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ORIGINAL ARTICLE

The impact of pretreatment with bolus dose of enteral glutamine on acute lung injury induced by oleic acid in rats

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Abstract
Purpose Both parenteral and enteral glutamine have shown beneficial effects in sepsis and ischemia/reperfusion-induced acute lung injury (ALI). Oleic acid (OA) has been used to induce ALI in experimental studies. In this study, we investigated the effects of pretreatment of a bolus dose of enteral glutamine on ALI induced by OA in rats.
Methods Twenty-eight adult female Sprague–Dawley rats weighing 240–300 g were divided into four groups, 7 in each. Group I and group II received normal saline for 30 days, group III and group IV received glutamine at a dose of 1 g/kg for 10 days by gavage, and in group II and group IV 100 mg/kg OA was administered i.v. Histopathological examination of the lung was performed with light and electron microscopy. Levels of protein carbonyl, malondialdehyde, superoxide dismutase, catalase, and glutathione peroxidase levels were measured in tissue samples. Levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, and total tissue oxidant status and total tissue antioxidant status were measured in serum samples.
Results Light microscopy showed that the total lung injury score of group IV was significantly lower than group II. Change in thickness of the fused basal lamina was not significantly different in groups II and IV under electron microscopy. TNF-α, IL-6, and IL-10 serum levels were higher in group II when compared to group I and significantly attenuated in group IV.
Conclusion Pretreatment with a bolus dose of enteral glutamine minimized the extent of ALI induced by OA in rats.

Keywords Glutamine · Lung injury · Oleic acid · Pneumocyte

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Introduction

Acute distress syndrome (ARDS), the most severe form of acute lung injury, remains a major problem with high mortality in intensive care. It was first described by Ashbaugh and Petty in 1967. Despite improvements in ventilator strategies and supportive care, the mortality of patients with ARDS, is still very high at 40–50 % [1]. Different treatment options have been investigated, such as using antibodies against inflammatory cytokines, inhibiting fibrin deposition, blocking vasoconstrictors such as endothelin, preventing the production of transcription factors, and enhancing alveolar fluid clearance by activating sodium channels [1, 2].

OA has been generally used to induce ALI in experimental animal models. Administration of intratracheal lipopolysaccharide, intratracheal HCl, and endotoxin infusion are other methods of inducing ARDS [3]. OA-induced ALI has become one of the best established models of ALI, characterized by increased permeability and pulmonary edema and alveolar infiltration of inflammatory cells [4, 5]. OA-induced ALI is relatively transient and resolves within hours. It was shown that it causes direct inhibition of surfactant function [6].

Glutamine is a nonessential amino acid, an important energy source for immune cells, and is crucial for optimal leukocyte activation and proliferation [7]. Numerous experimental and clinical studies have demonstrated that glutamine supplementation improves outcome [7–9]. Both enteral and parenteral glutamine have been shown to restore the integrity of gastrointestinal mucosa and decrease bacterial translocation [10, 11]. Enteral nutrition supplemented with glutamine shows beneficial effects in burn patients [10]. It has been previously reported that intravenous administration of glutamine has a beneficial effect on pulmonary inflammation in experimental models of sepsis [8, 12].

However, no previous study has evaluated the impact of an early enteral bolus dose of glutamine in the OA-induced ALI model. In the present study, we tested the hypothesis that an early bolus dose of enteral glutamine was associated with the improvement of lung morphology and reduction of inflammatory cytokines in OA-induced ALI in rats.

Methods

Twenty-eight adult female Sprague–Dawley rats weighing 240–300 g were divided into four groups, 7 rats in each. Animal care and handling were performed in accordance with the guidelines of the National Institutes of Health. The rats were housed in polypropylene cages lined with husks, renewed every 24 h, under a 12 h:12 h light/dark cycle at 22 °C, and had free access to water and food. The rats were fed a standard diet and only deprived of food 12 h before an experiment. The protocol of the experiment was approved by Kobay Animal Ethics Committee. The rats were divided into four groups, containing 7 rats in each. Group I: control rats received distilled water by gavage for 10 days and normal saline (1 ml/kg, s.c.) via the tail vein. Group II: rats received distilled water by gavage for 10 days in normal saline, and 100 mg/kg oleic acid (OA) was administered intravenously via the tail vein. Group III: rats received glutamine at a dose of 1 g/kg by gavage for 10 days. Group IV: rats received glutamine at a dose of 1 g/kg for 10 days by gavage and in normal saline, and a total amount of 100 mg/kg OA was injected via the tail vein. All the rats were kept in an oxygenated chamber for 4 h. Rats were anesthetized with intraperitoneal injection of ketamine 80 mg/kg and xzylazine 10 mg/kg. Serum and lung tissue homogenate were used for the estimation of biochemical markers and histopathological examination. At 4 h after OA injection, 2 ml intracardiac blood was withdrawn. The whole blood was centrifuged at 3,500 rpm for 15 min at 4 °C and plasma collected and stored at −80 °C. Cytokines (TNF-α, IL-6, IL-10), total tissue oxidant status (TOS), and total tissue antioxidant status (TAS) were measured at serum samples. Then, lung tissue samples were collected and the rats were sacrified. One lobe of the lung was divided into two pieces, one of which was used for light microscopy and the other for electron microscopic examination. One piece of the fresh tissue sample was rapidly fixed in 10 % buffered formalin, then dehydrated through graded alcohols and embedded in paraffin blocks. Sections (5 μm) were cut and stained with hematoxylin and eosin (H&E), according to standard protocols. The homogenate of the other piece of lung tissue was centrifuged at 4,000 *g* for 15 min and the supernatant analyzed. Malondialdehyde (MDA), protein carbonyl (PC), catalase, superoxide dismutase (SOD), and glutathione peroxide (GPX) were measured in tissue samples.

Measurement of cytokines

TNF-α, IL-6, and IL-10 levels were evaluated in plasma samples 4 h after OA injection. The assay was carried out by a colorimetric commercial kit (Calbiochem-Novabiochem, San Diego, CA, USA).

Measurement of MDA

The lipid peroxidation product and lung tissues were homogenized in 1.15 % KCl solution. A 100-μl aliquot of the homogenate was added to a reaction mixture containing 200 μl 8.1 % sodium dodecyl sulfate, 1,500 μl 20 % acetic acid (pH 3.5), 1,500 μl 0.8 % thiobarbituric acid, and

700 µl distilled water. Samples were then boiled for 1 h at 95 °C and centrifuged at 3,000 *g* for 10 min. The absorbance of supernatant was measured by spectrophotometry at 650 nm.

Measurement of SOD

SOD activity was evaluated by inhibition of nitro blue tetrazolium reduction by superoxide anion generated by the xhanthine/xhantineoxide system using a commercial assay kit (Nanjing Jiancheng, Nanjing, China.)

Measurement of catalase

Cayman's catalase assay kit was used to determine the activity of catalase. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced was measured calorimetrically in tissue homogenate.

Measurement of GPX

A Cayman GPX assay kit was used to measure the activity of GPX. The rate of decrease in A_{340} is directly proportional to the GPX activity in tissue samples.

Measurement of PC

Cayman's protein carbonyl colorimetric assay kit was used. The amount of protein hydrozone is quantified at an absorbance between 360 and 385 nm spectrophotometrically. The carbonyl content was standardized to protein concentration.

Measurement of TAS

TAS was measured using an Aeroset 2.0 analyzer and Cayman's total antioxidant status kit.

Measurement of TOS

TOS was measured with an Aeroset 2.0 analyzer and Cayman's TOS kit.

Light microscopic examination

One piece of lung was examined using a light microscope (DM6000B; Leica, Wetzlar, Germany) with a DC490 digital camera (Leica) and evaluated for acute lung injury according to the presence of interstitial edema, alveolar hemorrhage, interstitial mononuclear infiltration, intraalveolar neutrophils, intraalveolar macrophages and intraalveolar pneumocytes. Each lung specimen was given a score of 0–3 depending on whether the finding was absent (0), mild (1), moderate (2), or severe (3). Four randomly selected areas were evaluated under a light microscope (40× magnification) in each slide by two investigators blinded to group assignment of each animal. The mean value of the score of two investigators was used for graphic and statistical calculations. Total lung injury score was the sum of the average of the score of these six parameters (maximum score, 18).

Electron microscopic examination

The second piece of the fresh tissue samples was fixed in 2.5 % glutaraldehyde solution in phosphate buffer, pH 7.4, for 4 h, and postfixed for 1 h in 1 % osmium tetroxide solution in 0.1 M phosphate buffer. After washing in phosphate buffer, samples were dehydrated in a graded series of ethanol to absolute ethanol, treated with propylene oxide, and embedded in Araldite/Epon812 (Cat. No. 13940; EMS, Hatfield, PA, USA). After heat polymerization, sections were cut using a microtome. Semithin sections were stained with methylene blue-azure II and examined using a light microscope (Leica DM6000B) with a DC490 digital camera (Leica). Ultrathin sections (Leica Ultracut R) were double stained with uranyl acetate and lead citrate (Leica EM AC20). These sections were examined in a JEOL-JEM 1400 electron microscope and photographed by a CCD camera (Gatan, Pleasanton, CA, USA). The thickness of fused basal lamina in the air–blood barrier was measured in ten different areas in each group and the mean value for each group was calculated.

Statistical analysis

Statistical analysis was performed by using the Statistical Package for the Social Sciences version 17.0 for Windows (SPSS, Chicago, IL, USA) The data are expressed as mean ± standard deviation. A one-way analysis of variance (ANOVA) test was used in statistical analysis of parameters and for comparison of the groups. A post hoc Tukey test was used for secondary comparisons. $p < 0.05$ was considered as statistically significant.

Results

The histopathological changes in the study groups are given in Fig. 1. Open alveolar spaces and the alveolar wall composed of capillaries, flat squamous cells (type I pneumocytes), and round type II pneumocytes were examined by light microscopy of group I and group III (Fig. 1a, b). Severe lung injury was determined in group II when compared to group I and group III. Severe pulmonary

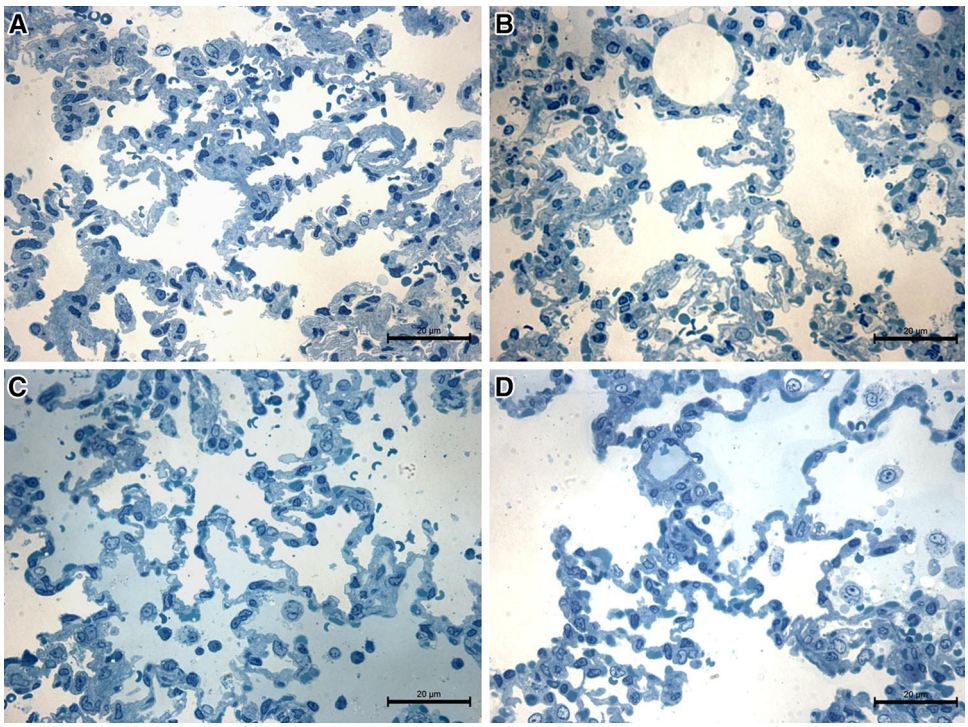

Fig. 1 Representative micrographs for the effects of oleic acid (OA) and glutamine in lung. Normal histological structure of lung with open alveolar spaces and type II and flat type I pneumocytes in group I (**a**) and group III (**b**). **c** Alveolar spaces filled with edematous fluid, intraalveolar inflammatory cells, and pneumocytes in group II. **d** Glutamine pretreatment attenuated the lung pathology seen with OA treatment. Methylene blue ×630

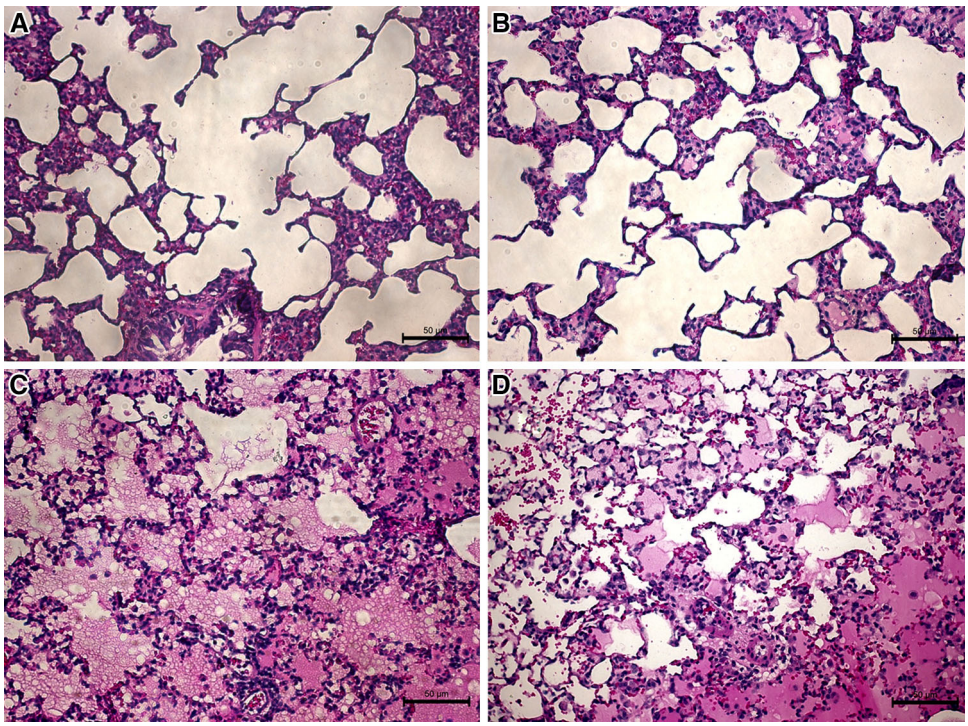

Fig. 2 Representative micrographs for total lung injury score: group I (**a**), group III (**b**), group II (**c**), group IV (**d**). Hematoxylin and eosin ×200

edema, perivascular edema, and both intraalveolar inflammatory cells and pneumocytes were found in group II (Fig. 1c). Some open alveolar spaces and intraalveolar neutrophils, macrophages, and pneumocytes were seen in group IV. Glutamine pretreatment reduced the pathological changes seen in the lung with OA (Fig. 1d).

Total lung injury score indicated that glutamine alone did not affect the lung injury score. There was no significant difference in the lung injury score between group I and group III. OA increased the total lung injury score, which was decreased with glutamine pretreatment in group IV when compared to group II (Figs. 2, 3).

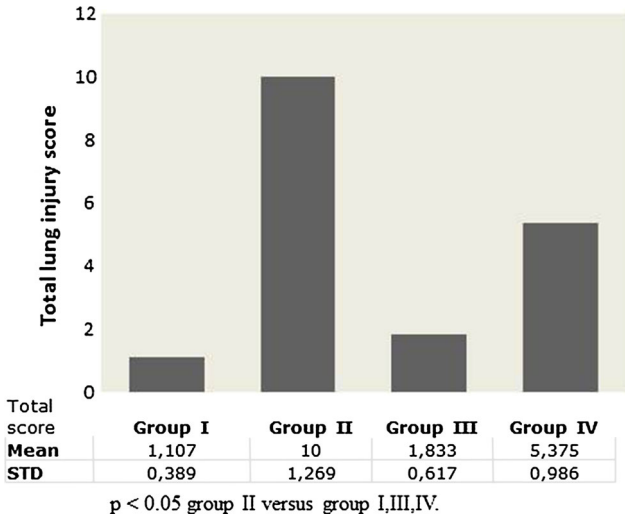

Fig. 3 Lung injury score in study groups. Total lung injury score was increased in group II. Glutamine pretreatment decreased the total lung injury score in group IV. Values are expressed as mean ± SEM ($p < 0.001$)

Electron microscopic examination of group III revealed normal morphology as in group I with fused basal lamina, endothelial cells, and thin cytoplasm of type I pneumocytes (Fig. 4a, b). OA resulted in swelling of type I pneumocytes especially at the side of the alveolar spaces that were filled with edematous fluid. Gaps were observed in the cytoplasm of type I epithelial cells in some areas. Hyaline membrane was observed in alveolar spaces. Platelets and inflammatory cells were observed in the capillary lumen. The inflammatory cells in the lumen of capillaries were apoptotic with condensed nuclei and widening of the perinuclear cisternae. Discontinuity and detachment of the endothelial cells from the basal lamina were observed in group II (Fig. 5). Endothelial cells in group IV showed normal morphology with pinocytotic vesicles, intact nucleus, and mitochondria besides the other organelles. Endothelial cells and type I pneumocytes were preserved by glutamine, but detachment of endothelial cells from the basal lamina, electron-lucent widening, disruption, and degeneration in the structural integrity of the basal lamina was observed in group IV (Fig. 6).The thickness of the fused basal lamina was increased in group II and group IV, but this change was not statistically significant. There was no statistical difference in the average thickness of basal lamina between the groups (Fig. 7).

TNF-α, IL-6, and IL-10 values were significantly higher in group II when compared to group I and attenuated in group IV (Fig. 8). There are no statistically significant differences in TAS,TOS, MDA, SOD, GPX, catalase, and PC levels between group II and group IV (Table 1).

Discussion

It is concluded in the current study that pretreatment with a bolus dose of enteral glutamine attenuated acute lung injury induced by OA in rats by reducing inflammatory cytokines.

Septic shock, intestinal ischemia/reperfusion, and ventilator-induced models are other clinically relevant animal lung injury models [13]. In this study, we used a direct and acute ALI model induced with OA. We obtained tissue and serum samples at 4 h after OA injection. An OA-induced acute lung injury was characterized histologically by thickening of alveolar septa, alveolar hemorrhage, and inflammatory cell infiltration [13, 14], causing severe alveolar damage with development of increased permeability of alveolar edema [15]. OA may promote alveolar and/or interstitial flooding and prevents fluid clearance by inhibiting active sodium transport [14]. Rylander et al. [5] speculated that in the OA-induced lung injury model the increase in total lung volume suggested a predominance of formation of edema over airway and alveolar collapse. The OA model described in the literature varies in terms of animals used, dose of OA, injury time period, and use of ventilator support. Only one intravenous injection of OA has been shown to exert its effects on lung within minutes or hours. McGuigan et al. [3] stated that with the doses of OA they used, the rats did not require ventilator support. We used also a single injection of OA at the same dose used in the previous study and did not ventilate the rats [3].

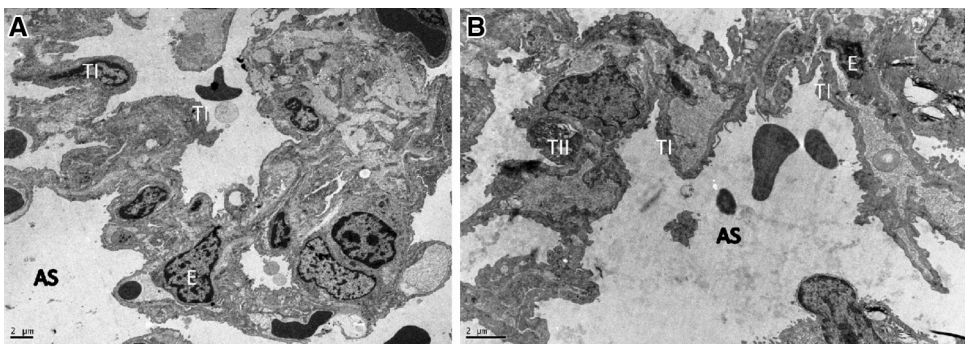

Fig. 4 Electron micrographs of lung from group I (**a**) and group III (**b**) with open alveolar spaces. *AS* alveolar space, *E* endothelial cell, *TI* type I pneumocyte, *TII* type II pneumocyte. Uranyl acetate–lead citrate. **a** ×4,000; **b** ×8,000)

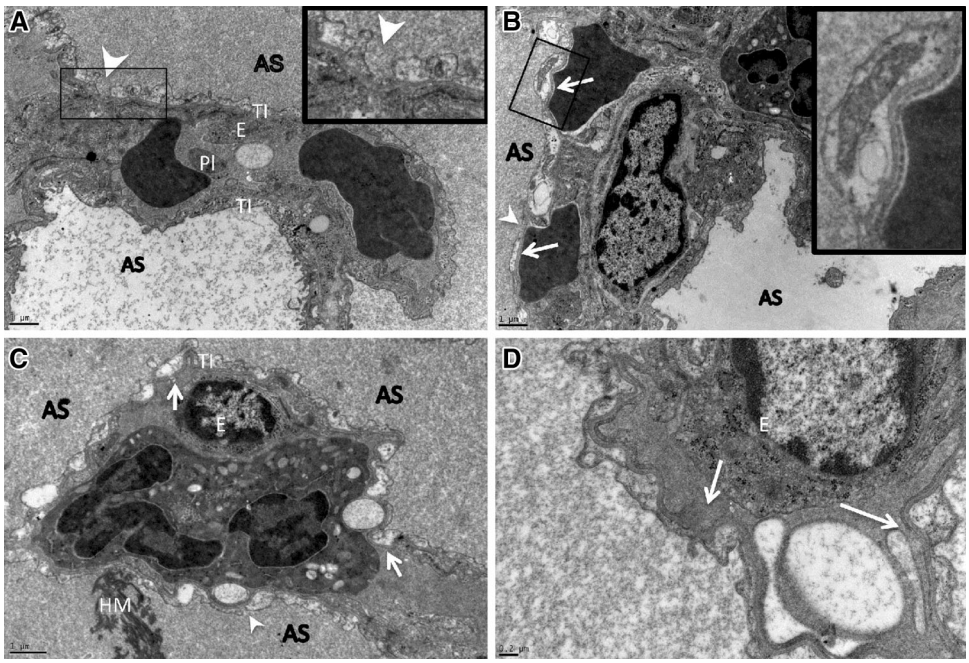

Fig. 5 Electron micrographs of lung from group II. **a** Gap in cytoplasm of type I pneumocytes (*arrowhead*) and platelet in capillary lumen. *Inset: rectangular* area represents discontinuity of type I pneumocytes, indicating air–blood barrier injury. **b** Swollen type I pneumocytes at side of alveolar spaces that were filled with edematous fluid, gap in cytoplasm of type I pneumocyte (*arrowhead*), and discontinuity in cytoplasm of endothelial cell (*arrows*). *Inset:* magnified view of *rectangular* area represents dashed appearance of the endothelial cell cytoplasm. **c** Apoptotic inflammatory cell in lumen of capillary. **d** Discontinuity in cytoplasm of endothelial cell indicating disruption of air–blood barrier. *AS* alveolar space, *E* endothelial cell, *TI* type I pneumocyte, *P* platelet, *HM* hyaline membrane. Uranyl acetate–lead citrate. **a**, **b** ×12,000, *inset* ***a*** ×24,000, *inset* ***b*** ×42,000, **c** ×15,000, **d** ×40,000)

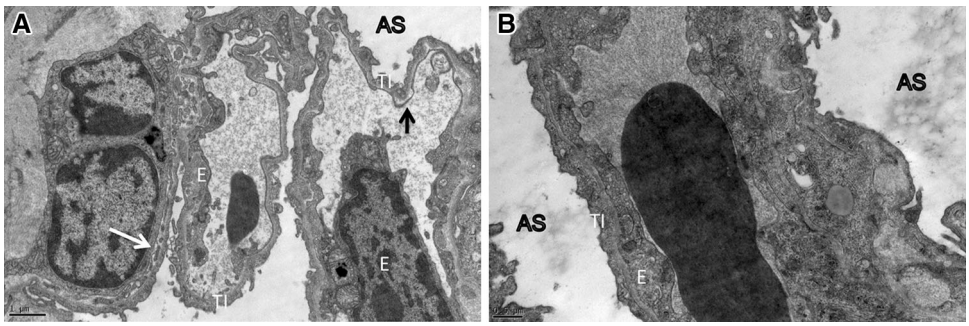

Fig. 6 Electron micrographs of lung from group IV. **a** Discontinuity in cytoplasm of endothelial cell (*white arrow*), electron-lucent widening, and disruption of fused basal lamina (*dark arrow*). **b** Degeneration in structural integrity of basal lamina. *AS* alveolar space, *E* endothelial cell, *TI* type I pneumocyte. Uranyl acetate–lead citrate. **a** ×15,000, **b** ×25,000)

Histopathological changes induced by OA are similar to the exudative phase of ARDS, which is characterized by interstitial and intraalveolar edema, intraalveolar hemorrhage, vascular congestion, and intravascular coagulation [3, 16]. Our findings were in agreement with results published in the literature.

Gaps between the cytoplasm of type I epithelial cells in some areas, and discontinuity and detachment of the endothelial cells from the basal lamina, showed deterioration of the blood–air barrier in group II rats by electron microscopy in our study. There was no difference in the thickness of the fused basal lamina between group II and group IV, but organelles of endothelial cells and type I pneumocytes were preserved in group IV.

The role of free radicals in OA-induced ALI is controversial in the literature [3, 17]. Liu et al. showed that reactive oxygen species increased immediately after OA injection and reached its peak value in 30 min [17]. Oxidative injury to the pulmonary capillary endothelial cells might be the first step in initiation of ALI induced by OA [17]. In contrast, McGuigan et al. demonstrated that free radicals do not contribute to the initial stages of OA-

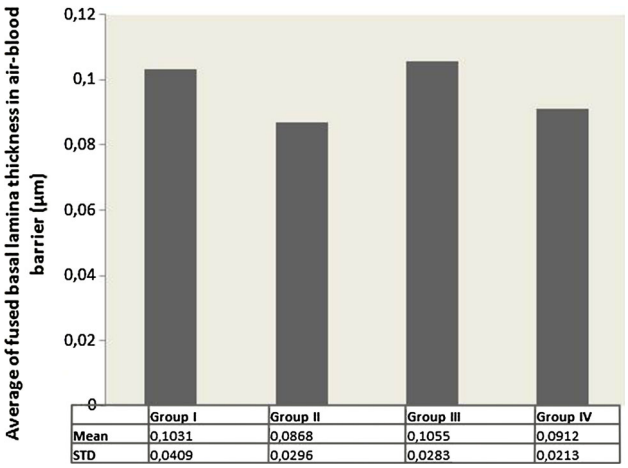

Fig. 7 Basal membrane thickness in air–blood barrier. There was no statistical difference in the average thickness of fused basal lamina among the groups. Values are expressed as mean ± SEM ($p = 1.0$)

induced lung injury, as seen in ethidium superoxide assay, up to 8 h. It would be more likely that the free radicals contribute to the longer duration of injury [3]. In our study we did not detect any difference in protein or lipid oxidation products between groups at the end of the 4th hour of OA injection.

It was shown that the level of TNF-α was directly correlated with the severity of ARDS and mortality [18]. Suppression of the production of TNF-α or blockade of its biological actions may have a potential therapeutic value against pulmonary diseases characterized by inflammation and cell death [19, 20]. Ito et al. [21] showed that urinastatin, a human urinary trypsin inhibitor, significantly improved the OA-induced histological changes by inhibiting the production of TNF-α. Mukhopadhyay et al. [19] stated that loss of lung surfactant during septic shock may be associated with increased TNF-α activity. Drugs widely

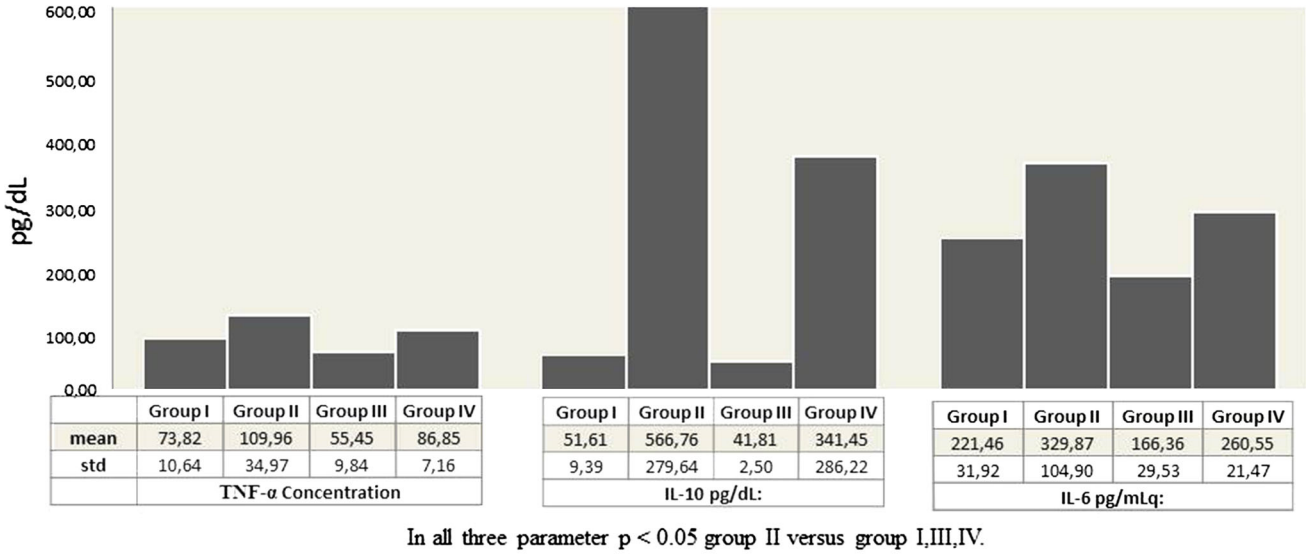

Fig. 8 Levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-10 by group

Table 1 Levels of superoxide dismutase (SOD), catalase activity, protein carbonyl (PC), malondialdehyde (MDA), glutathione peroxide in tissue samples (GPx), total tissue oxidant status (TOS), and total tissue antioxidant status (TAS) in serum samples

	Group I		Group II		Group III		Group IV	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
SOD (U/ml)	1.77	0.51	1.75	0.56	1.51	0.34	1.94	0.41
Catalase activity (nmol/min/ml)	154.17	10.27	140.67	38.99	122.03	13.37	147.91	14.84
Protein carbonyl (nmol/ml)	388.22	68.26	261.23	54.56	383.82	96.97	320.09	51.87
MDA ng/ml	3.73	0.04	3.42	0.07	3.67	0.29	3.57	0.15
GPx (nmol/min/ml)	81.93	54.39	164.66	58.57	102.68	199.70	272.39	160.20
TOS (mmol/l)	17.41	15.45	3.77	17.38	19.55	1.53	2.95	0.93
TAS (µmol/l)	1.00	0.14	1.06	0.14	0.68	0.15	0.65	0.11

used in the clinical area may offer a clinic approach for ALI/ARDS treatment. He et al. found that captopril (ACE inhibitor) prevented animals from severe lung injury induced by OA [13]. Chen et al. [22] showed that treatment of propofol attenuated the OA-induced inflammatory and pathological changes in conscious rats in the ALI model.

Data supporting the use of supplemental glutamine in critical care are not sufficient [23, 24]. The effect of glutamine on ALI in experimental models is also controversial. Hong et al. [25] demonstrated that pretreatment with glutamine-supplemented nutrition protects the liver after lethal hepatic injury, possibly by preserving glutathione stores. Glutamine may act against inflammatory injury by enhancing stress-inducible heat shock protein (HSP 70) expression, suppressing nuclear factor κB signal transduction activity, and reducing neutrophil infiltration and production of cytokines[26, 27].

Oliviera et al. [28] demonstrated, in an experimental model induced by cecal ligation and puncture surgery, that 0.75 g/kg i.v. glutamine reduced neutrophil infiltration, interstitial edema, and alveolar collapse. They found that glutamine attenuates epithelial cell apoptosis of the lung and distal organs. On the other hand, Hou et al. investigated the effects of dietary glutamine supplementation on neutrophil recruitment in a model of LPS-induced ALI. They found that the damage to the lungs was more severe in the glutamine group histopathologically. This result is in accord with the idea that glutamine leads to more superoxide anions activating and delaying the spontaneous apoptosis of neutrophils at 12 and 24 h after lipopolysaccharide (LPS)-induced injury, whereas it effectively eliminated superoxide anions at 6 h after LPS. In the same study, glutamine led to more proinflammatory cytokine production, accumulation of larger amounts of lipid peroxides in the lung, and more neutrophil recruitment in the early stage of LPS-induced ALI. It has also been shown in an in vitro study that glutamine increases the expression of NADPH oxidase, catalyzes superoxide anion production, and causes oxidative stress in neutrophils [7].

In our study, we only evaluated the effect of a bolus dose of enteral glutamine at 4 h (very early stage) after the OA injection. The discrepancies between the results of studies related to the effects of glutamine on ALI might arise from the differences in study methods, the path and dose of glutamine administration, ALI model, duration of follow-up, and time points at which histological and biochemical evaluations were made.

One limitation of our study is difficulty in extrapolating the results to clinical critical care. Another limitation is that high-dose glutamine was given before injury occurred and plasma glutamine levels were not analyzed.

In conclusion, pretreatment with a high bolus dose of enteral glutamine attenuated the ALI in this experimental model. Future studies are required to define the optimal pharmacotherapy and timing of administration to restore the overall balance between pro- and anti-inflammatory cytokines, oxidants, and antioxidants for ALI/ARDS. Glutamine might be a part of an early and appropriately provided enteral nutrition to critically ill patients at risk for ARDS in the intensive care setting.

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