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Effect of antioxidant in an acute lung injury animal model

Hyung-Sub Kang*, Hye-Min Park*, Hyeon-Kyu Go*, Shang-Jin Kim*, Jin-Shang Kim*, Seong-Jong Kim, and Gi-Beum Kim*,†**

*Department of Pharmacology, College of Veterinary Medicine, Korea Zoonosis Research Institute, Chonbuk National University, 664-14, 1-Ga Dukjin-dong, Dukjin-gu, Jeonju, Jeonbuk 561-756, Korea
**Division of Chemical Engineering, Chonbuk National University, 664-14, 1-Ga Dukjin-dong, Dukjin-gu, Jeonju, Jeonbuk 561-756, Korea
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Abstract−The objective of this study was to scrutinize the involvement of nitric oxide (NO) and the diagnosis of blood in ARDS in a rat model determined by the sequential exposure to lipopolysaccharide (LPS). Also, the present study was designed to evaluate the effects of taurine and dexamethasone on ARDS induced by LPS. Measurements of nitrite/nitrate were elevated in BAL of LPS challenged rats, indicative of an induction of the NO synthase. Taurine and dexamethasone abrogated the extent of endotoxin-induced ARDS, as evidenced by the decreases BAL nitrate/nitrite, BALF protein and lung pathology. T+L+D-group had higher PaO_2 and lower $PaCO_2$ values than L-group and T+L-group. But, ionized Ca^{2+} and Mg^{2+} both were not shown significant change. Also, T+L and T+L+D-group showed significant increase compared with L-group, but for the other side no significant difference was seen between T+L and T+L+D group. We suggest that taurine and dexamethasone may be a drug of choice for preventing ARDS.

Key words: Acute Respiratory Distress Syndrome (ARDS), Lung Injury, Lipopolysaccharide (LPS), Taurine, Dexamethasone, Nitric Oxide (NO)

INTRODUCTION

Acute lung injury (ALI) is a serious complication of major trauma occurring as a direct consequence of trauma to the lung or, more commonly, arising indirectly as a consequence of trauma elsewhere to the body. Acute respiratory distress syndrome (ARDS) can be associated with various disorders [1-5]. ARDS is a devastating disease characterized by an edematous reaction in the lungs, leading to defective gas exchange [6]. ARDS frequently develops after trauma, infections or a sequence of such events [7]. Endotoxemia (sepsis) refers to a heterogeneous class of syndromes caused by a systemic inflammatory response to infection. This condition may lead to severe shock and multiple organ failure [8], and is one of the leading causes of death in critically ill patients [9]. Acute lung injury has long been observed in animals and patients with sepsis or endotoxemia [10,11].

Taurine (2-amino-ethanesulfonic acid) has been reported to protect lung tissue from oxidant-induced damage in a variety of models involving inflammation as a pathogenic feature. Taurine is the principal free-amino acid in mammals, and is also detected in high concentrations in the brain, heart, eye, muscle, and liver. It can be synthesized from cysteic acid or hypotaurine, which is derived from methionine and cysteine within the body [12]. Either intracellularly or released into the extracellular medium, taurine may protect cells against attack by oxidants, and displays anti-inflammatory and immunomodulator activities [13]. In patients with ARDS, N-acetylcysteine (NAC) can lessen symptoms and shorten the symptom's duration, presumably by acting as an anti-oxidant and via the restoration of glutathione levels in cases of lung injury [14,15].

†To whom correspondence should be addressed.
E-mail: kgb70@jbnu.ac.kr

Based on these observations, it is expected that taurine will produce beneficial effects by reducing the lipopolysaccharide (LPS)-induced increases in biochemical factors, and by suppressing the release of pro-inflammatory cytokines and free radicals. Glucocorticoids (GCs) such as dexamethasone (DXM) are used in the treatment of ARDS. One study has shown that DXM alleviates LPS-induced ARDS in rats and upregulates pulmonary glucocorticoid receptors [16], attenuating ARDS-induced inflammation. Presently, the effect of sequential exposure to LPS on blood alterations in a rat model of ARDS was investigated. As well, the effects of taurine and DXM on LPS-induced ARDS were explored.

MATERIALS AND METHODS

1. Animals

Male Sprague-Dawley rats weighing 200-250 g were purchased from the Samtako Biokorea (Daejeon, Korea). Animals were housed four per cage at constant room temperature (22-27 °C) with alternating 12 hrs periods of light and dark. The animals were allowed free access to a standard rat pellet diet and tap water. All procedures in this study were performed in accordance with the National Institutes of Health Guide for the Care.

2. Experimental Protocols

Animals were randomly divided into four groups. The initial number of rats in each group exceeded nine. If death occurred during the observation period, additional experiments were performed to ensure that the number of rats in each group was 10. In the control group, rats were given an intratracheal (i.t.) infusion of water for injection (N-group) and phosphate buffered saline (PBS) (P-group). Sprague-Dawley rats (250-300 g, 10-12 weeks old) receiving *Salmonella enteritidis* LPS (Sigma-Aldrich, St Louis, MO, USA; dis-

solved in PBS prior to use) (L-group) were administered 7 mg LPS/kg body weight by a bolus i.t. injection over 1-2 min. The taurine+LPS treated group (T+L-group) were injected with 20 mM taurine during the seven days prior to the injection of LPS. The taurine+LPS+DXM treated group (T+L+D-group) were 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. Each group of animals was observed for seven days. The survivors were killed with an intravenous overdose of pentobarbital (100 mg/kg body weight). We collected arterial blood using 3 mL vacutainer (No additive), arterial blood was immediately recovered, centrifuged at 1,500 rpm for 5 min, and the supernatant used for blood gas and ion measurements. Ionized magnesium concentration [iMg^{2+}], ionized calcium concentration [iCa^{2+}], pH, hematocrit (Hct), hemoglobin (Hb) and some other blood gases were measured using a NOVA analyzer (NOVA Biomedical Corp., Waltham, MA, USA). The lung was immediately removed. Repeated bronchoalveolar lavage (BAL) of the airways was performed with a total of 3 ml PBS. The BAL fluids were centrifuged at 3,000 rpm for 5 min and supernatants used for nitrite/nitrate (NO_2^-/ NO_3^-) measurements.

3. Measurement of Nitrite/Nitrate

Levels of nitrite/nitrate in BAL fluids and sera were determined via the Greiss reaction [17]. NO synthesis was assessed indirectly by the measurements of nitrite (NO_2^-) and nitrate (NO_3^-): the stable derivatives from NO oxidation. Since the Greiss reagent does not react with nitrate, nitrate was converted to nitrite by incubating the samples and standards overnight with 1 U/mL of nitrate reductase from Aspergillus (Sigma, St. Louis, Missouri) and 1 mg/mL of NADPH (Sigma). The sample was mixed with Greiss reagent (1% sulfanilamide in 30% acetic acid mixed with 0.1% *N*-naphthylethylenediamine dihydrochloride in 60% acetic acid in 1 : 1 ratio) all from Sigma. Nitrite levels were determined by reading optical density using a wave length of 595 nm. Levels of nitrite were determined by comparison with a standard curve of sodium nitrate. The lowest concentration of nitrite/nitrate detectable is about 1 μM.

4. Measurement of LDH and Protein Concentration in Bronchoalveolar Lavage

Briefly, 0.2 mL of lavage fluid was added to 0.3 ml of Dri-STAT LD-L reagent (Sigma), pH 8.9, containing 51.6 mM L-lactate (Sigma) and 8.26 mM NAD (Sigma); absorbance was measured at 340 nm. One unit of activity is defined as that which produces 1 μM NADH/min under the condition specified by the manufacturer. Total protein levels were determined by modified Bradford procedure (kit from Sigma). Concentration of proteins is expressed in μg/mL.

5. Lung Pathology

After lung weight had been determined, lung tissue was taken for histological examination. Lung tissue was immersed in 10% formaldehyde fixative for 24 hrs and was then rinsed with tap water to remove the formaldehyde. For light microscopic examination, lung tissue was dehydrated with graded alcohol and then embedded in paraffin at 60 °C. A series of microsections (5 mm) was stained with haematoxylin and eosin.

6. Statistical Analysis

The results are expressed as the mean±standard error of the mean (SEM). The data was analyzed using a Student's t test and repeated-measures of analyses of variance (ANOVA) followed by a Bonferroni test. A p value <0.05 was considered significant.

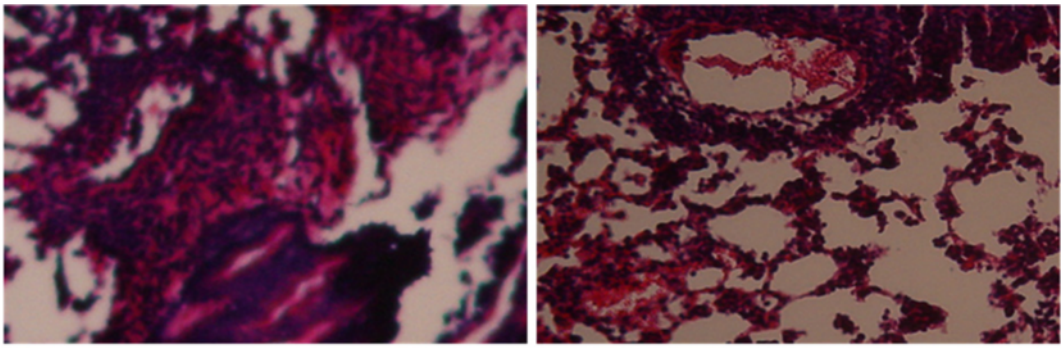
RESULTS

1. Lung Pathology

Lung pathology showed severe lung edema and inflammatory cell infiltration in a rat administered LPS (Fig. 1(a)). Histopathologic examination of lung tissue showed heavy perivascular and interstitial infiltration of large hyperchromatic white blood cells with abundant cytoplasm. Note the preventive effect of taurine on lung pathology (Fig. 1(b)). Pathological findings in ALI include diffuse alveolar damage with widespread alveolar wall thickening and infiltration by neutrophils and macrophages [18]. 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. Nitrate/Nitrite and BAL Protein Levels

BAL nitrate/nitrite, the metabolites of NO, was increased following LPS administration. 20 mM taurine and 10^{-7} M DXM treatment prior to the administration of LPS significantly decreased nitrate/nitrite (Fig. 2(a)). The peak NO concentration in BAL increased from 4.8±0.1 μM (in the control group) to 8.0±0.3 μM after LPS administration. Taurine and 10^{-7} M DXM reduced NO to 5.4±0.3 μM. Determination of BAL protein revealed the similar effects of LPS and LPS+taurine+DXM. The BAL protein values were 27.2±2.4, 48.1±3.8 and 37.0±3.7 mg/mL in the control, L- and L+T+D



(a) (b)

Fig. 1. Lung pathology. (a) Lung edema and inflammatory cell infiltration in a rat administered LPS, (b) Effect of taurine on lung pathology.

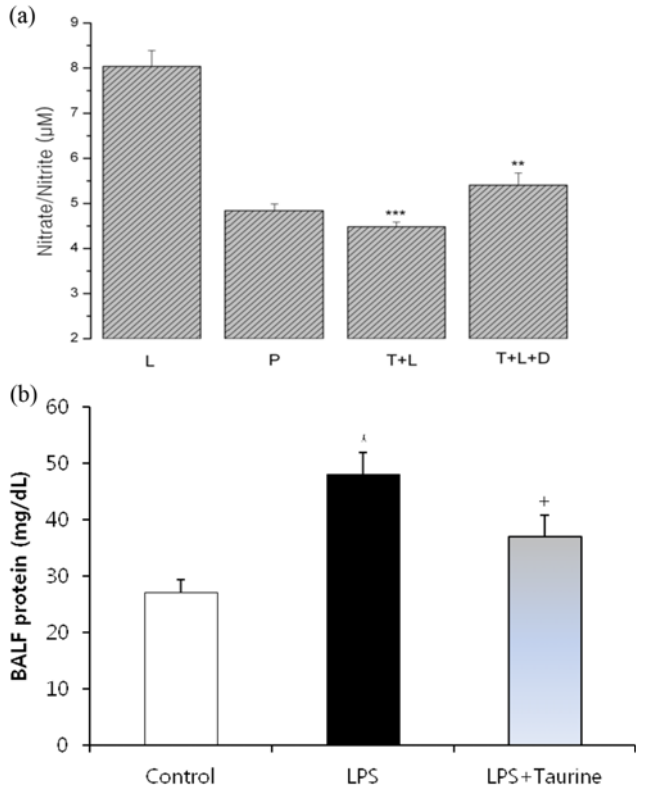


Fig. 2. Changes in (a) nitrate/nitrite level and (b) protein concentration in bronchoalveolar lavage fluid (BALF). Nitrate/nitrite level in BAL of rats that received injection of PBS or LPS (7 mg/kg) with or without taurine (20 mM before LPS). Lipopolysaccharide increased these parameters, whereas taurine diminished these values. L: LPS, P: PBS (control), T: taurine, D: dexamethasone. Data are the mean±SEM. *P<0.01 compared with vehicle; + P<0.01 compared with the LPS group.

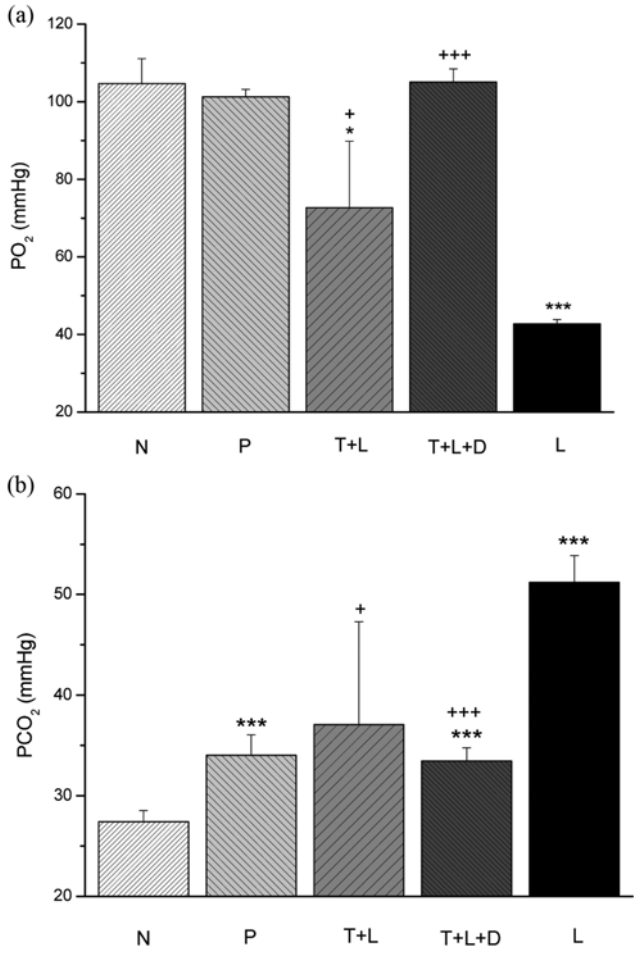


Fig. 3. The results of blood gas analysis in the rats. * p<0.05, ** p<0.01, *** p<0.005 vs. control, $^{+}$ p<0.05, $^{++}$ p<0.01, $^{+++}$ p< 0.005 vs. LPS (n=9). (a) Oxygen partial pressure (pO_2) (mmHg), (b) Carbone dioxide partial pressure (pCO_2) (mmHg).

groups, respectively (Fig. 2(b)). The LDH of BAL levels appeared as the same result. The LDH activities in BALF increased by 152± 0.6% after a 7 mg/kg LPS treatment. However, LDH rate of the increase was lower when the cells were treated in combination with taurine+DXM compared with LPS treatment alone (data not shown). In this study, we examined the involvement of NO in a rat model of ARDS. Challenge with LPS led to lung injury, as seen by increased LDH and protein level in BAL. Measurements of nitrite/nitrate were elevated in the BAL of LPS challenged rats, indicative of an induction of the NO synthase. Recent investigations in animals and patients have further demonstrated that NO production through iNOS synthesis is one of the mechanisms causing lung injury due to ischaemia/reperfusion, enterovirus 7 L and endotoxaemia [19-21]. Under septic or inflammatory conditions, iNOS in the endothelial cells, epithelial cells, macrophages, neutrophils, vascular smooth muscle cells and fibroblasts is activated [22,23]. The impact of NO on the development of inflammatory reactions is being scrutinized, as NO has clear pro-inflammatory properties, and is an important mediator of tissue damage [24]. In addition, NO impacts on function and cytokine release by inflammatory cells as well as cellular adhesion, which expands the role of this mediator as an important effect molecule in inflammation [25,26]. Agouridakis et al. found that the pro-inflammatory cytokines and adhesion molecules, such as TNF-a, IL-1, vascular adhesion molecule-1 and soluble intercellular adhesion molecule-1, were increased in the BAL in patients with ARDS [27]. We did not measure these factors in the BAL fluid in the present study. Further investigation is required to determine whether these factors are increased in airway lavage in animals subjected to ARDS.

2. Measurement of Blood Ion and Gas

Fig. 3 shows the results of blood gas analyses. N- and P-groups displayed oxygen partial pressure (PaO_2) in the normal range. The L-group exhibited severe hypoxemia with a significant decrease in PaO_2 (p<0.005). T+L-group showed a significant decrease in PaO_2 (p<0.05) compared with the N-group, but displayed significantly higher PaO_2 values than the L-group. No significant difference was evident in T+L+D-group, which had significantly higher PaO_2 values than both L- and T+L-groups. Carbon dioxide partial pressure ($PaCO_2$) increased significantly (p<0.005) from a baseline value of 27.4± 1.1 mmHg to 51.2±2.7 mmHg. L-group displayed a significant increase in $PaCO_2$ (p<0.005) compared with N-group rats. T+L-group displayed increased $PaCO_2$ compared with the N-group, but decreased $PaCO_2$ compared with the L-group. The T+L+D-group had lower $PaCO_2$ values than the L- and T+L-groups. In this study, we demon-

strated that taurine attenuated the lung damage induced by endotoxin (LPS administration). The taurine abrogated the extent of endotoxin-induced ARDS, as evidenced by the decreased BAL nitrate/nitrite, BALF protein and lung pathology. An earlier study by Bernard revealed that intracellular glutathione levels were reduced in patients with ARDS [14]. The taurine increased the cellular glutathione pool, thereby improving the chest radiograph oedema scores, pulmonary vascular resistance, static compliance, oxygen delivery and consumption in these patients. As a free radical scavenger and antioxidant, taurine abrogated the endotoxin-induced organ damage in conscious rats through suppression of the release of inflammatory cytokines and hydroxyl radicals. Kao et al. found that N-acetylcysteine (NAC) not only ameliorated the ALI, but also improved and shortened the LPS-induced systemic hypotension. This effect on the arterial pressure may be explained by the reduction in NO formation, as evidenced by the decrease in plasma nitrate/nitrite [28]. The taurine, 2-aminoethanesulfonic acid, can be synthesized from cysteic acid or hypotaurine, which is derived from methionine and cysteine within the body. NAC is the same line of taurine. So, sulfonic amino acids such as taurine and cysteine attenuated the symptom of ARDS by their anti-oxidative effects and inhibition of NOS. And anti-inflammation drug, dexamethasone attenuated NO synthase, with taurine.

Presently, we demonstrate that taurine attenuates lung damage induced by endotoxin (LPS). Taurine is an amino acid endowed with antioxidant, anti-inflammatory and immunomodulatory properties [29] that reduces oxidant-induced lung damage in a variety of experimental models [17,30]. When LPS was applied following treatment with 20 mM taurine, significantly higher PaO_2 values were evident in the rats, compared to those that did not receive taurine prior to LPS administration. The antioxidant properties of taurine may have, therefore, contributed to decreased pulmonary eosinophilia through an alteration of the oxidant-sensitive expression of cell adhesion molecules. In addition to the reduced numbers of inflammatory cells, taurine may lessen the oxidant burden by diminishing the generation by these cells of superoxide anion and other cytotoxic mediators [25,29], and could also protect airway cells from oxidative damage as demonstrated for other antioxidants [19]. Taurine increases the cellular glutathione pool, thereby improving the chest radiograph edema scores, pulmonary vascular resistance, static compliance, oxygen delivery and consumption. As a free radical scavenger and antioxidant, taurine abrogates the endotoxin-induced

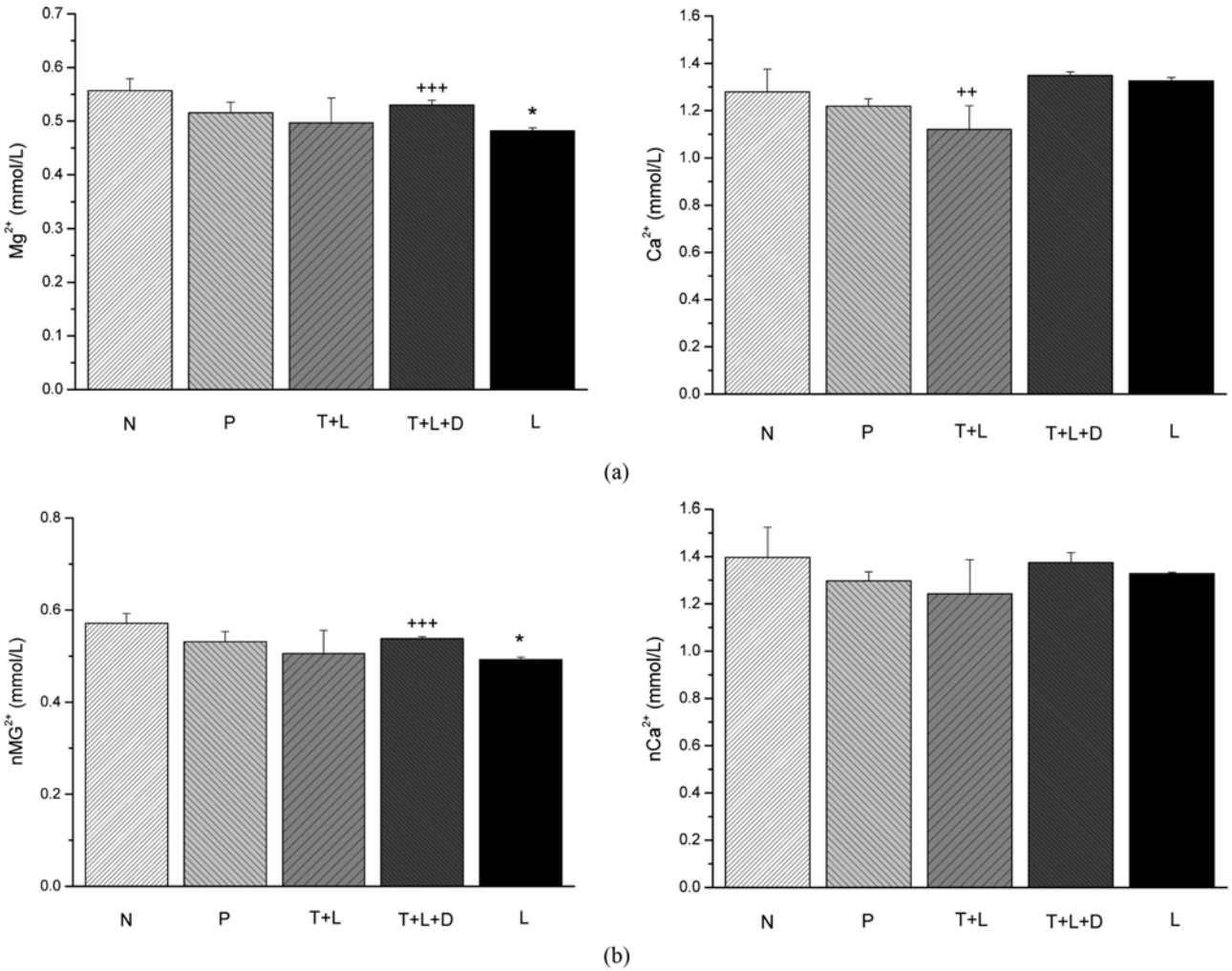


Fig. 4. The results of blood ions analysis in the rats. * p<0.05, ** p<0.01, *** p<0.005 vs. control, $^{+}$ p<0.05, $^{++}$ p<0.01, $^{+++}$ p<0.005 vs. LPS (n=9). (a) Ionized magnesium and calcium, (b) pH-normalized magnesium and calcium.

organ damage in conscious rats through suppression of the release of inflammatory cytokines and hydroxyl radicals. NAC not only ameliorates ALI, but also improves and shortens LPS-induced systemic hypotension [20]. The taurine can be synthesized from cysteic acid or hypotaurine, which are derived from methionine and cysteine within the body. NAC synthesis is similar.

This study describes the efficacy of taurine and DXM in a rat model of ALI. The taurine and DXM ameliorated LPS-induced lung injury and mortality. The patients with ALI are characterized by hypoxemia, bilateral infiltrates in chest radiographs and absence of an elevated pulmonary capillary wedge pressure [23]. Although LPS exposure and LPS-induced cytokines can directly produce tissue hypoxia by impaired oxygen metabolism [22,23], hypoxia in turn increases these LPS-stimulated responses [24,26]. Furthermore, lung tissue hypoxia can be induced by a variety of factors such as hypovolemia, reduced blood flow, inadequate delivery of oxygen and insufficient oxygen consumption [27]. These observations underscore the importance of the hypoxic stress response in protecting against LPS-induced ALI.

Fig. 4 shows the results of blood ion analyses. [iMg^{2+}] and pH-normalized Mg^{2+} of all experimental groups was decreased compared with the N-group. The L-group showed significantly decreased [iMg^{2+}] ($p<0.05$) compared with the N-group. The T+L-group showed no significant difference compared with the L-group, but [iMg^{2+}] in the T+L+D-group was increased compared with the L-group. [iCa^{2+}] in the P- and T+L-groups was decreased compared with the N-group, but was decreased as compared with the T+L+D- and L-groups. [iCa^{2+}] in the T+L+D-group was higher than the L-group, but not significantly so. Changes of blood pH affect the binding of these ions to plasma proteins, mainly albumin, because hydrogen ions compete with iCa^{2+} and iMg^{2+} for protein binding sites. So, pH-normalized [iMg^{2+}] and [iCa^{2+}] are very important; neither exhibited a significant change. Ionized Ca^{2+} and Mg^{2+} have many important physiologic functions, and measurement of [iCa^{2+}] and [iMg^{2+}] in plasma provides useful information for clinical diagnosis and management. [iCa^{2+}] and [iMg^{2+}] were measured in blood and the Ca^{2+} to Mg^{2+} ratio was calculated. Previous studies also have measured the ratio of iCa^{2+} and iMg^{2+} (iCa^{2+}/iMg^{2+}) as a standard. Both physiologically active ions are major determinants of various physiological states. We hypothesized that pre-treatment [iMg^{2+}] levels and/or iCa^{2+}/iMg^{2+} may have been confounding variables in this studies. Both elements share left/right-sided cell receptors and are discussed in associated pairs, as they function as an inseparable, interdependent unit and are essential to human health in physiological and pathological condition. Changes of pH in the specimen affect the binding of these ions to plasma proteins, mainly albumin, because hydrogen ions compete with Ca^{2+} and Mg^{2+} for protein binding sites [29]. Our results show that [iMg^{2+}] and pH-normalized Mg^{2+} of all experimental groups was decreased compared with the N-group. The L-group showed a significant decrease ($p<0.05$) compared with the N-group, but the T+L+D-group displayed a significant increase compared with the L-group. On a cellular level, magnesium sulfate modifies the formation of arachidonic acid metabolites, which are important mediators of ALI. Mg^{2+} may significantly affect lung structure and mediator reactions. Examples where high dose Mg^{2+} supplementation has been effective include the therapy of eclampsia and pre-eclampsia, tetanus, and severe bronchospasm [31-33].

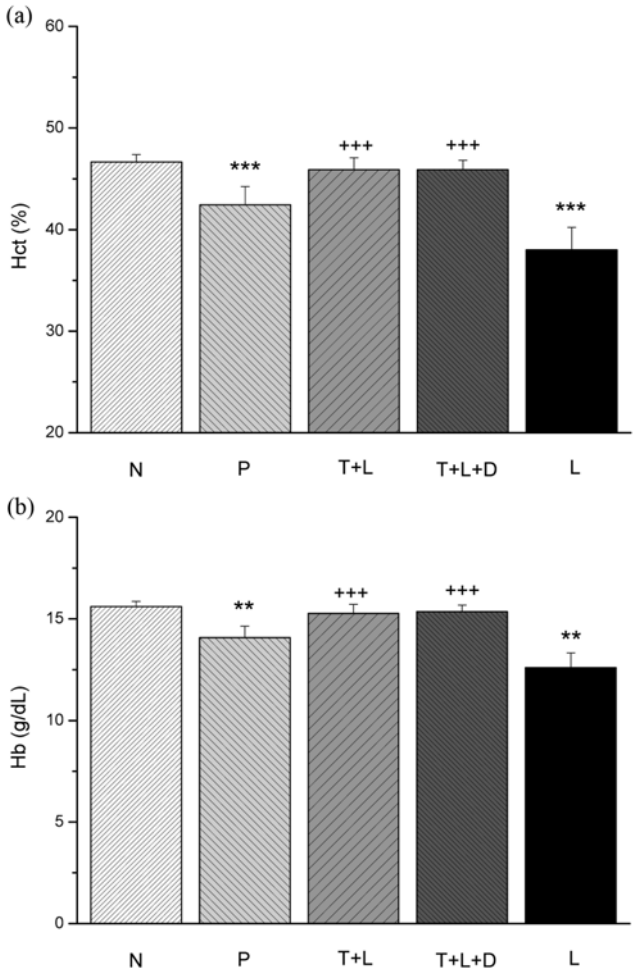


Fig. 5. The results of hematocrit (Hct) and blood hemoglobin (Hb) in the rats. * p<0.05, ** p<0.01, *** p<0.005 vs. control, $^{+}$ p<0.05, $^{++}$ p<0.01, $^{+++}$ p<0.005 vs. LPS (n=9). (a) Hematocrit (Hct), (b) Hemoglobin (Hb).

Hypomagnesemia tends to magnify the degree of hyperoxic lung injury, while high-dose Mg^{2+} therapy tends to attenuate the effects of hyperoxia [34]. So, in this animal model of diffuse alveolar damage, alterations in host serum Mg^{2+} levels may modulate the degree of lung damage. Appropriately, taurine and DXM abrogate the extent of endotoxin-induced ARDS by Mg^{2+} increase in serum. Our results suggest that the combination of taurine and DXM may be potentially beneficial in the treatment and prevention of ARDS.

Fig. 5 shows the results of hematocrit (Hct) and hemoglobin (Hb) concentration analyses. The L-group displays a significant decrease ($p<0.01$) compared with the N-group. But, the T+L- and T+L+D-groups show a significant increase compared with the L-group; no significant difference is evident between the T+L- and T+L+D-groups.

CONCLUSION

Challenge with LPS led to lung injury, as seen by increase LDH and protein level in BAL. Measurements of nitrite/nitrate were elevated in BAL of LPS challenged rats, indicative of an induction of the NO synthase. But, taurine+dexamethasone abrogated the extent

of endotoxin-induced ARDS, as evidenced by the decreased BAL nitrate/nitrite, BALF protein and lung pathology. So, our results suggest that taurine+dexamethasone may be a drug of choice for preventing ARDS.

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