoneally and sacrificed after 6, 12 and 24 h respectively under ketamine anaesthesia (75 mg/kg). The blood samples were collected and serum was separated. Lung tissues were collected, washed with cold saline and used as stated below.

**Myeloperoxidase (MPO) assay**

MPO, a heme–containing enzyme found primarily in polymorphonucleocytes (PMNs) was used as an indirect measure of PMN infiltration into the tissues. MPO assay was performed as described by Egan et al. with minor modifications. Briefly, 50 mg of the lung tissue was weighed, homogenized in 1.0 ml of solution B \( [0.021\% \text{ K}_2\text{HPO}_4, 0.663\% \text{ KH}_2\text{PO}_4 \text{ and } 0.5\% \text{ HTAB (hexadecyltrimethyl ammonium bromide) in distilled water.} \) The homogenates were freeze-thawed twice and centrifuged at 3000 rpm for 10 min. To 0.1 ml of supernatant, 2.9 ml of freshly prepared solution C \( [0.0105\ g \text{ K}_2\text{HPO}_4 \text{ and } 0.3315\ g \text{ KH}_2\text{PO}_4 \text{ in } 40\ ml \text{ of distilled water and } 5\ ml \text{ of solution A (0.017\% solution of dianisidine in methanol) and } 5\ ml \text{ of } 0.006\% \text{ H}_2\text{O}_2 \text{ in distilled water}] \) was added. The change in absorbance was observed every minute for 10 min, at a fixed wavelength of 460 nm using spectrophotometer. One unit of MPO was defined as that degrading 1 µmole of peroxide/min at 25°C and calculated per g of tissue \( (\Delta \text{OD/weight of tissue used/0.0113 = MPO/g of tissue, where 0.0113 = constant).} \)

**BALF collection and cells analysis**

After removing the extraneous tissue, trachea were cannulated using 5 ml syringe and lungs filled to its capacity with ice-cold saline. Lavage effluents were collected 5-times. Combined effluents were spun at 3000 rpm for 10 min to get BALF and BALF cells. The obtained BALF cells were resuspended in 0.5 ml of PBS. Total and differential cell counting was performed using a standard hemocytometer technique.

**Protein concentration of BALF (PCBALF)**

The PCBALF was estimated according to Bradford using crystalline bovine serum albumin (BSA) as a standard.

**Determination of serum marker enzymes for organ function**

Blood samples were collected from all animals \( (n = 6 \text{ for each group}) \) and centrifuged (2000 rpm for 5 min at 4°C) to separate serum. All serum samples were analyzed within 24 h by using standard laboratory techniques according to manufacturer’s protocols. The following marker enzymes were measured in the serum as biochemical indicators of multiple organ injury/dysfunction: serum glutamate oxaloacetate transaminase (SGOT), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), amylase activities were determined respectively...
for liver, lung, heart, pancreatic damage. Kidney dysfunction was analyzed by creatinine and blood urea nitrogen (BUN) (an indicator of reduced glomerular filtration rate and hence renal failure) levels.

Biochemical parameters
Prior to biochemical analysis, each lung sample (100 mg/ml buffer) was homogenized in 50 mM phosphate buffer (pH 7.0) and centrifuged at 10,000 rpm for 15 min. The supernatant obtained was used for biochemical analysis. All lung parameters were expressed as activity per mg protein. Protein content\(^{12}\), lipid peroxidation products\(^{13}\), reduced GSH\(^{14}\) and ascorbic acid\(^{15}\) and enzymatic antioxidants, such as superoxide dismutase (SOD)\(^{16}\), catalase (CAT)\(^{17}\) and glutathione peroxidise (GPx)\(^{18}\) activities were estimated.

Histopathological examination
Conventional techniques of paraffin-wax sectioning and haematoxylin-eosin staining were used for histological studies\(^{19}\).

Statistical analysis
Statistical analysis was performed using one-way analysis of variance (ANOVA) and a least significant difference (LSD) post-hoc test was used to compare individual means (SPSS for WINDOWS 11.5; SPSS Inc., Chicago). The results were expressed as the mean ± SD of six values in each group, and a statistical probability of \(p<0.001\) was considered to be significant.

Results

LPS administration leads to lung neutrophil sequestration
A significant increase in MPO activity was observed in rats receiving LPS (Groups II, III & IV) treatment and the increase was in a time-dependent manner, when compared to control (Group I) rats (Fig. 1). MPO is primarily found in neutrophil and LPS administration led to neutrophil sequestration.

Effect of LPS on BALF neutrophil content and protein concentration of BALF (PCBALF)
A marked increase in the mean neutrophil content was observed in differential count of BALF cells in LPS-treated rats (Groups II, III & IV) compared to control (Group I) rats (Fig. 2a). LPS also induced a time-dependent increase in accumulation of PCBALF in lavage fluid and the concentration of PCBALF showed significant differences (\(p<0.001\)) when compared to control ones (Fig. 2b).

LPS exposure increases the serum marker enzymes and depicts multiple organ failure
To determine the effect of LPS on multiple organ function, serum marker enzymes, LDH, SGOT, CPK

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**Fig. 1—Lung MPO activity in ARDS-induced rats** [Rats were exposed either to LPS or saline and were sacrificed at various time intervals. After collecting the lavage fluid (BALF), the lungs were homogenized and MPO activity was measured. The values were expressed as mean ± SD. Six animals were used in each group. Group I - control 24 h saline treated; LPS in the following groups was treated for different time intervals, Group II – 6 h; Group III – 12 h; Group IV – 24 h. \(*P\) value \(<0.001\), LPS against control was significant. MPO activity was increased seven-fold in group IV rats, when compared to control rats]

**Fig. 2—Effect of BALF cell neutrophil content and protein concentration of BALF (PCBALF) in ARDS induced rats** [Rats were treated with saline alone or LPS at various intervals and euthanized, subjected to BALF as stated in ‘Materials and Methods’, (a) BALF cell neutrophil content, (b) protein concentration of BALF was evaluated to assess the pulmonary vascular leakage. Each bar represents the mean ± SD. Six animals were used in each group. Group I - control 24 h saline-treated; LPS in the following groups was treated for different time intervals, Group II – 6 h; Group III – 12 h; Group IV – 24 h. \(*P\) value \(<0.001\), LPS against control was significant]
and amylase, besides creatinine and blood urea levels were measured to see the effect on lung, liver, heart, pancreas and kidney respectively. In LPS-treated rats, a significant increase in all the enzymes and creatinine and blood urea levels was observed in a time-dependent manner, when compared with control rats, indicating multiple organ dysfunction (Fig. 3).

### Levels of lipid peroxidation and non-enzymatic antioxidants

Effect of LPS on lipid peroxidation in ARDS-induced rats was investigated from lung homogenates of treated and control rats. Our data showed that lipid peroxidation end product MDA increased significantly \((p<0.001)\) in LPS-treated rats when compared to control rats. The oxidant level was 2-fold higher in group IV rats when compared to group I rats. The elevated MDA levels provided an evidence for massive oxidative stress. To validate the protective role of non-enzymatic antioxidants, the levels of ascorbic acid and glutathione were also measured in LPS-treated rats; their levels decreased significantly \((p<0.001)\) when compared to control rats (Table 1).

### Effect on enzymatic antioxidants SOD, CAT and GPx

To evaluate the defensive role of enzymatic antioxidants against LPS toxicity, the effect of enzymatic antioxidants, namely SOD, CAT and GPx on ARDS-induced rats was investigated in lung homogenates of LPS-treated and control rats. The activities of SOD, CAT and GPx declined significantly \((p<0.001)\) in ARDS-induced rats when compared to control rats.
compared with control rats (Table 1). The steady decrease in values were observed and the trend was Group IV > Group III > Group II > Group I.

**Lung histology of ARDS rats**

Lungs from LPS-treated and saline-treated rats were investigated for histological abnormalities. Control animals, treated with saline had no histological abnormalities in their lungs (Fig. 4a). LPS-treated rats showed thickening of the alveolar capillary membrane, shedding of bronchiolar epithelium, and diffused alveolar damage, which was characterized by alveolar and interstitial hemorrhage and edema as well as extensive interstitial and alveolar infiltration with neutrophils in group II rats (Fig. 4b). A similar array of progressive lung abnormalities was more predominant in group IV LPS-treated rats (Fig. 4d), followed by group III rats (Fig. 4c).

**Discussion**

Exposure of LPS is a well-known method of introducing acute lung inflammation and ARDS. LPS activates alveolar macrophages and causes neutrophils to infiltrate and damage the lungs. Accumulation of neutrophils occurs during acute lung injury. Understanding the molecular basis of the disease will reveal exciting new biomarkers that will present a promise for use in the clinical practice.

MPO, a well known marker enzyme for lung neutrophil sequestration increased seven-fold in LPS-treated rats (group IV) when compared to control rats (group I). BALF cell analysis also revealed that total cell count increased significantly in LPS-treated rats when compared with control rats (data not shown). During differential count, the neutrophil content in group IV rats was significantly \( p<0.001 \) elevated, when compared to control rats, which also supported the neutrophil infiltration. The excessive activation and migration of circulating neutrophils from blood to the alveolar airspace is one of the key events in the early development of ALI. It has been reported that LPS results in activation of the p38 mitogen activated protein kinase (MAPK), and inhibition of pulmonary MAPK activity abrogates LPS-induced TNF production, bronchoconstriction, neutrophil recruitment into the lungs and broncho-alveolar space.

Protein concentration of BALF was increased significantly in LPS-treated rats (group II 122.32 ± 31.54 mg/dl, group III 221.57 ± 26.97 mg/dl and group IV 280.47 ± 35.46 mg/dl), when compared with normal rats (Group I 29.68 ± 14.97 mg/dl). This clearly showed the presence of pulmonary vascular leakage. Clinical studies have reported that IL-1\( \beta \) is one of the most biologically active cytokines in the airway of patients with acute lung inflammation. IL-1\( \beta \) has been shown to inhibit fluid transport across the distal lung epithelium to cause surfactant abnormalities and to increase protein permeability across the alveolar-capillary barrier.

LPS-induced oxidative stress in lung is well established and it was characterized by increased MDA, pulmonary MPO, BALF neutrophil content and BALF protein concentration. Hence to start with, it was necessary to determine the basic parameters to confirm oxidative stress and our results were in agreement with the afore-said previous reports. Previous in vivo studies using animal models that principally involved oxidative damage to the lung have usually shown severe stages of injury with marked changes in lung dysfunction. However, in most studies, levels of antioxidants along with serum marker enzymes for organ dysfunction were not measured simultaneously, but our study correlated both.

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**Fig. 4**—Effect of LPS on lung tissue damage. Animals exposed to either LPS (10 mg/kg body weight) or saline were euthanized and their lungs were examined histopathologically with hematoxylin-eosin stain. The representative photomicrographs of lung sections from control 24 h saline-treated (a), 6 h LPS-treated (b), 12 h LPS-treated (c) and 24 h LPS-treated (d). Magnification × 40. Panels b, c and d showed progressive patchy consolidation, dense infiltration predominantly by neutrophils in the alveoli, bronchial wall and interstitium denoting maximum diffusion and alveolar damage in group IV rats treated with LPS. The changes in morphology was denoted as follows: * - alveoli; N – neutrophils; arrow marks – thickening of bronchial wall and edema formation. Scale bars: In Fig. 4a, 1 cm = 238.38 µm; in Fig. 4b, 1 cm = 237.02 µm; in Fig. 4c, 1 cm = 237.56 µm; in Fig. 4d, 1 cm = 240.96 µm.
Activity, and diminished GPx and SOD activities have been reported in serum of ARDS patients. Increased lipid peroxidation and CAT activity has been reported in serum of ARDS patients with ARDS. Lipid peroxidation products has been reported in pulmonary EC-SOD in a time and dose-dependent manner. The LD₅₀ value of E. coli LPS is reported to be ~50 mg/kg. We showed dysfunction of lung, liver, heart, kidney and pancreas at a much lower dose of 10 mg/kg of LPS, even after a short exposure of 6 h. Hence it was clearly shown that LPS-induced ARDS resulted in multiple organ failure that led to inflammation and tissue damage.

The status of oxidants in lung tissue, represented by TBARS levels increased significantly (p<0.001) in group IV rats (0.3583 ± 0.02 µmol/mg protein of MDA) when compared to control rats (0.1747 ± 0.018 µmol/mg protein of MDA). The elevated TBARS levels provided evidence for massive oxidative stress.

The SOD activity decreased by 57%, CAT activity by 46% and GPx activity by 39% in group IV (24 h LPS-treated) rats, when compared with control rats. LPS administration has been shown to result in a rapid and significant loss of more than 80% of pulmonary EC-SOD in a time and dose-dependent manner. The non-enzymatic antioxidants also declined in LPS-treated rats when compared to control rats. Ascorbic acid level decreased by 39, 68 and 72% respectively in groups II, III and IV rats. Similar results have been reported in plasma of patients with sepsis or septic syndrome. GSH activity was decreased from 19% in group II to 49% in group IV rats. A significant decrease in plasma levels of glutathione, ascorbate and elevated levels of lipid peroxidation products has been reported in patients with ARDS.

An increase in the levels of antioxidant enzyme activities has been reported in serum of ARDS patients and only a few in vivo animal studies are available. Increased lipid peroxidation and CAT activity, and diminished GPx and SOD activities have been reported in lung tissues of animals inhaling 0.5 mg/ml LPS (3,000,000 EU/mg) dissolved in 2 ml of saline using an inhalation chamber daily for 5 days. Improved oxidants and CAT activity after 180 min, and declined activity of SOD have been observed in guinea pigs with 0.7 mg/kg of LPS, when given intraperitoneally. Increased TBARS level and reduced SOD activity with no alterations in GPx activity is also reported, when LPS is administered to rats (10 mg/100 g) intravenously.

In our present study, after LPS exposure, lungs showed a significant decrease in the levels of SOD, CAT, GPx and non-enzymatic antioxidants like ascorbic acid and GSH in group IV (24 h LPS-treated) rats, when compared with control rats. This pattern contradicted with the previous reports. Increased levels of antioxidant enzyme activities were expected to compensate for the increased oxidative stress, so as to defend the animal against ROS. The average half-life of lung epithelial cells is 17 months. The differing results observed might be explained by the fact that the life-span of lung cells is much longer than the observation interval or induction of ARDS and the resulting alterations were studied for a short span (0-24 h) and hence there was not sufficient time for adaptation to occur. Furthermore, it might be due to the downregulation of NF-kappaB signaling, which is linked to decreased antioxidant levels, increased oxidative stress and enhanced cell death. Histopathological examinations also clearly revealed the effect of LPS on lung tissue damage.

In conclusion, the present study demonstrated that intraperitoneal administration of a relatively lower dose of LPS (10 mg/kg body weight) led to ARDS in rats. We found that increased levels of ROS-mediated oxidative stress and antioxidant deficiency attenuated the lung inflammation and tissue damage. Serum marker enzymes responsible for organ failure such as lung, liver, heart, pancreas and kidney showed significant alterations, leading to pathogenic conditions. Thus, the study clearly showed that LPS-induced ARDS in rats resulted in multiple organ failure, leading to increased mortality. However, both short and long-term studies by exposing the rats to different doses of LPS might provide us a better understanding on the adaptive mechanism of antioxidant enzymes at both the enzyme and expression levels.
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