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Dual hit lipopolysaccharide & oleic acid combination induced rat model of acute lung injury/acute respiratory distress syndrome

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***Background & objectives*: Despite advances in therapy and overall medical care, acute lung injury (ALI)/ acute respiratory distress syndrome (ARDS) management remains a problem. Hence the objective of this study was to develop a rat model that mimics human ALI/ARDS.**

***Methods*: Four groups of Wistar rats, 48 per group were treated with *(i)* intratracheal (IT) lipopolysaccharide (LPS) (5 mg/kg) dissolved in normal saline (NS), *(ii)* intravenous *(iv)* oleic acid (OA) (250 µl/kg) suspension in bovine serum albumin (BSA), *(iii)* dual hit: IT LPS (2 mg/kg) dissolved in NS and iv OA (100 µl/kg) and *(iv)* control group: IT NS and iv BSA. From each group at set periods of time various investigations like chest X-rays, respiratory rate (RR), tidal volume (TV), total cell count, differential cell count, total protein count and cytokine levels in bronchoalveolar lavage fluid (BALF), lung wet/dry weight ratio and histopathological examination were done.**

***Results*: It was noted that the respiratory rate, and tumour necrosis factor-α (TNF-α) levels were significantly higher at 4 h in the dual hit group as compared to LPS, OA and control groups. Interleukin-6 (IL-6) levels were significantly higher in the dual hit group as compared to LPS at 8 and 24 h, OA at 8 h and control (at all time intervals) group. IL-1β levels were significantly higher in LPS and dual hit groups at all time intervals, but not in OA and control groups. The injury induced in dual hit group was earlier and more sustained as compared to LPS and OA alone.**

***Interpretation & conclusions*: The lung pathology and changes in respiration functions produced by the dual hit model were closer to the diagnostic criteria of ALI/ARDS in terms of clinical manifestations and pulmonary injury and the injury persisted longer as compared to LPS and OA single hit model. Therefore, the ARDS model produced by the dual hit method was closer to the diagnostic criteria of ARDS in terms of clinical manifestations and pulmonary injury.**

Key words ALI - ARDS - endotoxaemia - fat embolism - lipopolysaccharide and oleic acid (OA) combination - rat model - septicaemia

Acute lung injury (ALI) and its most severe manifestation, acute respiratory distress syndrome (ARDS), is a clinical syndrome defined by acute hypoxaemic respiratory failure, bilateral pulmonary infiltrates consistent with oedema and normal cardiac filling pressures[1]. Despite strides made in understanding the pathogenesis of ALI/ARDS in intensive care, the mortality rates persist between 30 and 60 per cent[2]. The main treatment approach includes ventilatory care and cardiovascular support based on recognition of the clinical picture. Other therapeutic measures for the treatment of ALI/ARDS include glucocorticosteroids and surfactant replacement therapy and inhaled nitric oxide[3].

Research into the pathogenesis of this devastating condition is crucial for the development of novel therapies that target specific disease mechanisms[4]. Unfortunately, no single animal model of ALI/ARDS replicates the complex pathophysiological changes seen in patients and there is a difficulty in developing reliable, reproducible ALI/ARDS model in which the pathologic triad of alveolar neutrophilia (indicating inflammation), hyaline membrane deposition (indicating disruption of the alveolar/capillary barrier) and microthrombi (indicating endothelial injury) can be reproduced[5]. To improve the outlook for patients with ALI/ARDS, advancement in disease model is a necessity. Improvements in disease model should take into account the key pathophysiological features and may require the use of multiple modalities to mimic multiple features of acute lung injury.

One of the most common underlying aetiology for development of ALI/ARDS in humans is septicaemia with an estimated 18-42 per cent of patients developing ARDS with a mortality rate of approximately 50 per cent[6]. While, the effect of intravenous (iv) administration of oleic acid (OA) on the lungs of several animal species is well established as a model of acute diffuse lung injury resembling the initial phase of ARDS. Theoretically, the OA model is closely related to the subset of ARDS caused by fat embolism[7]. The cellular injury induced by lipopolysaccharide (LPS) appears to be related to apoptosis, in contrast to the OA model, in which the injury is due to necrosis at the endothelial level[2,8]. Although animal models of ALI/ARDS induced by LPS, OA and other chemicals have been well documented, they do not mimic the ALI/ARDS syndrome in humans completely[4]. The establishment of an animal model that satisfies the diagnostic criteria of ARDS remains a significant requirement to ARDS research[9].

Table I. Various dose combinations of lipopolysaccharide (LPS) & oleic acid (OA) investigated in pilot study

Dose of LPS (mg/kg)	Dose of OA (µl/kg)
5 mg in 0.15 ml normal saline (NS)	250 µl in 250 µl bovine serum albumin (BSA)
3 mg in 0.1 ml NS	200 µl in 160 µl BSA
2 mg in 0.1 ml NS	100 µl in 180 µl BSA

In this study, we report a dual hit LPS and OA combination induced animal model of ALI/ARDS with refractory hypoxaemia and its comparison with single hit models using LPS alone or OA alone.

Material & Methods

The study was conducted at the Bombay Veterinary College, Mumbai, India. The study protocol was approved by the Animal Care Ethics Committee of the Bombay Veterinary College, Mumbai, India. Male Wistar rats (180-220 g) obtained from Haffkine Institute, Mumbai, India, were kept at a regular 12 h light: dark cycle, with a temperature of 22±3°C. Food and water were given *ad libitum*.

LPS from *Escherichia coli* (O111:B4), was obtained from Sigma Aldrich, USA. OA, bovine serum albumin (BSA) and phosphate buffer saline (PBS) were obtained from Hi-media Laboratories, Mumbai. Interleukin (IL) - 1β, 6 and tumour necrosis factor (TNF)-α enzyme-linked immunoassay (ELISA) kits were purchased from Krishgen BioSystems, India.

Selection of dose and administration of LPS and OA: Rats were anaesthetized by intraperitoneal injection of ketamine (40 mg/kg) and xylazine (5mg/kg). LPS was prepared in normal saline (NS) and given intratracheally (IT) while OA was suspended in 0.1 per cent BSA and given intravenously. In pilot study combination of LPS and OA was administered to animals (3 rats per dose combination) in various doses and concentrations. Step down in doses and volumes of combination were done till rats survived at least for 24 h without any intervention (Table I).

The doses of LPS and OA which induced ALI/ARDS in rats with minimum number of mortalities within 24 h were found to be 2 mg/kg LPS in 0.1 ml NS given (IT) and 100 µl/kg OA in 180 µl BSA given iv and this was used in subsequent experiments.

Experimental design: Four groups with 48 animals per group were treated with *(i)* IT LPS (5 mg/kg) dissolved

in normal saline (NS), *(ii)* intravenous OA (250 µl/kg) suspension in 0.1 per cent BSA, *(iii)* dual hit with IT LPS (2 mg/kg) dissolved in NS and iv OA (100 µl/kg) and *(iv)* control group with IT NS and iv 0.1 per cent BSA. Each group was divided into four subgroups of 12 rats each, for assessing respiratory functions at 4, 8, 24 and 30 h after induction of injury.

Assessment of respiratory function: Animals were observed for cyanosis, signs of respiratory distress and mortality. At each time interval, of the surviving animals, two were subjected to chest X-rays (Siemens digital X-ray equipment, India) and in six animals plethysmograph (Hugo Sachs Elektronik- Harvard Apparatus, Germany) readings and bronchoalveolar lavage fluid (BALF) collection was done. Remaining surviving animals were sacrificed using cervical dislocation method and left lung was subjected to measuring wet/dry weight ratio while in right lung histological examination was performed.

For the assessment of respiratory functions chest X-rays were taken of anaesthetized animals at exposure of 2.5 kV and 42 mA for a fraction of 0.4 seconds. Animal was placed in a double chamber plethysmograph to measure respiratory rate and tidal volume as indirect indicators of hypoxaemia[10].

Lungs were lavaged *in situ* by infusion of 15 ml (in 5 ml aliquots) sterile normal saline solution via a cannula ligated in the trachea. The BALF was collected into plastic tubes on ice. BALF was centrifuged at 371 g at 4°C for 10 min. After centrifugation, two samples of 2 ml were taken from the supernatant and were stored at -20^0 C for analysis of cytokines IL-1β, IL-6 and TNF-α[11]. Total cell count, differential cell counts and total protein concentration were determined from cytocentrifuge precipitate.

Levels of IL-1β, IL-6 and TNF-α in the BALF were determined using ELISA kits. Samples and standards were assayed in duplicate spectrophotometrically using a microplate reader. Results were recorded as optical densities, plotted against the linear portion of the standard curve, and expressed as picograms of cytokine/millilitre of BALF.

To determine lung wet/dry weight ratio animals were sacrificed and thorax was opened. The trachea was separated from thymus and oesophagus and cut just below the larynx. The lungs still connected to the heart were dissected. The left lung was excised and immediately weighed using a precision balance, then re-weighed after being dried for 24 h in an incubator at 90°C[12]. The wet/dry weight ratio was calculated by dividing the wet weight by the dry weight.

Histopathological analysis of lungs: Right lungs were immersed in 10 per cent formaldehyde fixative for 24 h and were then rinsed with tap water to remove the formaldehyde. For light microscopic observation, tissues were dehydrated with graded alcohol and then embedded in paraffin[13]. A series of microsections (5µm) were stained with haematoxylin–eosin using standard histological techniques and microscopic examination was done at a magnification of ×100 for evidence of inflammatory changes.

Statistical analysis: The significance of the difference between experimental groups and time points was calculated using two-way ANOVA test followed by post hoc Bonferroni correction.

Results

Pilot study: Combinations of LPS and OA were administered in various concentrations. Acute severe tachypnoea (> 200 breaths per minute) was noted in rats within minutes of induction of injury using LPS (5 mg/kg - IT) and OA (250 µl/kg - iv). The breathing became progressively laboured and by 4 h shallow ataxic breathing was followed by apnoeic spells with 100 per cent mortality in the group. Rats treated with a combination of LPS (3 mg/kg - IT) and OA (200 µl/kg - iv) showed increasing tachypnoea, respiratory distress, ataxic breathing and apnoeic spells with 100 per cent mortality noted around 8 h after injections. This group and the prior group were not considered for further studies.

In rats injured using LPS (2 mg/kg - IT) and OA (100 µl/kg - iv) in combination, deaths were observed in each subgroup (4, 8, 24 and 30 h) and mortality increased with time. Thus acute and sustained injury was evident in this group. This dose combination was used in subsequent study.

Experimental study: Table II gives comparative analysis of mortality of rats in various groups. In group *(i)*, respiratory rate was significantly increased (130-140 breaths per minute) 8 h after LPS (5 mg/kg) stimulation. Rats developed respiratory distress and cyanosis early within 8 h. Maximum mortality of 25 per cent was seen at 8 h and less (8%) mortality was

Table II. The mortality in rats in various experimental groups

Group	Time (h) after stimulation [N(%), mortality out of 12 rats]			
	4	8	24	30
(i) LPS group (5 mg/kg)	2 (16.7)	3 (25.0)	1 (8.3)	0
(ii) OA group (250 µl/kg)	1 (8.3)	2 (16.7)	3 (25.0)	1 (8.3)
(iii) LPS (2 mg/kg) + OA (100 µl/kg)	1 (8.3)	2 (16.7)	3 (25.0)	3 (25.0)
(iv) Control group	0	0	0	0

LPS, lipopolysaccharide; OA, oleic acid

observed at 24 h. Surviving rats in this group gradually recovered from these symptoms and returned to normal respiration 24 h post-stimulation. Hence readings at 30 h post-injury were not taken for this group.

Rats in group *(ii)* were tachypnoeic (85-135 breaths per minute), developed obvious respiratory distress and cyanosis at 24 h post-OA (250 µl/kg) stimulation. Injury in this group was delayed as compared to group *(i)* with peak of mortality (25%) at 24 h. Most of the respiratory symptoms gradually subsided 24 h after the stimulation in surviving rats.

The respiratory manifestations of rats in group *(iii)* injured using LPS (2 mg/kg - IT) and OA (100 µl/kg - iv), symptoms of respiratory distress started at 4 h, peaked at 8 h and were still evident 24 h post-stimulation. Rats in control group *(iv)* were in good general condition, with a normal respiratory rate of 60-70 breaths per minute. No respiratory distress, cyanosis or death was observed.

Table III gives comparison of various parameters used in the assessment of respiratory function in LPS, OA and combination of LPS & OA treated animals. It was noted that respiratory rate (Fig. 1) and TNF-α levels (Fig. 2) were significantly higher at 4 h in combination group as compared to LPS (P<0.01), OA and control (P<0.001) group. IL-6 levels were significantly higher in dual hit group as compared to LPS (P<0.05 at 8 and 24 h), OA (P<0.05 at 8 h) and control (P<0.01 at 4 and 30 h and P<0.001 at 8 and 24 h) group. IL-1β (Fig. 3) levels were significantly higher in LPS and dual hit group, each with P<0.001 at all time intervals as compared to OA and control group.

Injury induced, as reflected by all above parameters along with chest X-rays, tidal volume, total leukocyte count (Fig. 4), neutrophil per cent, total protein level and histopathological examination (Fig. 5), in dual hit group was earlier (4 h versus 8 h) and more sustained (30 h versus 24 h) as compared with LPS group. The injury in LPS group decreased by 24 h as against sustained injury seen in dual hit group at 24 h.

Discussion

The development of an appropriate ARDS animal model is a prerequisite for the research and product development for clinical treatment of the condition. LPS has frequently been used to develop ARDS animal model as it has been reported that 70 per cent of clinical ARDS cases are associated with sepsis and LPS is known to cause ARDS after sepsis[14,15]. It has also been reported that intratracheal injection of LPS can induce ARDS like symptoms in rats[10,16]. At the initial stage of this study, we investigated the effects of intratracheal injection of LPS in the rat. It was found that intratracheal instillation of LPS resulted in tachypnoea, elevated total protein concentration in the BALF, destruction of alveolar structure and infiltration of pulmonary tissue by large number of inflammatory cells (primarily neutrophils). These pathological changes were consistent with those of ALI, but the pulmonary injury was not evenly distributed within the lung, *i.e.* injury with different pulmonary lobes. Also hypoxaemia was not sustained, and animal started to recover 8 h post-stimulation. All these suggested that this method induced temporary (mild) hypoxaemia that mimicked ALI, but not ARDS.

Similarly intravenous injection of OA has been reported to mimic ARDS in animal models[7,17]. In this model lung injury was associated with damage of the pulmonary vascular endothelium accompanied by local haemorrhage and oedema. However, we found that infiltration of inflammatory cells was comparatively less, injury was delayed as compared to LPS, the degree of pulmonary injury was relatively milder and pathologically no pulmonary oedema formed although

Table III. Comparison of various parameters in different experimental groups

Parameter	Model	Time (h) post stimulation			
		4	8	24	30
Tidal volume (ml) N = 6	LPS	0.93 ± 0.19§	0.50 ± 0.14**£	1.02 ± 0.17£§	Not done
	OA	1.14 ± 0.32	1.03 ± 0.25	0.97 ± 0.24	1.15 ± 0.57
	LPS+OA	1.33 ± 0.24	0.95 ± 0.14	0.82 ± 0.15	0.93 ± 0.19
	Control	1.07 ± 0.17	1.11 ± 0.11	1.01 ± 0.08	1.07 ± 0.08
PMN (%) N = 6	LPS	82.17 ± 5.98***£	84 ± 7.15***£	72.33 ± 3.5**	Not done
	OA	64.67 ± 8.09**	66.33 ± 6.09***	70.17 ± 6.49**	47.17 ± 9.28
	LPS+OA	80.67 ± 5.05***£	78.5 ± 6.55***	71.83 ± 11.89**	37.67 ± 11.25
	Control	39 ± 11.37	39.33 ± 10.80	44.25 ± 12.39	29.5 ± 16.00
Total protein (g/dl) N = 6	LPS	0.6 ± 0.35**	0.63 ± 0.10***	0.35 ± 0.10£§	Not done
	OA	0.42 ± 0.07	0.58 ± 0.07**	0.62 ± 0.19*	0.3 ± 0.09
	LPS+OA	0.55 ± 0.10*	0.5 ± 0.07*	0.57 ± 0.14*	0.3 ± 0.13£
	Control	0.27 ± 0.08	0.23 ± 0.05	0.3 ± 0.13	0.23 ± 0.10
IL-6 (pg/ml) N = 6	LPS	2321.5 ± 1168.27*	3383.36 ± 1179.14***§	1761.58 ± 1037.92§£	Not done
	OA	1678.15 ± 873.70	2697.47 ± 1132.76**§	3040.7 ± 1064.83***	1179.23 ± 545.49
	LPS+OA	2628.1 ± 1049.28**	4325.57 ± 1245.57***£	4143.87 ± 1924.89***£	2577.2 ± 1611.38**£
	Control	54.97 ± 19.46	54.1 ± 19.01	44.45 ± 9.30	39.22 ± 14.47
Lung wet/dry weight ratio	LPS	4.98 ± 0.39 N = 4	6.47 ± 0.65 N = 3	3.9 ± 0.64 N = 5	Not done
	OA	4.39 ± 0.69 N = 5	4.92 ± 1.13 N = 4	5.36 ± 0.54 N = 3	2.89 ± 0.67 N = 5
	LPS+OA	4.63 ± 0.47 N = 5	5.38 ± 0.78 N = 4	3.81 ± 0.68 N = 3	3.9 ± 0.69 N = 3
	Control	3.3+0.88 N = 6	3.65+1.25 N = 6	3.50+1.00 N = 6	3.87+0.48 N = 6

All values expressed are mean ± SD. $P^*<0.05$, $^{**}<0.01$, $^{***}<0.001$ compared to control group. § indicates $P<0.05$ compared to LPS+OA group. £ indicates $P<0.05$ compared to OA group. (Using two-way ANOVA followed by post-hoc Bonferroni correction)
LPS, lipopolysaccharide; OA, oleic acid; PMN, polymorphonuclear leukocytes; IL, interleukin

the dose of OA administered was higher than that given in most of the earlier studies[18,19]. Thus ARDS could not be reliably induced in the rat using intravenous injection of OA alone.

We tried to develop a quick onset and sustained lung injury model using a combination of intratracheal lipopolysaccharide and intravenous OA in rats. Conventional animal model dose of LPS (5 mg/kg) and OA (250 µl/kg) when used in combination, proved lethal within 4 h of induction of injury. Hence, step down of the doses was tried. A dose of LPS of 2 mg/kg combined with 100 µl/kg of OA produced acute and sustained lung injury. In this model rats developed clinical, radiological, biochemical and histopathological changes which correlated with development of ALI/ARDS. These changes were seen within 4 h of injury, peaked around 8 h and recovery was noted 30 h after injury. This model was associated with the pathological injuries at both the epithelium and endothelial level mimicking the human ALI/ARDS model very closely. Epithelial injury was induced by intratracheal LPS[20] while the endothelial injury was due to intravenous OA[21]. The combined synergistic effect led to an early and sustained response. As one

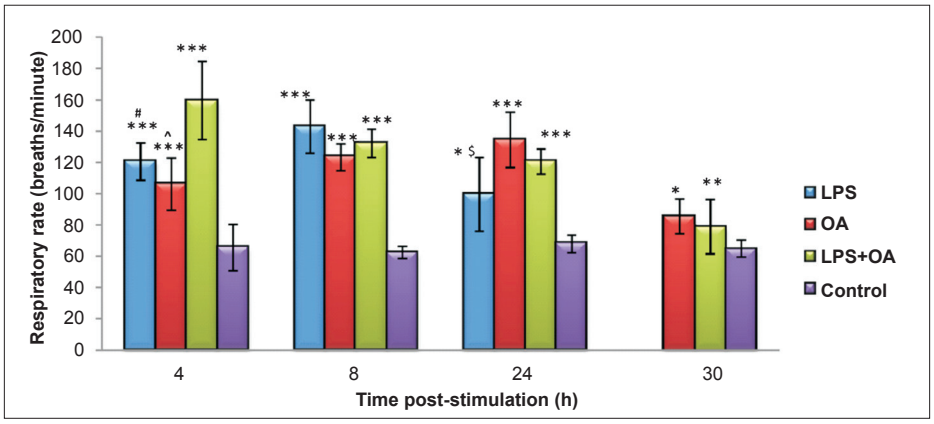

Fig. 1. Comparison of respiratory rate in different experimental groups. All values are expressed as mean ± SD (N=6). P^{*}< 0.05, **<0.01, ***<0.001 compared to control group. $^{\#}P$<0.01 and ^ indicates P<0.001 compared to LPS+OA group. $^{\$}$ indicates P < 0.01 for LPS group compared to OA group.

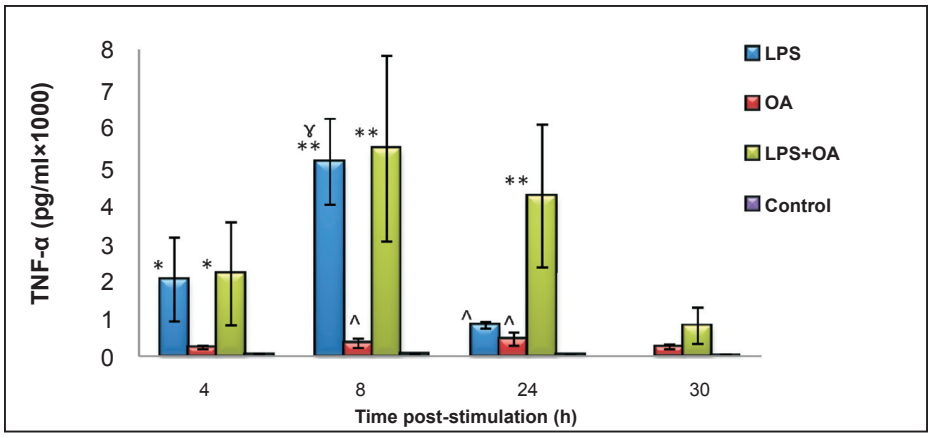

Fig. 2. Comparison of TNF- α in different experimental groups. All values are expressed as mean ± SD (N=6). P^{*}<0.05, **<0.01 compared to control group. ^ indicates P<0.001 compared to LPS+OA group. γ indicates P < 0.001 for LPS group compared to OA.

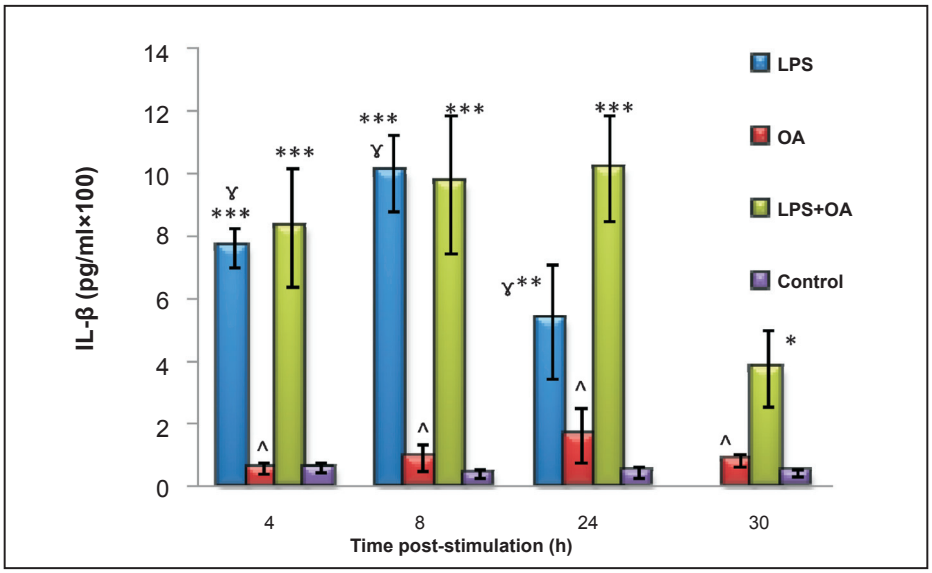

Fig. 3. Comparison of IL-1β in different experimental groups**.** All values are expressed as mean ± SD (N=6). P^{*}< 0.05, **<0.01, ***<0.001 compared to control group. ^ indicates P<0.001 compared to LPS+OA group. γ indicates P<0.001 for LPS group compared to OA.

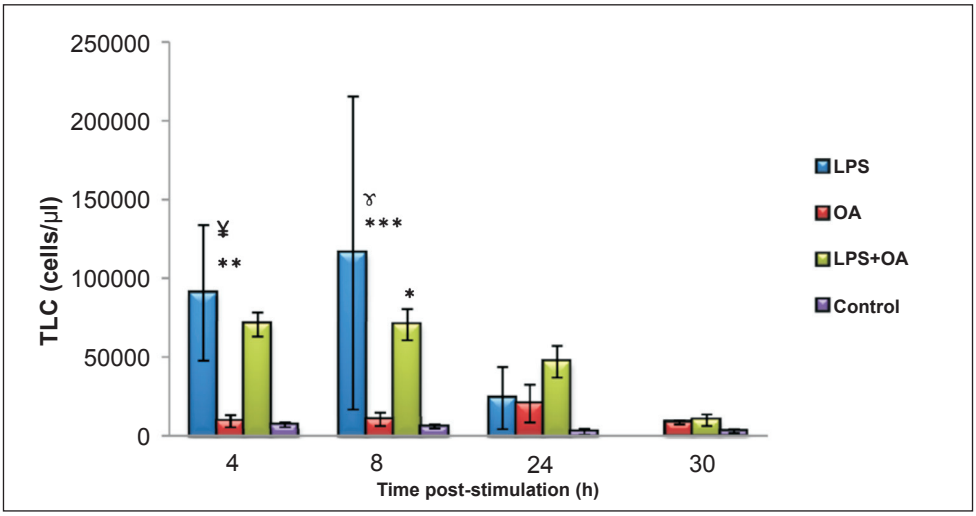

Fig. 4. Comparison of total leukocyte count (TLC) in different experimental groups. All values are expressed as mean ± SD (N=6). $P^{*}<0.05$, $^{**}<0.01$, $^{***}<0.001$ compared to control group. ¥ indicates $P<0.01$ and γ indicates $P<0.001$ for LPS group compared to OA.

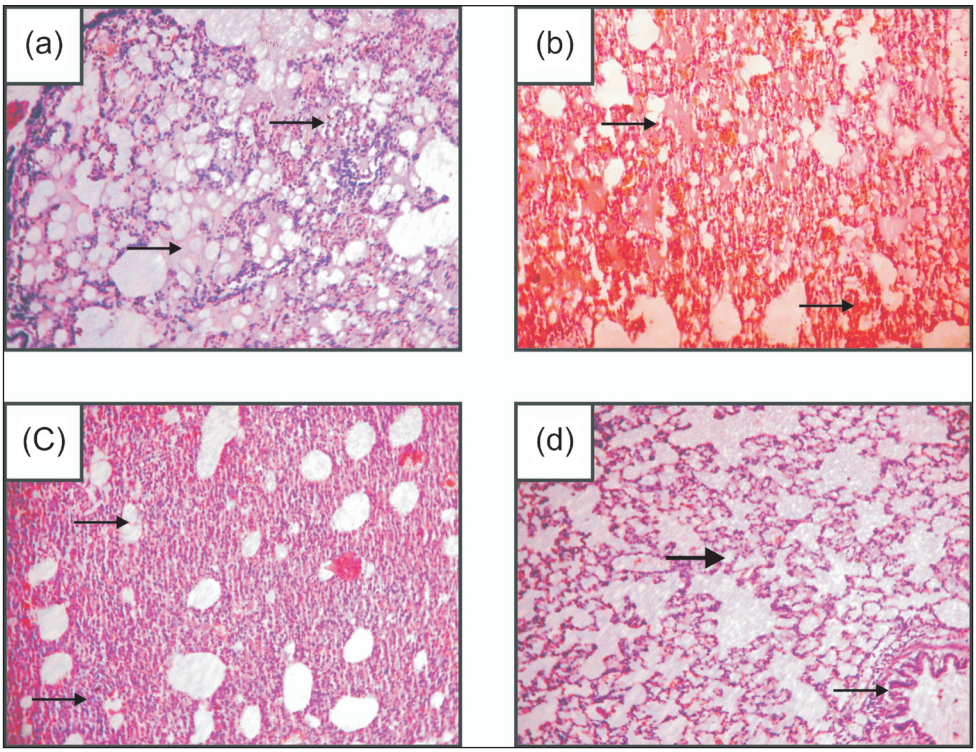

Fig. 5. Comparison of histopathology in rat lungs 24 h post injury in various experimental groups. (Magnification: x100; haematoxylin and eosin staining). **(a)** LPS group: The alveolar epithelium was severely damaged. Large volumes of exudates containing neutrophils and lymphocytes (arrow) were found in the alveolar cavity. **(b)** OA group: Pulmonary congestion, oedema and haemorrhage (arrow) are apparent. The lung tissue was infiltrated with lymphocytes. **(c)** LPS and OA combination group: The lungs showed severe degree congestion, oedema and infiltration with inflammatory cells. The alveolar cavity contained remarkable amount of inflammatory exudates and protein fluid (arrow). The entire lung was filled with plasma – like fluid, suggesting severe injury to alveoli. **(d)** Control group: Histopathology of rat lungs in control group. The alveolar walls were very thin and the majority of the alveoli contained no cells demonstrating normal architecture of lungs. Two different arrows are pointing towards (1) thin alveolar walls in the lungs (thick arrow) and (2) bronchioles (thin arrow).

of the diagnostic criteria for ALI/ARDS is bilateral pulmonary infiltrates, rat chest X-rays were taken at periodic time intervals to demonstrate development of lung injury. Bilateral pulmonary infiltrates were noted in all the three animal models developed using LPS, OA and combination of LPS and OA. Van Helden *et al*[10] also reported a marked alveolar consolidation in all quadrants of the lungs in LPS treated rats at the end of 24 h.

In the present study, we demonstrated that the dual hit stimulation not only elicited symptoms of respiratory distress in rats and caused mortality closer to that of clinical ARDS, but also produced a refractory hypoxaemia that persisted for at least 30 h. In this model, the pulmonary injury was more severe than in any single hit model. Li and Wei[20] studied LPS two hit induced refractory hypoxaemia ARDS rat model. Dual hit was achieved by injecting LPS intraperitoneally followed by intratracheal LPS instillation after 16 h. They found that a two hit LPS stimulation induced prolonged hypoxaemia and specific pulmonary injury in rats. Zhou *et al*[22] similar to our study, used combination of endotoxin and OA to compare physiologic, biochemical, histopathologic, and imaging manifestations in models of acute lung injury in mice. These both studies concluded that the rat ARDS model produced by double hit method was more stable and closer to the diagnostic criteria of ARDS.

In summary, we propose that intratracheal instillation of LPS followed by intravenous injection of OA induces persistent hypoxaemia and specific pulmonary injury in rat that is close to the diagnostic criteria of clinical ARDS. Dual hit better mimics the pathological process of ARDS than LPS or OA alone.

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***Conflicts of Interest*:** None.

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