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Effectiveness of antioxidant and membrane oxygenator in acute respiratory distress syndrome by endotoxin

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Abstract−This study evaluated the effectiveness of antioxidants in animal models with damaged lungs such as inflammatory mediator-induced acute respiratory distress syndrome (ARDS) and established an ARDS therapy technique by suppressing active oxygen with membrane oxygenator. When inflammatory mediator that has an endotoxin, such as LPS, was injected directly into the airway, inflammatory pulmonary edema developed in the lung, and could induce ARDS. To treat such endotoxin-induced ARDS, the antioxidants such as taurine and dexamethasone were injected and their antioxidant effects were evaluated. They turned the blood pH to the normal condition, increased blood hemoglobin and hemocrit concentration and oxygen partial pressure (PO_2), and improved survival. When a membrane oxygenator was used alone on the animal models with ARDS, similar antioxidant effects were identified. When a membrane oxygenator and antioxidants were used simultaneously, synergistic antioxidant effects were revealed. Therefore, the simultaneous use of antioxidants and membrane oxygenator is more effective than the use of either of them in treating the animal models with ARDS. This result indicates that bilateral use of antioxidants and membrane oxygenator may be useful as a potential therapy technique for the treatment of acute respiratory distress syndrome.

Key words: ARDS, Membrane Oxygenator, Endotoxin, Antioxidant, Dexamethasone

INTRODUCTION

Acute respiratory distress syndrome (ARDS), being a severe pulmonary inflammatory disease which is related to multiple organ dysfunction syndrome (MODS) or systemic inflammatory response syndrome (SIRS), is a disease, which has pulmonary edema caused injury of intrapulmonary capillary vessel and alveolarbarrier, as the main complaint. In 1967, Ashbaugh [1] defined this disease, which has dyspnea occurring by various causes such as multiple trauma, inflammation and high inspired oxygen as main symptom, as the cryptogenic respiratory distress syndrome in the adult [1,2]. Incidence was reported to be 3.5 to 75 among 100,000 individuals and in the United States 100,000-150,000 patients occur with 40,000-70,000 dying every year. Causes of occurrence are related to various risk factors such as direct lung injury and injury caused by an indirect mechanism that emits haematogenous inflammatory media. Main causes are sepsis, aspiration pneumonia, injury and blood transfusion [3,4]. The presently available best therapy of the dyspnea is extracorporeal membrane oxygenation (ECMO) [5-7], a device that can deliver oxygen through blood circulation. However, the mechanically aided respiration for the dyspnea patients requires a long-term use that may cause complications and high cost [8-10].

Artificial lung assist device designed to be installed within thoracic cavity is similar to the extracorporeal device, but studies on new surgery are required [11]. This device has disadvantages in that it necessitates precise surgery and has low material transfer efficiency. According to the recent studies, the intravenous lung assist device is limited by various restrictions such as blood compatibility, gas permeability and limited space for its clinical application. Therefore, studies to solve these restrictions are required [12-16].

ARDS is a generalized disease. The reason of its high death rate is the accumulated complication of multiple organ system failure (MOSF). The MOSF means an inclusive damage in the microcirculatory system, and involves insufficiency syndrome in various organs. ARDS is an MOSF that appears in the lung. What play an important role in MOSF causes are neutrophil and tumor necrosis factor (TNF) [17-19]. Changes in plasma that are not related to the cells are affected by various media such as complement system, coagulation-fibrinolysis system, kinin system and cytokine, media-

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tors, which are generated in the cells, such as oxidants, proteolytic enzymes, nitric oxide, growth factor, neuropeptide and nuclear factor B that causes protein production [20]. Inflammatory media in lung or blood produced by various cells induce inflammatory pulmonary edema, and consequently cause acute lung damage in the ARDS. Production of inflammatory media and development of pulmonary edema induces rise in pulmonary capillary pressure and increases the development of pulmonary edema. Oxidants can cause damage to the cells, and antioxidant inactivates oxidants. But even as the increase of antioxidants can induce pulmonary damage, it is important to maintain an appropriate balance between oxidants and antioxidants.

In the early stage of ARDS neutrophil, macrophage and complement are involved in pulmonary damage. Macrophage-like neutrophil also produces protease or oxygen metabolites such as hydrogen peroxide, peroxides, hypochlorous acid, N-chloroamines and hydroxyl radical. It also mobilizes and activates neutrophil isolating thromboxane or leulotriene and activates other inflammatory cells. These oxygen metabolites can affect the cellular molecules such as lipids, proteins and nucleic acid, leading to a lung injury [21-23].

A clinically important cause of the mechanism of pulmonary tissue injury according to oxidative stress of neutrophil is sepsis, with the gram-positive bacterial sepsis is a main cause. A great many studies on the causes of sepsis-induced ARDS have been conducted, but their fundamental mechanism has not yet been identified. Damage to tissues by the oxygen free radical is one of sepsis-induced ARDS [9]. According to the recent studies, various subtypes of phospholipase A2 (PLA2) are involved in the development of sepsis-induced ARDS incidence, and these PLA2 subtypes are mostly activated under the presence of calcium ion [21-23].

Our aim was to develop animal models for ARDS and ARDS therapy technique that can suppress active oxygen, a risk factor of ARDS, using antioxidants and membrane oxygenator.

MATERIALS AND METHODS

1. Experimental Animal

In this study, male SD-rats weighing 230-270 g were used. They were grouped into 6. In the control group, rats were given an intratracheal (i.t.) infusion of phosphate buffered saline (PBS) (G1). The taurine+LPS treated group (G2) was injected with 20 mM taurine during the seven days prior to the injection of LPS. The taurine+LPS+DXM treated group (G3) was injected with 20 mM taurine during seven days prior to LPS administration and intraperitoneally (i.p.) with 10^{-7} M DXM starting one day prior to LPS administration. Sprague-Dawley rats (250-300 g, 10-12 weeks old) receiving *Salmonella enteritidis* LPS (Sigma Chemical Co., St. Louis, MI) (G4, LPS only was injected) were administered 7 mg LPS/kg body weight by a bolus i.t. injection over 1-2 min. G5 was oxygenator-treated group (after LPS was injected, membrane oxygenator only was used), and G6 was taurine+LPS+DEX+oxygenator-treated group (after LPS was injected, taurine, which is an antioxidant, dexamethasone and membrane oxygenator were simultaneously used).

2. ARDS Animal Model

SD rats weighing 230-270 g were used to develop inflammatory ARDS-inducing animal model protocol using inflammatory mediators. Eight hours after endotoxin containing Pyrogen-free saline (LPS0111: B4; Sigma) was injected into abdomen at 50 mg/kg, *N*-formyl-methionyl-leucylphenylalanine (FNLP, Sigma) was injected at 10 mg/kg.

3. Bronchoalveolar Lavage Fluid

Repeated bronchoalveolar lavage (BAL) was conducted in the lung of dead rats with 3 mL of PBS, the resulting BAL fluid was centrifuged for 10 minutes at 350 G, and the supernatant was used to measure nitrite/nitrate and protein & lactate dehydrogenase (LDH). Survival rate and the number of cells in the BAL fluid were estimated with trypan blue exclusion. To remove the blood in the lung of rats, 10 mL of Hank's balanced salt solution (HBSS) was injected into the circulatory system to treat the system. Serum was collected from the rats of each group by sinus orbital bleeding.

4. Nitrite/Nitrate Level

The level of nitrite/nitrate in the BAL fluid and serum was obtained through Greiss reaction, and the synthesis of NO was indirectly evaluated by measuring nitrite and nitrate (a stable derivative of NO). Because Greiss reagent does not react with nitrate, nitrate reductase (Sigma) of 1 U/mL obtained from *Aspergillus* and NADPH (Sigma) of 1 mg/ml were added, and the resulting solution was incubated overnight. All the samples were mixed with Greiss reagent (Sigma, 1 : 1 mixture of 1% sulfanilamide contained in 30% acetic acid and 0.1% *N*-naphthyl-ethylenediamine dihydrochloride contained in 60% acetic acid) used, and nitrite level was determined with the optical density at 595 nm. Nitrate level was determined with the standard curve of sodium nitrate, and the nitrite/nitrate level was adjusted to be higher than detectable one, 1 μM.

5. LDH and Protein Activity

0.2 ml of BAL fluid was added to 0.3 ml of Dri-STAT LD-L reagent (Sigma, pH 8.95) containing 1.6 mM L-lactate (Sigma) and 8.26 mM NAD (Sigma). Absorbance was measured at 340 nm. Activity of 1 unit was defined as the production of NADH at 1 μM NADH/min under the condition optimized by the manufacturer. Total protein level was determined by the modified Bradford procedure (kit from Sigma).

6. Test Equipment

Blood was analyzed with NOVA analyzer (NOVA Biomedical Corp., Waltham, MA, USA). Servo Ventilator (900C, SIEMENS-ELEMA, Solna, Sweden) was used for the ventilation of rats. Anesthesia of animals was induced with halothane vaporizer (Draegerwerk GmbH, Lubeck, Germany).

7. Histological Test

Lungs and livers were fixed in 10% buffered formalin, and specimens were prepared and then stained with hematoxilineosin for histological analysis.

8. Survival of ARDS Animal

To evaluate the survival of the ARDS animals developed in this study, intravascular lung assist device [13-16] developed in previous studies was restructured to the present laboratory animals. The effective membrane area for the gas exchange of the restructured lung assist device was adjusted to 0.05 m^2, and priming volume was adjusted to 4 mL. A system for the measurement of survival rate was structured as in Fig. 1. The whole system was composed of venous blood container, membrane oxygenator, heat exchanger and roller pump, and to connect the membrane oxygenator to the animal, sterilized 4 mm-diameter silicone tube was inserted to the venous

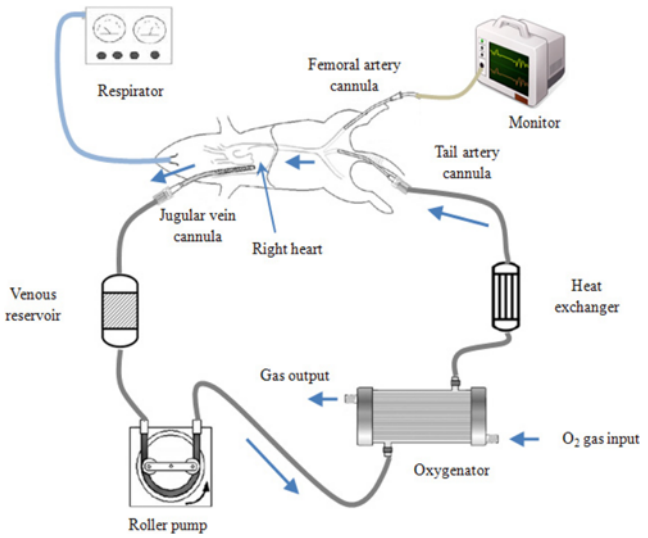

Fig. 1. Schematic diagram of surgical procedure and rat oxygenation apparatus.

vessel and 1.6 mm-diameter silicone tube was inserted to the arterial vessel. The animal temperature was maintained at 36.6-38.3 °C using temperature controlled experimental table for small-sized animals.

9. Statistical Analysis

Student's t test and ANOVA (analyses of variance) were used for the data analysis, and the data was regarded as statistically significant if the *p* value was less than 0.05 (±SEM).

RESULTS AND DISCUSSION

1. ARDS Animal Model

Fig. 2 represents the results of the ARDS models developed using LPS and the X-ray images (Fig. 2(A)) of the rats 42 that died hours

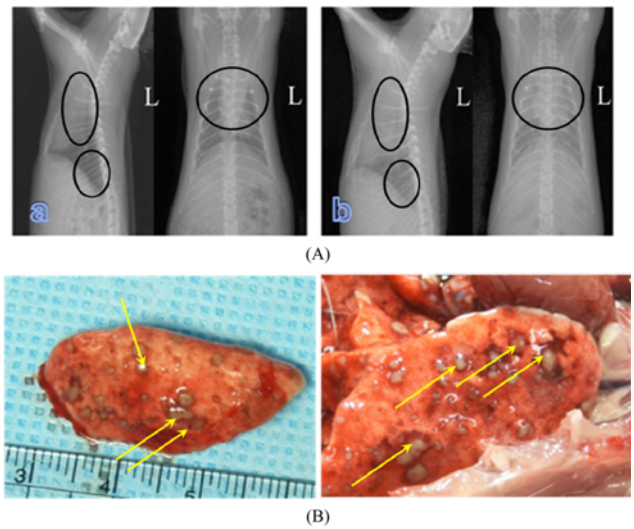

Fig. 2. Chest radiography and necropsy of acute respiratory distress syndrome (ARDS) rat (A) Chest radiography of rat. (a) Control, (b) LPS induced ARDS model, (B) Necopsy of rat: Diffuse abscess over the area of lung.

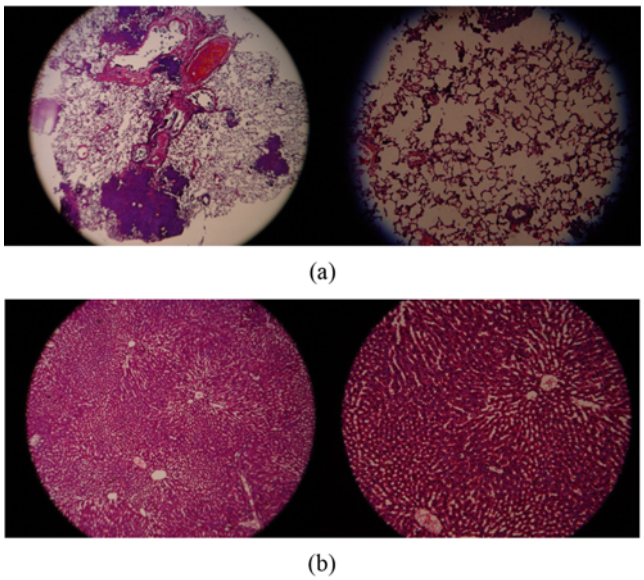

Fig. 3. Photomicrographs of (a) lung and (b) liver after injection LPS.

after LPS injection to the lung of rats via airway at 7 mg/ml/g. ARDS incidence was identified with X-ray reading, and all the rats died within 5 days showing abrupt decrease in respiration. We did not see infiltration in the control group (circles, Fig. 2(A-a)). However, in the LPS treatment group, we were able to see extensive diffuse infiltration in bilateral lung fields. This result proved that LPS injection induced ARDS rats (circles, Fig. 2(A-b)). Fig. 2(B) was the images of extracted lungs of the died rats. In this result, we performed a necropsy on the ARDS rat. Disseminated abscess was found over the area of ARDS rat's lung (arrows). As seen in the images, inflammation had developed in the lung. This inflammation is supposed to be a cause of ARDS incidence.

Fig. 3 represents the results of examination for the lung and liver biopsy of the rats that died from LPS injection. In the figure it was found out that epithelial cell of the lung was destroyed. Lung pathology showing severe lung edema and inflammatory cell infiltration in a rat administered LPS. Histopathologic examination of lung tissue shows heavy perivascular and interstitial infiltration of large hyperchromatic white blood cells with abundant cytoplasm. Pathological findings in ALI include diffuse alveolar damage with widespread alveolar wall thickening and infiltration by neutrophils and macrophages [24]. In the current study, LPS-treated SD-rat developed histological findings compatible with ALI and displayed hypoxemia. LPS-induced hypoxemia is known to accompany microvascular endothelial and epithelial injuries of the lung.

2. Bronchoalveolar Lavage Fluid

Fig. 4 represents the results of the measurement of the nitrate (NO_3^-) (Fig. 4(a)) and nitrite (NO_2^-) (Fig. 4(b)) in the BAL fluid collected from the lungs of ARDS-induced animals using LPS. According to the experimental results, the nitrate concentration of the LPS-injected group (G4) increased compared to that of PBS-injected group (G1), the control group.

However, the nitrate concentration of the taurine-injected group (G2) and taurine+dexamethasone-injected group (G3) decreased significantly ($p<0.05$). Also, the nitrate concentration of the mem-

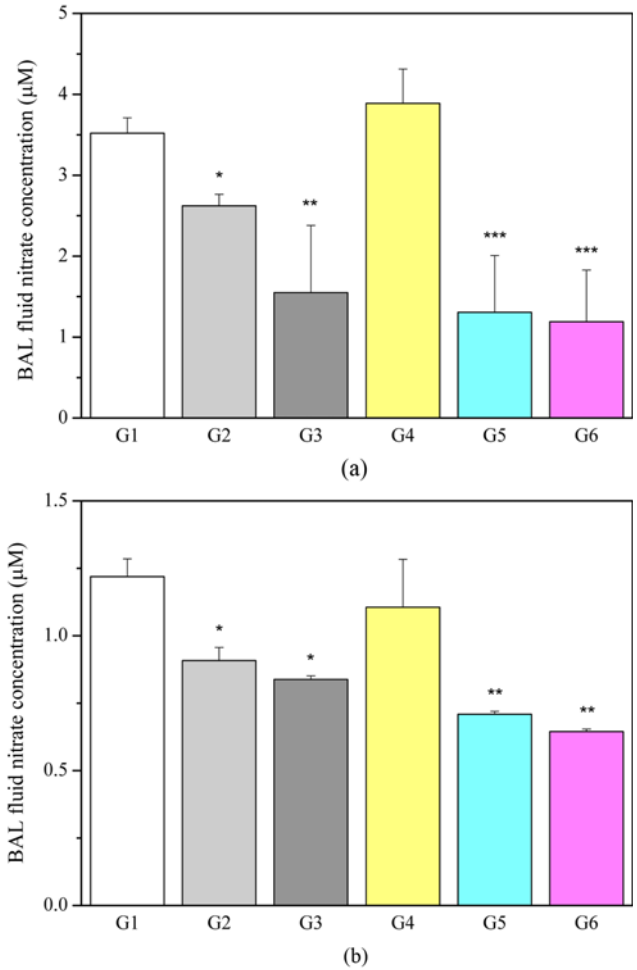

Fig. 4. Nitrate/nitrite levels in BAL of rat (a) Nitrate level (b) Nitrite level *p<0.05, **p<0.01 vs. G4.

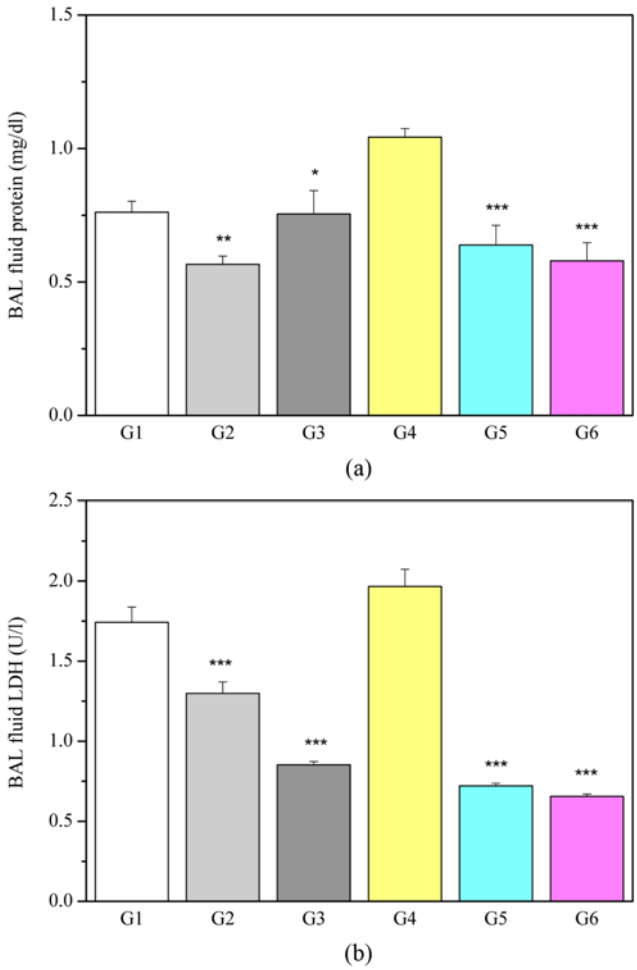

Fig. 5. Protein and LDH levels in BAL of rat (a) Protein level (b) LDH level *p<0.05, **p<0.01, ***p<0.005 vs. G4.

brane oxygenator-used group (G5, G6), decreased significantly (p< 0.005). In addition, according to the experimental results, the nitrite concentration showed similar trends to that of nitrate. From these results, we could identify that in the ARDS incidence by toxic substances such as LPS, taurine and dexamethasone have an antioxidation effect. In case where a membrane oxygenator was used, too, as the membrane oxygenator supplies sufficient oxygen, an antioxidation effect could be identified [25,26].

Fig. 5 represents the results of measured protein and LDH concentration in the BAL fluid collected from the lungs of the ARDS-induced rats using LPS. Protein and LDH concentration changes also showed similar trends to the above stated results. Through these experiments, it was found that antioxidants and membrane oxygenator are effective for the ARDS.

3. Oxygen and Carbon Dioxide Pressure in Blood

Fig. 6 represents oxygen (PO_2) and carbon dioxide (PCO_2) partial pressures in the blood when membrane oxygenator and antioxidants (taurine and dexamethasone) were used in the ARDS-induced rat models using LPS. According to the results, PO_2 of the LPS-injected group (G4) reduced remarkably and PCO_2 appeared to increase. However, the taurine-injected group (G2) showed lower PO_2 and higher PCO_2 than the control group (G1), but showed higher PO_2 than the LPS-injected group (G4). Also, the taurine+dexamethasone-injected group (G3) showed a similar PO_2 compared to the control group, but showed slightly higher PCO_2 than the control group. From these results, antioxidant therapy was found to be effective, since induced models are inflammatory mediator-induced ones in the ARDS models developed in this study. When the membrane oxygenator developed was applied to the animal models, oxygen partial pressure in the blood (G5, G6) was lower than that of control group (N), but the oxygenator could partially increase the PO_2 in the acute respiratory distress syndrome animal models (G4). In inflammatory mediator-induced acute respiratory distress syndrome animal models developed in this study, bilateral use of oxygenator and taurine+dexamethasone (antioxidants) showed a similar PO_2 in the blood to that of the normal rats. From this result it was found that bilateral use of antioxidants and membrane oxygenator is effective in inflammatory mediator-induced acute respiratory distress syndrome animal models.

4. Blood Characteristics

Fig. 7 represents the concentrations of arterial hematocrit (a) and hemoglobin (b). According to the experimental results, when antioxidants were used, blood hematocrit concentration and hemoglobin concentration increased. Also, use of oxygenator and bilateral use of membrane oxygenator and antioxidants increased the concentration of blood hemocrit and hemoglobin.

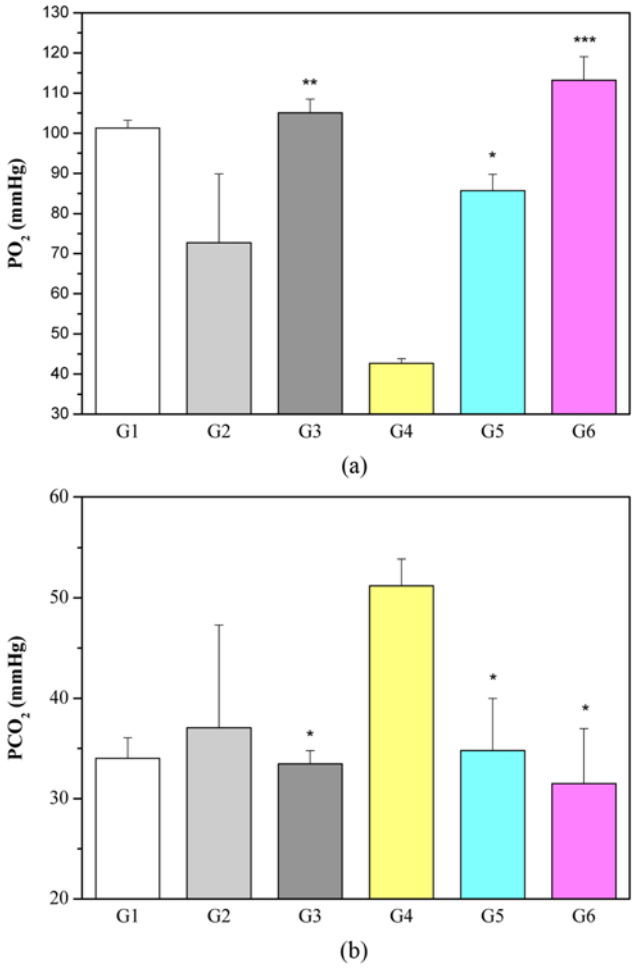

Fig. 6. The results of blood gas analysis in the rats (a) pO_2, (b) pCO_2 *p<0.05, **p<0.01, ***p<0.005 vs. G4.

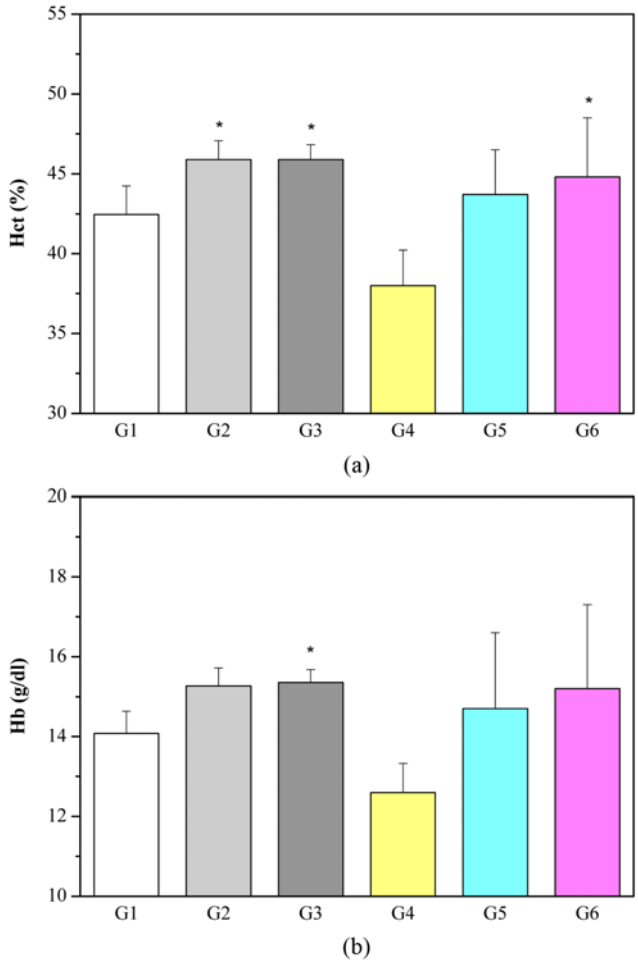

Fig. 7. The results of hematocrit (Hct) and blood hemoglobin (Hb) in the rats (a) Hematocrit (Hct), (b) Hemoglobin (Hb) *p< 0.05 vs. G4.

Fig. 8 represents the results of measured pH of the animals. The pH decreased when LPS was injected, because the LPS induced the acidification of the blood. However, when the antioxidants, taurine and dexamethasone, were injected, the blood acidified by the LPS could be maintained at normal condition. Use of the membrane oxygenator as well showed similar trends.

Fig. 9 represents the results of measured blood protein concentration. According to the results, protein concentration increased when LPS was injected. But protein concentration decreased when antioxidants and membrane oxygenator was used.

Fig. 10 represents the results of measured blood calcium ion concentration. According to the results, the concentration of calcium ion increased but with no statistical significance when LPS was injected. Also, the concentration of calcium ion decreased when the antioxidants, taurine and dexamethasone, were injected. However, the concentration of calcium ion decreased when membrane oxygenator and antioxidants were used simultaneously. As the blood calcium ion acts as a coenzyme of the two enzymes that produce fibrin, it is essential for blood coagulation. Also, calcium ion acts as a coenzyme of various intra- and extracelluar enzymes. Because calcium ion is bound to serum protein, the total calcium concentration increases or decreases as serum protein concentration increases or decreases. Because blood hydrogen ions compete with calcium ions

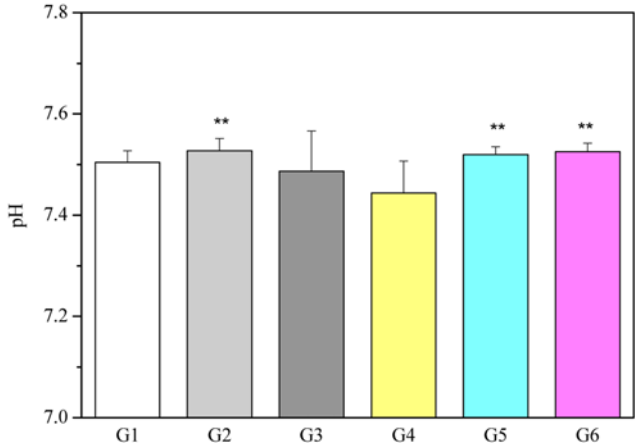

Fig. 8. Hydrogen ion concentration in blood **p<0.01 vs. G4.

for the binding site of protein, hydrogen ion concentration is greatly affected by the calcium that binds to protein. If blood hydrogen ion concentration decreases, calcium ion level decreases and proportion of calcium ions among total calcium increases [25,26].

Fig. 11 represents measured survival of ARDS animal models

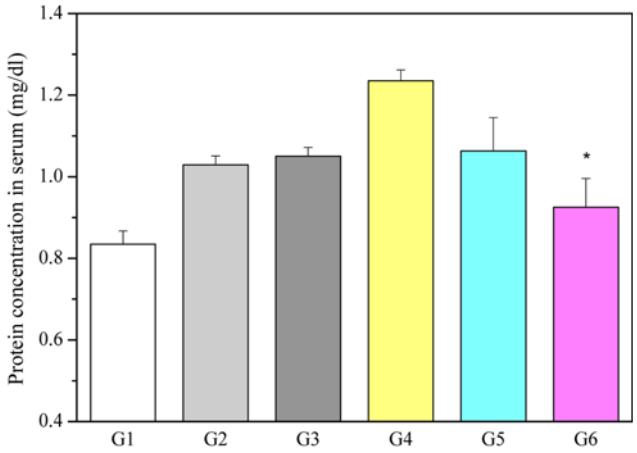

Fig. 9. Protein concentration in serum *p<0.05 vs. G4.

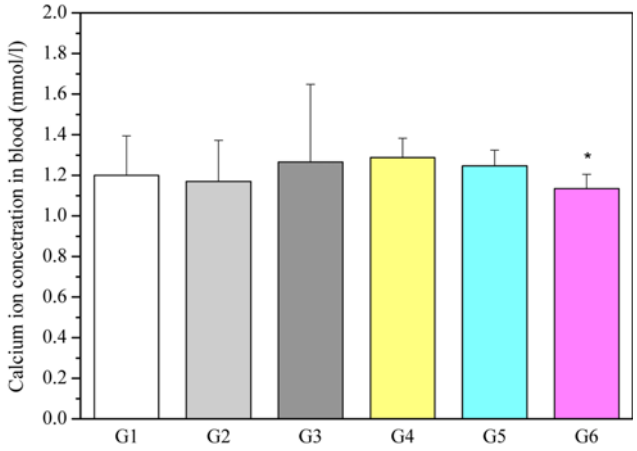

Fig. 10. Calcium ion concentration in blood *p<0.05 vs. G4.

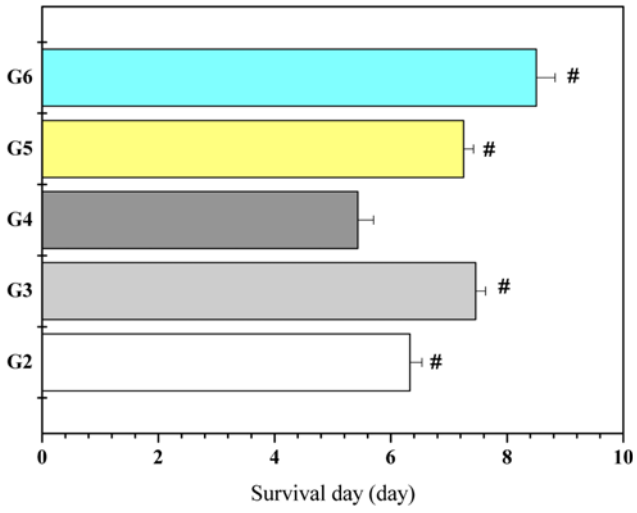

Fig. 11. Survival day in the rats #p<0.001 vs. G4.

when antioxidants and membrane oxygenator were used. LPS-injected group (G4) survived for 5.43 days on average, but in antioxidants-injected cases, taurine-injected group (G2) survived for 6.33 days and dexamethasone-injected group (G3) survived for 7.46 days, meaning that G3 survived longer than G2 by 1 day. In addition, membrane oxygenator-use group (G5) survived longer by 1-2 days. The group to which both antioxidants and membrane oxygenator were used simultaneously (G6) survived longer by 2 days or longer. From these results, it was found that antioxidans and membrane oxygenator effectively acted on LPS-induced ARDS, since they had an antioxidizing effect.

CONCLUSION

We evaluated the effectiveness of antioxidants, taurine and dexmethasone, in the animal models with damaged lungs such as inflammatory mediator-induced ARDS and established an ARDS therapy technique by suppressing active oxygen using membrane oxygenator [24,25]. Experimental results showed that when inflammatory mediator that has an endotoxin such as LPS was injected directly into the airway, it developed inflammatory pulmonary edema in the lung, and could induce respiratory distress syndrome. It induced respiratory acidosis as well reducing the blood pH. To treat such endotoxin-induced acute respiratory distress syndrome, the antioxidants, taurine and dexamethasone, were injected. As a result, they turned the blood pH to the normal condition, increased blood hemoglobin and hemocrit concentration and oxygen partial pressure (PCO_2), and could increase survival [25,26].

When a membrane oxygenator was used as well, similar effects were identified to those of antioxidants. When a membrane oxygenator and antioxidants were used simultaneously, better effects were identified. Therefore, it is concluded to be effective for the treatment of acute respiratory distress syndrome to use antioxidants and membrane oxygenator simultaneously when endotoxin-induced acute respiratory distress syndrome occurs.

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