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著者別名	荒井 サブリナ , 下條 信威, 河野 了, 水谷 太郎
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Assessment of circulatory and pulmonary endothelin-1 levels in a lavage-induced surfactant-depleted lung injury rabbit model with repeated open endotracheal suctioning and hyperinflation



Junko Kamiyama, Subrina Jesmin, Hideaki Sakuramoto, Nobutake Shimojo, Md. Majedul Islam, Tanzila Khatun, Masami Oki, Satoru Kawano, Taro Mizutani*

Department of Emergency and Critical Care Medicine, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan

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ABSTRACT

Aims: Endothelin-1 (ET-1) is a mediator of various physiological and pathological processes, including vascular inflammation, cell proliferation and vasoconstriction. Attenuation of ET action using ET-1 antagonists reduces pulmonary vascular leakage and inflammation in several models of lung injuries and experimental acute respiratory distress syndrome (ARDS). Based on these earlier reports, the current study investigates the patterns of ET-1 levels in circulation and pulmonary tissues in an experimental model of lavage-induced surfactant-depleted lung injury. Additionally, we also test the effects of open endotracheal suctioning (OES) and hyperinflation (HI) as recruitment maneuver following OES on ET-1 levels.

Main methods: Briefly, 24 Japanese white rabbits were anesthetized and intubated. Normal saline was instilled into the lung and washed mildly. After instillation, rabbits were ventilated at definite settings at a total duration of 3 hours. OES and HI were performed every 15 minutes from the beginning of the protocol.

Key findings: Here, we show that both circulatory and pulmonary ET-1 levels increased in models with lung injury induced by saline lavage compared to healthy control group. No further aggravation in expression of pulmonary ET-1 was seen after OES and HI, although OES and HI worsened arterial hypoxxygenation and severity of lung injury. In contrast, circulatory ET-1 levels significantly decreased after OES and HI but were not associated with blood pressure changes.

Significance: We conclude that in a saline lavage-induced lung injury model, both circulatory and pulmonary ET-1 levels increased. Further, OES and HI exerted differential effects on ET-1 expression at both circulatory and pulmonary levels.

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Introduction

Mechanical ventilation is an indispensable tool for providing basic life support and in major surgical procedures of intensive care patients. This is particularly the case for patients with acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). However, ventilator-induced lung injury (VILI) is a common complication in intubated and mechanically-ventilated patients (Dreyfuss and Saumon, 1998; Slutsky, 2005; Albaiceta and Blanch, 2011). The causes of VILI are multifactorial and it has been suggested that they may play a crucial role in the pathogenesis of the disease by releasing factors into the systemic circulation from the lung. Collectively, these events may

subsequently lead to distal organ failure (Wheeler and Bernard, 2007). In view of this, it has been proposed that development of protective ventilation strategies for VILI is key in the management of ALI and ARDS (The Acute Respiratory Distress Syndrome Network, 2000).

Endotracheal suctioning is one of the major procedures frequently performed by nurses for patients during mechanical ventilation. The purpose of this procedure is to directly clean the airway secretions, as patients in this state are unable to cough up the sputum. Specifically, open endotracheal suctioning (OES) is one of the methods of endotracheal suctioning, which requires disconnecting the ventilator from the patient before insertion of a suction catheter into the trachea (Maggiore et al., 2002). It is clear that more airway secretion is removed in OES than in closed endotracheal suctioning of both animal models and patients (Lindgren et al., 2004; Lasocki et al., 2006; Copnell et al., 2007). However, disconnecting from the mechanical ventilator in OES leads to severe hypoxxygenation and lung injury (Suh et al., 2002;

* Corresponding author at: Department of Emergency and Critical Care Medicine, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki, 305-8575, Japan. Tel.: +81 29 853 3210, 3081; fax: +81 29 853 3092.

E-mail address: mizutani@md.tsukuba.ac.jp (T. Mizutani).

Maggiore et al., 2003; Carames et al., 2006). Notably, the induced repeated recruitment and de-recruitment by OES can induce a cytokine response in ARDS patients (Ranieri et al., 1999).

Hyperinflation (HI) is one of the recruitment maneuvers (RM) used to stimulate a cough, recover oxygenation and improve compliance, following OES in mechanically-ventilated subjects (Hodgson et al., 2000; Patman et al., 2000; Berney and Denehy, 2007). Previous studies have demonstrated an association between RM and cytokine release (Talmor et al., 2007) and that RM directly promotes cytokine releases, increases lung stress and compromises lung function (Santiago et al., 2010). If indeed this is the case, the release of inflammatory mediators from the injured lung into the systemic circulation could lead to distal organ failure (Wheeler and Bernard, 2007).

Endothelin-1 (ET-1), a family of 21 amino acid peptide (three isoforms exist; ET-1, ET-2, ET-3), is a mediator of various physiological and pathological processes, including vascular inflammation, cell proliferation and fibrosis. The involvement of ET-1 in the pathogenesis of lung injuries, as well as in ARDS, has been well documented (Filip et al., 1991; Horgan et al., 1991; Jesmin et al., 2011). Furthermore, another study has demonstrated the involvement of ET-1 in VILI (Lai et al., 2010).

To date, there is no study that has examined the expression profile of ET-1 in circulation and pulmonary tissues of a saline lavage-induced surfactant-depleted lung injury model, with or without repeated OES or HI during mechanical ventilation. Thus, the current study attempts to use an experimental model of lavage-induced surfactant-depleted lung injury to investigate the pattern of circulatory and pulmonary expression of ET-1. In addition, we also tested the effects of OES and RM, such as HI, on ET-1 levels. Lastly, we explore whether ET-1 is also involved in lung injury, as well as arterial hypoxxygenation during mechanical ventilation, under conditions of repeated OES or OES and HI.

Methods

Animals

The study was performed using 24 Japanese White Rabbits, weighting between 2.5 and 3.2 kg and fed with a standard diet and water ad libitum. This study was pre-approved by the Ethics Committee of the Animal Resource Center of the University of Tsukuba and the rabbits were cared for in accordance with the guidelines of ethical animal research.

Animal preparation

Rabbits were sedated using 1.0% lidocaine solution (0.25 mg/kg, intramuscularly) and sodium pentobarbital (75–150 mg, bolus infusion) and then restrained in a supine position. The anesthesia was maintained during the experiment by a continuous infusion of sodium pentobarbital (5 mg/kg/h) and pancuronium (0.1 mg/kg/h) administered through the ear vein via an infusion pump. Also, the body fluid in the animals was maintained by a continuous infusion of normal saline (5 mL/kg/h).

The anterior neck was then carefully dissected, followed by a tracheotomy, insertion of an endotracheal tube (3.5 mm internal diameter) in the trachea and mechanical ventilation in pressure-controlled mode (LTV-1000 ventilator; Care Fusion, San Diego, CA). Since no lung lavage was induced, the ventilator settings for the baseline were as follows: inspired oxygen fraction (F_{iO_2}) 1.0; peak end-expiratory pressure (PEEP) of 2 cmH₂O; and inspiratory time of 0.5 second. Airway pressure and respiratory rate were adjusted to maintain constant expiratory tidal volume of 6 to 8 mL/kg (around the criteria range of protective ventilation strategy), and initial respiratory rate was set to achieve normocarbica. In the current study, the essential parameters of protective ventilation strategy were carefully followed, including: 1) low tidal volume (6 mL/kg), 2) expiratory plateau pressure <30 cmH₂O, 3) permissive hypercapnia, and 4) set up of PEEP to prevent alveolar collapse (The

Acute Respiratory Distress Syndrome Network, 2000). Mechanical ventilation was continued in the same manner throughout the experiment, except for the adjustments of PEEP level, described later.

In order to monitor systemic arterial pressure and sampling blood gas, a catheter was carefully inserted into the right carotid artery. Blood was then drawn using heparinized syringe for determining baseline levels of PaO₂, PaCO₂, arterial pH and lactate (ABL 720; Radiometer Copenhagen, Copenhagen, Denmark). Baseline readings for lung mechanics were also measured (baseline). Lastly, body temperature was monitored continuously and maintained between 38 °C and 39 °C using a rectal probe and a heating pad, respectively. When arterial blood pressure in the current experimental protocol dropped to life-threatening levels, intravenous saline infusion was gradually increased, up to a maximum of 1.5 times in order to restore the blood pressure back to normal.

Lung injury

After 30 minutes of stability, we recorded the baseline data of hemodynamics, gas exchange and lung mechanics. Acute lung injury was induced by lavaging the whole lung using a modified technique, described earlier by Lachman et al. (1980). With the rabbit in the supine position, warm sterile saline (28 mL/kg, average number of lavages were 3–4 times with some variations to obtain PaO₂ < 100 mmHg) was administered into the trachea via the endotracheal tube. The rabbits were then gently rocked from side to side in order to perform mechanical ventilation and the saline solution was gravity-drained and then actively suctioned with a suctioning catheter. The lavage process was repeated until adequate injury was evident (defined as PaO₂ < 100 mmHg) and each lavage was performed at 5 minutes intervals. After 30 minutes, the blood gas was sampled to monitor the stability of the animal before the experimental ventilation protocol was begun.

Experimental protocol

In the current study, 4 groups of animals were used. The healthy control group (control, $n = 6$) was mechanically ventilated but without saline lavage. The rest of the animals were first lavaged, and randomly assigned to three groups ($n = 6$ /each), namely: a) lung injury group (lung injury): in this group the animals with lung injury were mechanically-ventilated for 3 hours; b) open endotracheal suctioning group (OES): animals here were also mechanically-ventilated for 3 hours. However, in addition, OES was performed every 15 minutes after the protocol was started. For endotracheal suctioning, 6 French suctioning catheters were used (TrachCare, Ballard Medical products, Draper, Utah). Suction depth was of 2 cm (length of adapter) plus length of tracheal tube and a negative pressure of -150 cmH₂O was applied for 15 seconds, while withdrawing the catheter; c) hyperinflation group (HI): in this group suctioning was performed, as described earlier in the OES group. Further, animals were considered manually hyperinflated by changing the ventilator mode with PEEP of 0 cmH₂O and positive airway pressure of +5 cmH₂O for 1 minute. After the protocol was started, the rabbits were ventilated by pressure-controlled ventilation with a PEEP of 10 cmH₂O, inspiratory time of 0.5 second and inspired oxygen fraction of 1.0. Airway pressure and respiratory rate were adjusted to maintain constant expiratory tidal volume of 6 to 8 mL/kg, according to the protective ventilation strategy (The Acute Respiratory Distress Syndrome Network, 2000). The inspiratory pressure limit was set at 25 cmH₂O and respiratory rate limit was 55/min. Blood gas sampling as well as hemodynamics and pulmonary parameters data were performed every 30 minutes and blood samples (plasma and serum) taken every hour. Finally, the rabbits were euthanized with bolus injection of sodium pentobarbital and lungs were processed for morphological examination.

Table 1
Baseline characteristics of four experimental groups.

	Control	Lung injury	OES	HI	p Value
Body weight (kg)	3.00 ± 0.14	3.02 ± 0.11	2.86 ± 0.12	2.92 ± 0.17	0.223
The number of lavage (times)	–	3.04 ± 0.52	3.33 ± 0.52	3.35 ± 1.04	0.904
pH	7.40 ± 0.07	7.41 ± 0.06	7.42 ± 0.08	7.43 ± 0.06	0.870
PaCO ₂ (mmHg)	44.4 ± 9.11	45.1 ± 3.35	40.6 ± 8.24	40.4 ± 6.05	0.542
PaO ₂ (mmHg)	413 ± 81.9	421 ± 39.8	460 ± 45.9	444 ± 32.4	0.401
Lactate (mmol/l)	1.17 ± 0.41	0.75 ± 0.20	1.06 ± 0.60	1.36 ± 0.43	0.148
HR (bpm)	247 ± 58.0	278 ± 41.2	232 ± 59.4	245 ± 24.7	0.388
MAP (mmHg)	126 ± 20.1	126 ± 8.54	115 ± 15.4	113 ± 10.7	0.253
PIP (cmH ₂ O)	12.7 ± 0.95	12.8 ± 0.40	13.6 ± 2.33	12.6 ± 1.36	0.218

HR, heart rate; MAP, mean arterial blood pressure; PIP, peak inspiratory pressure; OES, open endotracheal suctioning; HI, hyperinflation. Control group (without lavage), lung injury group only (without OES and HI), OES group (without HI) and HI group (OES + HI). All values were mean ± SD.

Summary of blood and lung tissue sampling

Blood sampling for blood gas analysis was performed at the following time points: a) at baseline before lavaging; b) at the end of lavages; c) and thereafter every 30 minutes for 3 hours, bringing the total blood sampling for gas analysis to eight (times). Blood sampling for serum and plasma preparation was performed thrice, i.e., at baseline before the onset of lavaging, after completion of lavaging and finally 3 hours after OES and HI, and following induction of lung injury. Lung samples were taken at the end of 3 hours, when OES and HI were completed.

Enzyme-linked immunosorbent assay for plasma and pulmonary ET-1 level

The left lung was harvested, snap frozen in liquid nitrogen and stored at –80 °C. Lung tissues were homogenized with 10 vol. of 20 mM TrisHCl (pH 7.4), 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM Na₃VO₄, 2 mM dithiothreitol, 20 mM glycerophosphate, 0.6% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 60 µg/mL aprotinin, and 1 µg/mL leupeptin on ice using a homogenizer. The homogenate was gently rotated for 30 min at 4 °C and then centrifuged at 13,000 g for 10 min at 4 °C, and the protein concentration of the resulting supernatant was determined. Concentration of ET-1 in plasma and pulmonary tissue extracts was determined using a Quantikine ET-1 Enzyme Immuno Assay Kit (R&D Systems, MN, USA). A 4.5 hour solid phase enzyme-linked immunosorbent assay (ELISA) was used, and contained synthetic ET-1 and antibodies raised against synthetic ET-1. This immunoassay has been shown to accurately quantitate synthetic and naturally occurring ET-1. A monoclonal antibody specific for ET-1 was pre-coated onto a microplate. Standards and samples were pipetted into the wells and if present, ET-1 antigen was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific to ET-1 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and a color developed in proportion to the amount of ET-1 bound in the initial step. The color development was stopped and its intensity measured. All assays were performed in duplicate. The antibody used in this assay has 23.4% cross-reactivity to human endothelin-2 and 0.5% cross-activity to human/rat endothelin-3.

Western blotting

Ice-cold lung tissue was minced with scissors, homogenized, centrifuged and then the concentration of the protein (supernatant) was determined using the bicinchoninic acid protein assay (Pierce Biotechnology). Samples were boiled in reducing SDS sample buffer for 5 minutes, loaded onto an SDS-PAGE (4–15% polyacrylamide) gel, subjected to electrophoresis, and electrophoretically transferred to polyvinylidene difluoride filter membrane. To reduce non-specific binding, the membrane was blocked for 2 hours at room temperature with 5% non-fat milk in PBS

(137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄) containing 0.1% Tween 20, incubated overnight at 4 °C with primary antibodies in PBS-Tween buffer, washed three times with PBS-Tween buffer, and then the membrane was incubated with a suitable secondary antibody coupled to horseradish peroxidase for 60 minutes at room temperature. The blots were then washed three times in PBS-Tween buffer and subsequently visualized with an enhanced chemiluminescence detection system (Amersham) and exposed to an X-ray film (Fuji Photo Film). The intensity of total protein bands per lane was evaluated by densitometry. Negligible loading/transfer variation was noted between samples. Moreover, beta-actin was used as loading control. Commercially available and well-characterized anti-endothelin-1 rabbit IgG affinity purified polyclonal antibody was used in the present study (IBL, 18201) and this antibody has been shown to have cross reactivity with many species including bovine, canine, human, mouse, porcine, rat and others. Its cross-reactivity of other endothelin family is 1.0% to endothelin-2, 0.4% to endothelin-3, 100.0% to big endothelin-1, 0.2% to big-endothelin-2, and 0.4% to big endothelin-3. For detecting endothelin B receptor, we used rabbit polyclonal anti-endothelin B (ETB) receptor antibody C-terminal which has already been proven effective for immunoblotting (Alomone Labs, Jerusalem, Israel).

Histological analysis

The right lung was removed and inflated with 4% formaldehyde to a pressure of 20 cmH₂O via trachea, and then fixed in 4% formaldehyde for over 24 hours. Subsequently the lungs were divided into 4 sections and each section was stained with hematoxylin-eosin. Researchers unaware and blinded to the nature and characteristics of the sample examined the hematoxylin-eosin stained slides microscopically using a quantitative evaluation system. Lung injury scores were performed, based on a thorough histopathological evaluation, including interstitial edematous alterations, inflammatory details, hyaline membrane injury/degeneration and the extent/severity of bronchiolar injury (0 = not present, 4 = severe and present throughout), as described previously (Suh et al., 2002). Total lung injury score was calculated by summing each score obtained from the 4 evaluation categories, described above.

Statistical analysis

Intergroup differences were compared by one-way ANOVA, and the concentrations of ET-1 and lung injury score were analyzed by Kruskal-Wallis test. Parametric data were expressed as mean ± SD, whereas non-parametric data were expressed as median (interquartile range). The effects of time course on gas exchange variables in four groups were analyzed with repeated measures ANOVA with Bonferroni's correction for multiple comparisons. All tests were performed using IBM-SPSS version 21.0 software (IBM-SPSS Inc., Chicago, IL, USA).

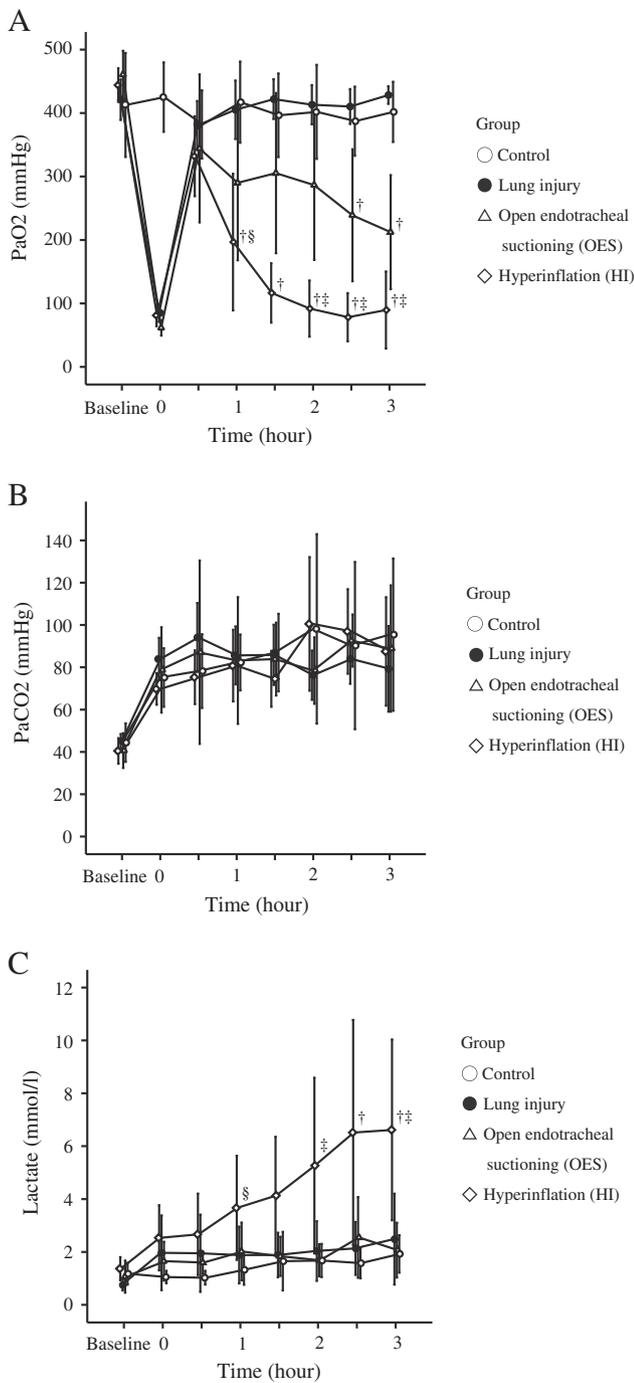


Fig. 1. Sequential transition of blood gas analysis; A) PaO₂, B) PaCO₂ and C) lactate in control group (without lavage; open circle), lung injury group only (without OES and HI; closed circle); OES group (without HI; open triangle); and OES + HI group (open rhombus). OES = open endotracheal suctioning; HI = hyperinflation. †*p* < 0.05 vs. lung injury group; ‡*p* < 0.05 vs. OES group; §*p* < 0.05 vs. previous adjacent time point in the same group.

Results

Baseline characteristics of experimental animals

No differences were observed in the baseline characteristics among the rabbits, with respect to body weight (Table 1). No differences were found in the following parameters, prior to performing lung lavages: pH, PaO₂, PaCO₂, hemodynamics variables and lung mechanics.

Gas exchange

In three groups treated with saline lavage, PaO₂ levels dropped below 100 mmHg after inducing lung injury but increased over 300 mmHg after PEEP levels increased to 10 cmH₂O (Fig. 1A). PaO₂ level stabilized at about 400 mmHg in the lung injury-induced group for the duration of the present study, while it decreased continuously in both the OES and HI groups, particularly in the HI group (Fig. 1A). The levels of PaO₂ at 3 hours were found to be at its lowest in the HI group (*p* = 0.001). In contrast to PaO₂, levels of PaCO₂ did not show any significant difference at 3 hours among all the study groups and did not display any distinct time-dependent change (Fig. 1B). In addition, HI group had further elevated levels of lactate compared to lung injury group and OES group without HI (Fig. 1C).

Hemodynamic variables and respiratory mechanics (Table 2)

No significant differences were observed in the following hemodynamic and respiratory variables among the four groups of experimental animals: heart rate (HR), mean arterial pressure (MAP), pH, respiratory rate (RR) and peak inspiratory pressure (PIP).

Plasma and pulmonary ET-1 level

There was no significant difference observed in the levels of plasma ET-1 among four experimental groups at baseline (Fig. 2A) before lavaging (*p* = 0.988). After induction of lung injury but before the start of OES and HI interventions, plasma ET-1 levels of the three groups treated with saline lavage were elevated significantly (median, interquartile range) (lung injury group; 1.84 (1.59–2.16) pg/mL, OES group; 1.74 (1.59–1.93) pg/mL, HI group; 1.81 (1.43–1.93) pg/mL) compared to the healthy control group (no lung injury); 0.94 (0.81–1.20) pg/mL (Fig. 2B). In contrast, the plasma concentration of ET-1 decreased in both OES (1.86 (1.62–2.13) pg/mL) and HI (1.74 (1.53–2.38) pg/mL) groups at the end of 3 hours (after the completion of OES and HI interventions), compared to the lung injury group without OES and HI (Fig. 2C). ET-1 levels in the lungs were significantly higher in the three groups induced with lung injury compared to the control group, consistent with levels of the plasma ET-1 (Fig. 3A). However, there was no significant change in the ET-1 levels of lung tissues in the OES (6.70 (5.65–9.42) pg/mg) and HI groups (8.30 (5.56–11.1) pg/mg) compared to the lung injury-induced group without OES and HI (7.14 (5.34–9.09) pg/mg, Fig. 3A). The pattern of expression in pulmonary ET-1 levels was also supported and complemented by the data from immunoblotting studies (Fig. 3B) and the molecular weight of band was same as observed in previous study (Yuan et al., 2013).

Broncho alveolar lavage fluid (BALF) ET-1 level

The concentration of ET-1 in BALF was also found to be significantly higher in the three experimental groups treated with saline lavage and induced with lung injury (lung injury group; 2.22 (2.10–2.62) pg/mL, OES group; 2.07 (1.57–2.62) pg/mL, HI group; 2.46 (1.72–3.45) pg/mL) compared to the control group (0.63 (0.56–0.76) pg/mL, Fig. 4A).

ETB receptor expression level in lung tissues

As shown in Fig. 4B, contrary to the data of pulmonary ET-1 levels in lung tissues, the protein expression level of pulmonary ETB receptor was significantly decreased in lung injury group (*p* = 0.001) compared to control group in the present experimental setting. Repeated OES caused further downregulation in pulmonary ETB receptor expression in lung injury animals compared to the lung injury group without undergoing OES (*p* = 0.001); further downregulation trend in ETB receptor protein expression was still continued in OES group receiving HI but

Table 2
Sequential changes of hemodynamics and pulmonary parameters of experimental animals.

	Baseline	0 h	1 h	2 h	3 h
HR (bpm)					
Control	247 ± 23.3	270 ± 23.5	253 ± 20.9	240 ± 17.5	246 ± 21.9
Lung injury	278 ± 19.1	235 ± 19.2	244 ± 17.0	273 ± 14.3	244 ± 17.8
OES	232 ± 19.0	216 ± 19.2	211 ± 17.1	236 ± 14.3	223 ± 17.8
HI	245 ± 19.1	193 ± 19.2	210 ± 17.0	212 ± 14.3	192 ± 17.8
MAP (mmHg)					
Control	127 ± 6.82	106 ± 6.58	113 ± 7.21	105 ± 7.34	95.5 ± 11.2
Lung injury	126 ± 5.57	110 ± 5.37	104 ± 5.89	93.6 ± 5.99	103 ± 9.19
OES	115 ± 5.57	100 ± 5.37	103 ± 5.89	104 ± 5.99	99.7 ± 9.19
HI	113 ± 5.57	113 ± 5.37	112 ± 5.89	93.5 ± 5.99	83.4 ± 9.19
pH					
Control	7.40 ± 0.03	7.26 ± 0.05	7.17 ± 0.05	7.19 ± 0.04	7.19 ± 0.03
Lung injury	7.41 ± 0.03	7.14 ± 0.04 ^a	7.13 ± 0.03	7.19 ± 0.03	7.13 ± 0.03
OES	7.42 ± 0.03	7.18 ± 0.04 ^a	7.18 ± 0.03	7.15 ± 0.03	7.14 ± 0.03
HI	7.43 ± 0.03	7.21 ± 0.04 ^a	7.15 ± 0.03	7.10 ± 0.03	7.07 ± 0.03
RR (times/min)					
Control	23.0 ± 2.66	30.0 ± 3.83 ^a	31.5 ± 4.21	40.5 ± 4.66	39.8 ± 5.24
Lung injury	21.6 ± 1.88	33.7 ± 2.71 ^a	36.4 ± 2.98	35.0 ± 3.30	31.8 ± 3.71
OES	23.4 ± 1.88	36.8 ± 2.71 ^a	38.0 ± 2.98	39.6 ± 3.30	40.5 ± 3.71 ^a
HI	24.3 ± 1.88	35.2 ± 2.71	36.6 ± 2.98	39.5 ± 3.29	42.3 ± 3.71 ^a
PIP (cmH ₂ O)					
Control	12.8 ± 0.75	17.3 ± 1.22 ^a	18.5 ± 0.98	19.7 ± 1.09	19.2 ± 1.16
Lung injury	12.8 ± 0.61	20.8 ± 0.99 ^a	21.6 ± 0.80	22.3 ± 0.89	20.1 ± 0.94
OES	13.6 ± 0.61	19.1 ± 0.99 ^a	20.8 ± 0.80	21.3 ± 0.89	22.0 ± 0.94
HI	12.6 ± 0.61	20.1 ± 0.99 ^a	21.0 ± 0.80	22.3 ± 0.89	23.5 ± 0.94

HR, heart rate; MAP, mean arterial blood pressure; RR, respiratory rate; PIP, peak inspiratory pressure; OES, open endotracheal suctioning; HI, hyperinflation. Control group (without lavage), lung injury group only (without OES and HI), OES group (without HI) and HI group (OES + HI). All values were mean ± SD.

^a $p < 0.05$ vs. the previous value in same group.

this downward trend did not reach at the statistically significant level ($p = 0.673$).

Histological analysis

Fig. 5A shows the representative lung images taken from the experimental groups, with the summary of quantitative evaluation. Histological analysis of lung tissue revealed some morphological changes, i.e., compared to the control group without saline lavage (1.0 (1.0–2.0)), all the other three groups induced with lung injury (2.5 (2.0–4.5)), irrespective of intervention by OES (5.0 (3.0–6.0)) or both (HI group; 7.0 (4.0–8.0)), demonstrated a significant and progressively worsening total lung injury score by 3 hours (Fig. 5B). This implies that our current experimental model had significant lung injury (Fig. 5B). OES caused further morphological damage to the lungs, as was evident from the total lung injury score (Fig. 5B). The score of HI group was the worst among the four groups of animals investigated in current study ($p = 0.001$, Fig. 5B).

Discussion

This is the first study demonstrating aberrations in levels of plasma and pulmonary ET-1 in a rabbit model of lung injury induced by saline lavage with surfactant depletion. Further, we demonstrate here the effects of repeated OES and HI on ET-1 levels using the same model. Specifically, we show that: a) saline lavage-induced lung injury model rabbits have significantly higher levels of both circulatory and pulmonary level of ET-1 compared to the control group without lavage; b) circulatory levels of ET-1 are down-regulated in OES and HI groups compared to the lung injury group without OES and HI, and; c) pulmonary ET-1 levels are unchanged in OES and HI groups compared to the group of lung injury without OES and HI. These changes in the OES and HI groups did not correlate with changes in blood pressure of the lung injury model. However, the lung injury scores were more pronounced in OES and HI groups that also had progressively worsening conditions in arterial oxygenation compared to the animals induced with lung injury but without OES and HI.

In the current study, we found that at approximately 3 hours after saline lavage, the pattern of arterial PaO₂ in lung injury group was found to be altered and decreased to less than 100 mmHg. Thus, the current model could be a good model for lung injury/ARDS since the blood-oxygenized index (PaO₂/F_IO₂) in the present study, did not get higher than 200 mmHg, which correlates with the clinical criteria of ARDS. Thus, based on both model validation and duration of experimental protocol (3 hours), we were able to generate a lung injury model that closely resemble or simulate clinical ARDS, thus fulfilling the clinical criteria of the ARDS guidelines. In addition, besides the decrease of PaO₂ in the lung injury model (Fig. 1A), we were also able to demonstrate or reveal a significant presence of lung injury using histological analysis (Fig. 5B). Also, we uncovered significant elevated levels of circulatory and pulmonary tumor necrosis factor (TNF)-α in the current lung injury model compared to those of the healthy control group without saline lavage (Kamiyama et al., unpublished observation, 2013).

The current data showing elevated levels of circulatory and pulmonary ET-1 levels in saline lavage-induced lung injury model complement data from the earlier report by Nakano et al. (2007), that showed increased production of lung ET-1 in acute lung diseases, such as hypoxia-induced respiratory failure or ALI/ARDS. In addition, serum and pulmonary levels of ET-1 were reported to increase in large tidal volume compared with small tidal volume, as well as in a canine ARDS-like model induced by oleic acid (Lai et al., 2010) and a rat ALI-like model induced by intravenous infusion of lipopolysaccharide (Pan et al., 2012). It is worth noting that although the methods of generating the lung injury models are different between the current study and the earlier studies (Lai et al., 2010), most of the studies reported higher levels of circulatory and pulmonary ET-1 level in lung injury/ARDS models. More specifically, our current data are consistent with findings of other earlier studies (Deja et al., 2004) that used the saline lavage-induced surfactant-depleted ARDS model, which has been well characterized and a frequently used experimental model. Specifically, Deja et al. (2004) showed that inhalation of the ET_A receptor antagonist prevented hypoxigenation and increased intrapulmonary shunt after saline lavage. Thus as evident from present study and also from other studies, in lavage-induced surfactant depleted ARDS/lung injury models there was a significant alteration of circulatory and pulmonary ET-1

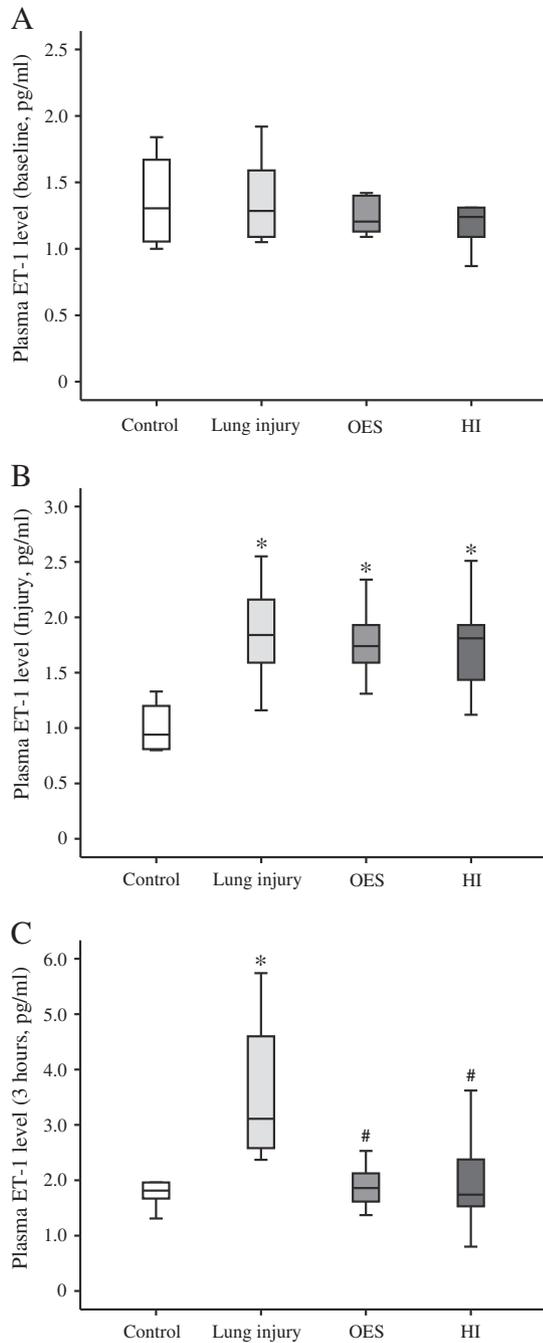


Fig. 2. The expression of circulatory ET-1 level by ELISA; A) baseline (prior to the lavage induction), B) just after the generation of lung injury (after lavage) and C) at the end of 3 hours experimental protocol with OES and HI, in four experimental groups namely control group (without lavage), lung injury group only (without OES and HI), and OES group (without HI) and HI (OES + HI) group. OES = open endotracheal suctioning; HI = hyperinflation. The end of boxes indicates the 25th and 75th percentiles, and the line in the bar indicates the median value. **p* < 0.05 vs. control group; #*p* < 0.05 vs. lung injury group.

levels compared to the healthy control subjects. In other words, it can be stated that in a lung injury model (induced by saline lavage) simulating the criteria and conditions of human and experimental ARDS, ET-1 level was upregulated in both lung tissues, BALF samples and at plasma levels. In contrast, previous study by Wenz et al. (2000) demonstrated that acute change of oxygenation did not induce short-term effect on pulmonary ET-1 in ARDS subject (Wenz et al., 2000). Thus, more studies will be required here to explore the specific mechanism and effects of ET-1 involvement in lavage-induced surfactant depleted lung injury model.

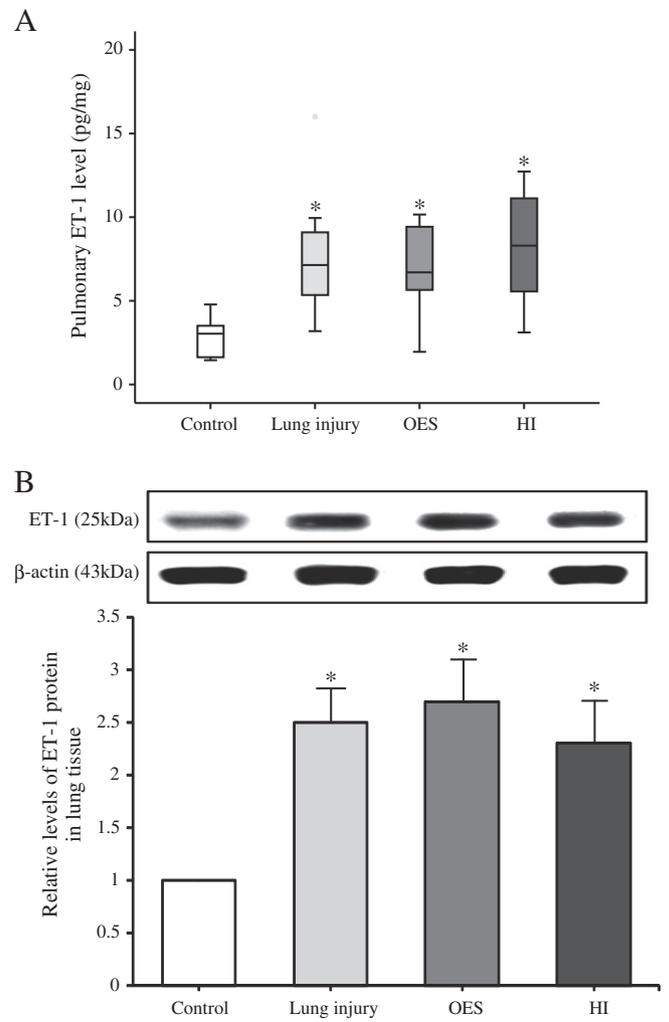


Fig. 3. (A) The expression of pulmonary ET-1 level by ELISA, and (B) relative levels of ET-1 in lung tissue as demonstrated by immunoblotting from control group (without lavage), lung injury group only (without OES and HI), OES group (without HI) and HI (OES + HI) group at the end of 3 hours experimental protocol since the generation of lung injury model. OES = open endotracheal suctioning; HI = hyperinflation. (A) The end of boxes indicates the 25th and 75th percentiles, and the line in the bar indicates the median value. (B) For immunoblot analysis, in each of the experiments, the band obtained in the control group is normalized to 1.0. The values are expressed as means ± SD. **p* < 0.05 vs. control group.

The most important finding of the present study is the effects of OES and HI on the pulmonary levels of ET-1 in lung injury animals undergoing mechanical ventilation. Performance of OES and HI failed to further up-regulate levels of the pulmonary ET-1 in lung injury-induced animals, based on the present experimental settings. While ET-1 seems to be involved in the pathogenesis of lung injury, as reported previously (Filip et al., 1991; Horgan et al., 1991; Jesmin et al., 2011), OES and HI could not aggravate levels of pulmonary ET-1 in the current lung injury model. On the other hand, both the levels of arterial desaturation and lung injury score worsened in the lung injury animals after repeated OES and HI (Figs. 1A and 5B). In several previous studies, it has been shown that OES worsens hypoxxygenation and hemodynamic instability (Suh et al., 2002; Carames et al., 2006; Maggiore et al., 2003; Johnson et al., 1994) in ARDS subjects, consistent with the current observations. However, there is lack of evidence on the effects of OES on pulmonary ET-1 level, although ET-1 has been shown to be potentially associated with hypoxxygenation and hemodynamic change (Comellas and Briva, 2009; Kohan, 2013). We assumed that OES and HI would cause further aggravation in pulmonary ET-1 level in current study design. The cause of the deterioration of oxygenation and lung injury in HI group might be

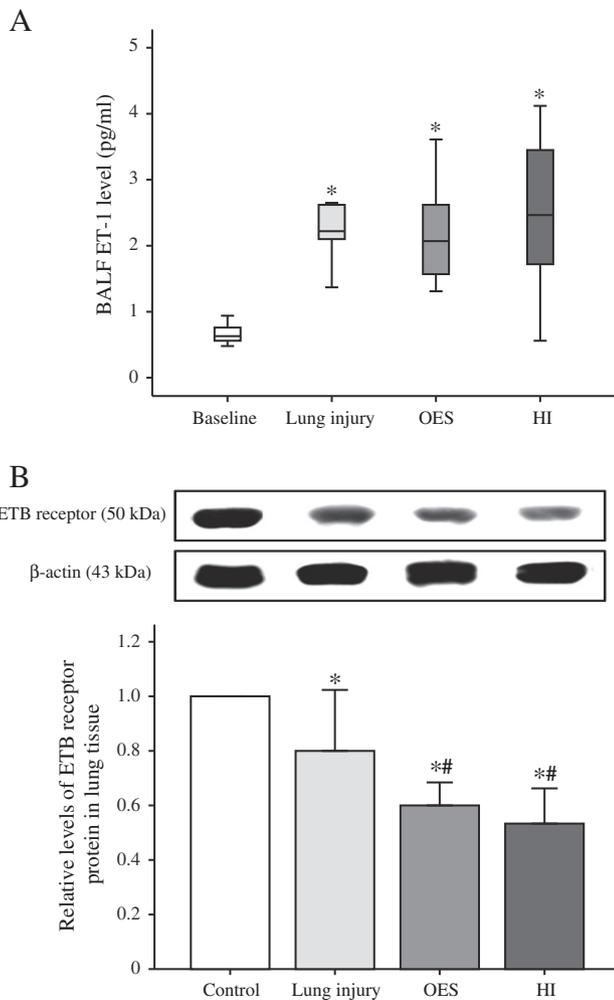


Fig. 4. (A) The expression of bronchoalveolar lavage fluid (BALF) ET-1 level by ELISA and (B) relative levels of ETB receptor in lung tissue as demonstrated by immunoblotting from control group (without lavage), lung injury group only (without OES and HI), OES group (without HI) and HI (OES + HI) group at the end of 3 hours experimental protocol since the generation of lung injury model. OES = open endotracheal suctioning; HI = hyperinflation. (A) The end of boxes indicates the 25th and 75th percentiles, and the line in the bar indicates the median value. (B) For immunoblot analysis, in each of the experiments, the band obtained in the control group is normalized to 1.0. The values are expressed as means \pm SD. * $p < 0.05$ vs. control group; # $p < 0.05$ vs. lung injury group.

due to an increase in lung volume and the repeated cyclic recruitment and de-recruitment. In the previous study, plasma and pulmonary levels of ET-1 were reported to increase in large tidal volume compared with small tidal volume in ARDS subjects (Lai et al., 2010; Pan et al., 2012). Thus, if there was an increase in lung volume after HI, plasma and pulmonary levels of ET-1 could be elevated in HI group. The discrepancies between the present data with these earlier reports for now cannot be convincingly explained here. In fact, in our most recent observation (Kamiyama et al., unpublished observation, 2013), using the same model and time points as in the present study, we have revealed further up regulation of serum TNF- α in lung injury model after repeated OES and HI. Based on this fact, it can be extrapolated from the current data that pulmonary ET-1 level may not be affected by OES and HI in lung injury subjects undergoing mechanical ventilation at the current setting. The cause of reduction of oxygenation after HI with OES may not be vasoconstriction, but lung injury itself because we did not observe any change in hemodynamic variable and ET-1 levels after HI. A longer time frame of investigation in future study designs may be essential in order to develop much clearer conclusions on this issue.

Another novel finding of the present study is the down regulation of plasma ET-1 level in the lung injury animal by OES and HI. However, the

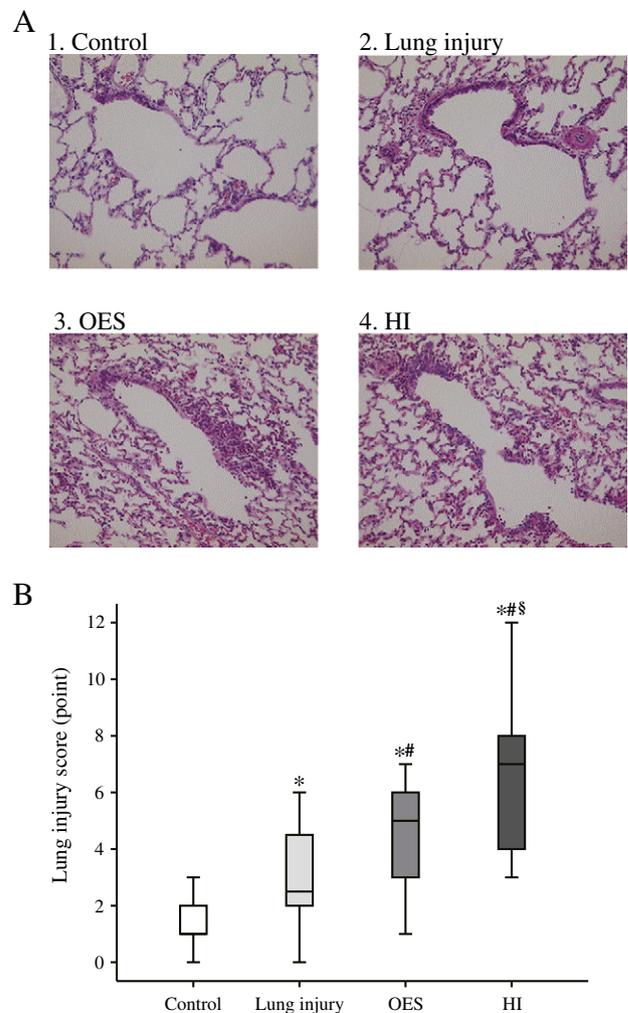


Fig. 5. (A) Representative images of lung morphology from hematoxylin–eosin staining and (B) quantitative analysis of lung injury score, obtained from control group (without lavage), lung injury group only (without OES and HI), OES group (without HI) and HI group (OES + HI). OES = open endotracheal suctioning; HI = hyperinflation. The end of boxes indicates the 25th and 75th percentiles, and the line in the bar indicates the median value. * $p < 0.05$ vs. control group; # $p < 0.05$ vs. lung injury group; § $p < 0.05$ vs. OES group.

underlying mechanism of this observed change cannot currently be explained. In addition, the clinical impact of the observed change in levels of circulatory ET-1 by OES and HI in the present study cannot be fully interpreted, thus warranting detailed investigations in the future. The differential effects of OES and HI on circulatory and pulmonary ET-1 levels in the present study have led us to investigate the pulmonary expression pattern of ETB receptor, generally known as the receptor of vasodilation and clearance for ET-1, in the present study. Consistent to our speculation, we found the inverse expression pattern of ETB receptor in lung tissues in current lung injury model compared to the expression level of circulatory and pulmonary ET-1. Pulmonary ETB receptor expression was downregulated in lung injury models in the current study compared to that of control animals. In fact, lung ETB receptor is important to determine the circulating level of ET-1, because it works as a clearance receptor for ET-1 (Fukuroda et al., 1994). Also, lung is an important organ for ET-1 clearance (Fukuroda et al., 1994). Indeed, consistent with a major role for ETB in the clearance of ET-1, selective ETB antagonist increases plasma ET-1 concentration. Furthermore, ETB genetic mutation models have increased plasma ET-1 level (Garipey et al., 2000). In addition, increased levels of ET-1 and endothelin-A (ETA) and decreased ETB expressions in the lungs have been suggested to be involved in the lung dysfunction of broiler chickens with

developmental ascites (Gao et al., 2013). In the present study, we cannot explain the cause and effects of inverse expression pattern of circulatory and pulmonary ET-1 and pulmonary ETB in lung injury models compared to control animals. In addition, consistent to our speculation, we found further downregulation of pulmonary ETB receptor expression in lung injury models undergoing OES and HI compared to the lung injury animals without OES and HI in the present study. Thus, from the present study we state that OES and HI differentially affect circulatory and pulmonary ET-1 level as well as pulmonary ETB receptor expression in lung injury subjects. At moment, we have no adequate explanation underlying this observed differential expression pattern of ET-1 and ETB receptor induced by OES and HI in lung injury models. Future studies are highly required to explore and clarify the functional and clinical consequences as well as mechanistic insights underlying the differential expression patterns of ET-1 and ETB receptor induced by OES and HI in lung injury subjects as found in the present study.

Throughout the experiment, no significant change in blood pressure was observed, although circulatory levels of ET-1 were increased in animals with lung injury animals but later down-regulated in the OES and HI groups. Because ET-1 is a potent vasoconstrictor that has been implicated in the pathogenesis of hypertension (Yanagisawa et al., 1988), many studies have been able to show the relationship between blood pressure and ET-1 change. For now, the mechanisms underlying the selective and tissue-specific decrease in ET-1 levels in plasma but not pulmonary tissues as observed here remains unclear. One would expect that if levels of plasma ET-1 had already been sequestered into the lung by the third hour post-induction, pulmonary ET-1 levels would have been elevated. However, we did not observe any significant changes in ET-1 levels in the pulmonary tissue. Thus, the findings of the current study seem to be based on blood pressure independent mechanisms.

Mechanical ventilation is basically an invasive process (Chiumello et al., 1999; Ranieri et al., 1999; Rothen et al., 1998), and several parameters related to this process may induce or enhance the magnitude of lung injury, in addition to an increase in pulmonary and systematic inflammatory responses (Wheeler and Bernard, 2007). We also know that hyperventilation and factors such as recruitment maneuver, can also lead to deterioration of hemodynamic instability (Moran et al., 2011) in ARDS. If these entire factors are aligned, the magnitude of microvascular endothelial cell impairment in the lung or in the whole body may be exacerbated. It is therefore important that future studies focus on understanding the more specific roles of ET-1 in the current ARDS model, which clinically closely simulate human ARDS. In addition, studies with much longer study duration might be required in future. Another drawback of the present study is that we could not here present the actual Pplat, Crs and tidal volume applied during recruitment. Thus, the effects of HI on these parameters should also be determined using appropriate, specific devices and technologies in future studies. The current experimental design can be also utilized in other established ARDS/lung injury models to further validate the current experimental results. Lastly, a detailed evaluation of pulmonary microcirculation, as well as the pattern in expression of downstream signaling factors of ET-1 system should also be assessed.

Conclusion

In conclusion, we report that ET-1 levels of both the circulatory and pulmonary tissues were found to be elevated in a saline lavage-induced surfactant-depleted lung injury model undergoing mechanical ventilation. OES and HI did not lead to further up-regulation of ET-1 in the pulmonary tissues of the current model. However, lung injury severity and arterial desaturation was exacerbated after OES and HI.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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