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Biomedicine & Pharmacotherapy 118 (2019) 109344

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

Partial liquid ventilation–induced mild hypothermia improves the lung function and alleviates the inflammatory response during acute respiratory distress syndrome in canines

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ARTICLE INFO

Keywords:
Acute respiratory distress syndrome
Perfluorocarbon
Partial liquid ventilation
Mild hypothermia
Inflammatory factors

ABSTRACT

Background Acute respiratory distress syndrome (ARDS), which is the severest form of pulmonary injury, is the leading cause of death in critical care. At present, the mortality remains high in ARDS. Partial liquid ventilation (PLV) using perfluorocarbon (PFC) has been proven to improve gas exchange and respiratory dynamics of the lungs during ARDS. However, PLV has not been shown to reduce the mortality of ARDS. Some studies have shown that mild hypothermia therapy can reduce lung injuries in animal models of ARDS by reducing inflammatory cytokine levels in lung tissues. However, hypothermia cannot produce a lung protection effect alone, and it may have a synergistic effect with other protective measures. To explore the possible role of PLV combined with mild hypothermia in the treatment of ARDS, in this study, we used PFC liquid ventilation to induce mild hypothermia in dogs suffering from ARDS and analyzed the effects of PFC liquid ventilation-induced mild hypothermia on the levels of inflammatory factors and lung histopathology in dogs with ARDS. The experimental dogs were randomly divided into conventional mechanical ventilation (CMV), normal temperature PFC liquid ventilation (NPLV), hypothermic PFC liquid ventilation (HPLV), and mechanical ventilation (MV) groups. After induction of ARDS, the CMV group was treated with CMV for respiratory support, the HPLV group was treated with PLV-induced mild hypothermia using 15 °C PFC and maintained the rectal temperature at 34–36 °C, the NPLV group was treated with PLV using 36 °C PFC and maintained the rectal temperature at 36–38 °C. The MV group served as the control group. Analyses of the pulmonary pathology, partial pressure of oxygen in the blood, and lung wet-dry weight ratio (W/T) of each dog revealed that PLV-induced mild hypothermia significantly increased the PaO_2 values and attenuated lung injury, and there were no adverse effects on hemodynamics. Furthermore, treatment with PLV-induced mild hypothermia significantly increased the expression of the anti-inflammatory factor IL-10 in bronchoalveolar lavage fluid (BALF) and attenuated the expression of interleukin (IL-6) and tumor necrosis factor-α (TNF-α) in peripheral blood and in lung BALF. Moreover, the results showed that the expression of myeloperoxidase (MPO) and NF-κB p65 in lung tissues was significantly decreased by PLV-induced mild hypothermia compared with NPLV and CMV. Our results indicated that PLV combined with mild hypothermia can provide protection against oleic acid-induced ARDS in dogs.

1. Introduction

Acute lung injury/acute respiratory distress syndrome (ALI/ARDS), first described by Ashbaugh in 1967 [1], presents as diffuse alveolar-capillary membrane damage caused by severe infection, trauma, shock, acidosis, and various intrapulmonary and external pathogenic factors and is characterized by progressive respiratory failure and refractory hypoxemia [2]. ARDS is usually accompanied by diffuse alveolar inflammation and alveolar interstitial edema syndrome, and its mortality is still greater than 40% [3]. The traditional treatment of ARDS is comprehensive therapy based on mechanical ventilation (MV). Partial liquid ventilation (PLV) with perfluorocarbons (PFCs) is a novel therapeutic strategy in the treatment of ARDS in recent years. This technique of combined liquid-gas ventilation is involves the intratracheal instillation of PFC in volumes up the functional capacity of the lung during conventional mechanical ventilation (CMV). A large number of studies [4,5] have demonstrated that PFC-mediated PLV can significantly improve the gas exchange function and respiratory dynamics

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https://doi.org/10.1016/j.biopha.2019.109344
Received 3 May 2019; Received in revised form 2 August 2019; Accepted 7 August 2019

of lungs during ARDS. However, PLV has not been shown to reduce the mortality of ARDS. Mild hypothermia has an organ protective effect by delaying proinflammatory cytokine production in human peripheral blood mononuclear cells [6]. Some studies have shown that mild hypothermia therapy can reduce lung injuries caused by ventilators in animal models of ARDS [7]. However, hypothermia cannot produce a lung protection effect alone, and it may have a synergistic effect with other protective measures. It is established that the lungs are not only a place for gas/blood exchange but also a site for heat exchange. A good cooling effect can be achieved through the lungs [8,9]. A novel method using cold fluorocarbon PLV-induced hypothermia is convenient and feasible and may produce a synergistic effect. We induced mild hypothermia by using cold PFC-mediated PLV to explore the effect of fluorocarbon PLV combined with mild hypothermia on an ARDS model. However, the mechanisms by which PLV-induced mild hypothermia influences inflammatory regulators and pulmonary histopathology have not been clarified. Therefore, in this study, we investigated the effect of PLV combined with mild hypothermia on the inflammatory factors and pulmonary histopathology in dogs with ARDS. Our results demonstrated that PFC-mediated PLV combined with mild hypothermia could significantly improve gas exchange, attenuate inflammatory reactions, and relieve oleic acid-induced ARDS.

2. Materials and methods

2.1. Experimental animals and treatment

This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Nanchang University and followed the guidelines for the protection and use of laboratory animals of Nanchang University. A total of 36 healthy, male, mongrel dogs, weighing from 9.5 to 11.8 kg, with an average weight of 10.15 ± 0.89 kg were provided by the Animal Department of Nanchang University Medical College and were maintained in a normal environment. Animals were weighed and intramuscularly injected with 10 mg/kg ketamine hydrochloride and 0.4 mg/kg midazolam to achieve general anesthesia. Each dog was placed in the supine position on a bench; an ID 7.0 mm endotracheal tube was placed, and the dog received MV using a ventilator (PB840 ventilator, Puritan-Bennett, USA). During the experiment, 0.2–0.3 $mg{\cdot}kg^{-1}{\cdot}h^{-1}$ intravenous midazolam and 0.1–0.2 $mg{\cdot}kg^{-1}{\cdot}h^{-1}$ vecuronium were used to maintain anesthesia and as a muscle relaxant, respectively, and the dogs received intravenous infusions of 5–10 $ml{\cdot}kg^{-1}{\cdot}h^{-1}$ Ringer's lactate solution. Heart rates were continuously monitored by an ECG attached to the left and right forelimbs of the animals and an intimate electrode attached to the left hind limb (MP20 multifunction monitor, Philips, the Netherlands). The internal jugular vein was separated, and a Swan-Ganz catheter (Edward, USA) was inserted into the jugular vein. The room temperature was controlled at 22 °C (average of 22 ± 0.5 °C).

2.2. Preparation of animal models

The preparation of the oleic acid-induced ARDS animal model was based on the method described by Nakazawa et al [10]. High purity, 0.07–0.12 ml/kg oleic acid (Sigma, USA) was uniformly infused through the right jugular vein over a 20-minute period, with samples taken 3 times. The blood gas parameters were analyzed every 20 min after the airway pressure began to increase. The detection of arterial blood gas parameters was repeated until the oxygenation index (PaO_2/FiO_2) was ≤ 200 mmHg (1 mmHg = 0.133 kPa). The preparation of the ARDS model was considered successful if the oxygenation index could be maintained at ≤ 200 mmHg for 30 min. If the blood gas parameters did not reach the above standard after 1 h, additional oleic acid was injected at 0.02 ml/kg. The average modeling time was 1.6 ± 0.6 h. The ARDS model was successfully achieved in 36 experimental dogs.

2.3. Experimental grouping and processing

Experimental animals were divided into 4 groups according to the random number table method, with 9 animals in each group. The animals were grouped as follows. 1) The CMV group: MV was supplied by the ventilator after successful ARDS modeling. Respiratory parameters: tidal volume (V_T): 8 ml/kg; respiratory rate (f): 20 bpm; oxygen concentration (FiO_2): 0.8; inspiratory to expiratory ratio (I:E): 1:2; positive end-expiratory pressure (PEEP): 5 cmH_2O (1 cmH_2O = 0.098 kPa). The experimental bench was covered with a heating blanket. The dog's rectal temperature was maintained at 36.5–37.9 °C. 2) The normal temperature PFC liquid ventilation (NPLV) group: after the ARDS model was successfully achieved, mechanical ventilation was provided, and 10–15 ml/kg PFC at a normal temperature of 36 °C was slowly injected into the lung through a side hole in the tracheal intubation. The ventilator parameter settings and temperature were the same as those for the CMV group. 3) The hypothermic PFC liquid ventilation (HPLV) group: after successful ARDS modeling, each dog was provided with MV, and PLV was performed using 10–15 ml/kg 10 °C PFC injected through the tracheal tube to reduce the core temperature of the dog. The rectal temperature was maintained at 34–36 °C using 15 °C PFC ventilation. 4) The MV group was used as the control group. After anesthesia, the same amount of saline was injected through the jugular veins of all dogs, and all dogs received ventilation. The ventilator parameters for all dogs were set to those described for the CMV group.

2.4. Data collection and specimen retrieval

The following time points were used for data collection: after anesthesia was stabilized for 0.5 h (T0), when successful ARDS modeling was achieved (T1), and at 1 h (T2), 2 h (T3), 3 h (T4), and 4 h (T5) after successful modeling was achieved. The MV group was also sampled at corresponding time points. At each time point, 0.5 ml of blood from the femoral artery was sampled for blood gas analysis using a GEM premier 3500 blood gas analyzer (GEM, USA). An electronic temperature probe was inserted 6 cm through the anus to monitor the rectal temperature. Cardiac output (CO) was measured at T1-T5 by using a Swan-Ganz catheter. When measuring CO, 10 mL of ice-cold saline (4 °C) was injected within 4 s. The measurement was repeated 3 times, the average value was calculated, and heparinized anticoagulation was performed. The pressure sensor was connected to a multifunction monitor to continuously measure arterial pressure and mean arterial pressure, and the heart rate was measured with the multifunction monitor. At T0, T1, T3, and T5, 5 ml of peripheral venous blood was sampled. The sample was centrifuged at 2000 rpm for 10 min, and the supernatant was stored at -80 °C. An enzyme-linked immunosorbent assay (ELISA) was used to measure the concentrations of IL-6 and TNF-α in peripheral venous blood supernatants. At T5, animals were sacrificed by excessive anesthesia, the thoracic cavity was quickly opened, and the right bronchus was ligated. The left lung was lavaged 3 times with 5 ml/kg normal saline using bronchoalveolar lavage, and bronchoalveolar lavage fluid (BALF) was collected and centrifuged. The supernatant was stored at −20 °C, and the concentrations of TNF-α, IL-6 and IL-10 in BALF were measured by ELISA (Abcam, UK). Right middle lobe tissue was collected, and 1 g of tissue was rinsed with normal saline and dried with filter paper. The wet weight was determined using an electronic analyzer. The dry weight was obtained after drying in an 80 °C electric oven for 72 h until a constant weight was achieved. The wet weight/dry weight ratio (W/D ratio) of the lung tissue was then calculated. The right lower lobe tissue was removed and stored at -80 °C after removing surface blood and water. The specimens were fixed with 10% formalin, embedded in paraffin, and sliced. Hematoxylin and eosin staining and light microscopy were performed on the slices. The reference method [11] was used to estimate the degree of intra-alveolar hemorrhage, intra-alveolar edema, interstitial edema, pulmonary interstitial hyperemia, neutrophil infiltration, and hyaline membrane thickness. Each

field was scored as 0, 1, 2, 3, or 4, which corresponded to normal (no injury), mild (injury < 25% field of vision), moderate (injury < 50% field of vision), severe (injury < 75% field of vision), or extremely severe (injury ≥ 75% field of vision), respectively. Each slice was observed for 10 fields, and the cumulative average lung pathological injury score was calculated.

For determination of myeloperoxidase expression in lung tissue, after the dogs were sacrificed, the right lower lobe of the lung was isolated and snap-frozen. The expression of myeloperoxidase (MPO) in the lung was detected using an ELISA kit (Bluegene, China) according to the manufacturer's instructions. Briefly, the lung tissue was homogenized and centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatants were added into a microtiter plate (100 μl /well) precoated with a murine anti-MPO mAb, and then 10 μl balance buffer and 50 μl enzyme conjugate were added to each well. After incubation for 1 h at 37 °C, the plate was washed 5 times, followed by the addition of the substrate and stop solution. Optical density (OD) at 450 nm was measured using a microplate reader. All samples were assayed in triplicate.

The expression of NF-κB p65 in the lung was determined by immunostaining. After baking, deparaffinization, and rehydration, paraffin sections were placed into a pressure cooker containing antigen retrieval buffer (0.01 M citrate buffer, pH 6.0) and cooked with full pressure for 2 min to unmask antigens. Nonspecific blocking was performed for 20 min, and then, an antibody against NF-κB p65 was added (Boster-Bio, USA) and incubated overnight at 4 °C. Immunostaining was performed by incubating the sections with an NF-κB p65 biotin-conjugated secondary antibody (Boster-Bio, USA) at 37 °C for 1 h. PBS was used to fully elute all antibodies, and 3,3-diaminobenzidine (DAB, Bioson-Bio, China) was then used to visualize the immunohistochemical staining. Cell nuclei were counterstained with hematoxylin, slices were washed with hydrochloric acid, and alcohol dehydration was performed. Then, slice were sealed and subjected to microscopic examination and photography. Images were obtained with a microscope (Olympus BX51, Japan), and the proportion of positively stained cells was analyzed with Image-Pro plus 5.1 software. The expression of NF-κB p65 in lung tissue is presented as the mean optical density (MOD).

For histology and immunostaining analysis, the slides were renamed using Arabic numbers and subjected to a double-blinded examination by two pathologists.

2.5. Statistical analysis

Data were analyzed using Statistical Product and Service Solutions (SPSS) statistical software version 18.0 (SPSS Inc., USA) and are expressed as the mean ± standard deviations (SD). Comparisons between groups were performed using an independent sample *t*-test. Two-way ANOVA was used to compare repeated measures at different time points within each group. P values of less than 0.05 were considered statistically significant.

3. Results

3.1. Changes in rectal temperatures (°C) among the 4 experimental groups ($\bar{x} \pm s$, $N = 9$)

From T2–T5, the rectal temperature of the dogs in the HPLV group was 35 ± 1 °C, and that of the NPLV, MV and CMV groups was 37 ± 1 °C (Fig. 1).

3.2. Arterial blood gas analysis

PaO_2 is a measurement of the partial pressure of oxygen dissolved in plasma, which indicates how much oxygen is available in the alveoli to be dissolved in blood. We collected blood from the femoral artery and analyzed PaO_2 values. We found that the PaO_2 values in the CMV, NPLV, and HPLV groups were significantly reduced compared to that in the control group ($P < 0.05$) at the T1–T5 time points. However, the PLV-induced mild hypothermia treatment significantly increased the PaO_2 values in the HPLV group compared with those in the CMV group after 2, 3 and 4 h of ARDS modeling ($P < 0.05$) (Fig. 2). The PaO_2 values were significantly ($P < 0.05$) higher in the HPLV group than in the NPLV group at the T4 and T5 time points, which may be due to improvements in O_2 transport caused by PFC and reductions in oxygen consumption caused by mild hypothermia.

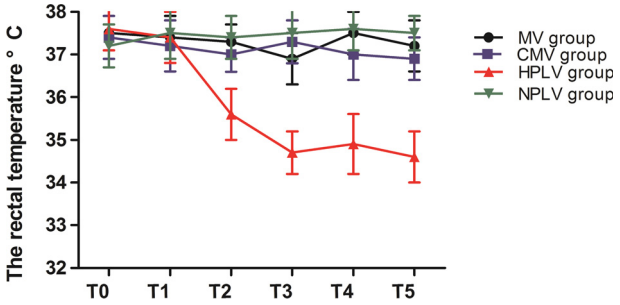

Fig. 1. Changes in rectal temperature among the 4 experimental groups ($\bar{x} \pm s$, $N = 9$).
Abbreviations: MV group, mechanical ventilation after anesthesia and an injection of normal saline; CMV group, mechanical ventilation after ARDS; HPLV group, hypothermic PFC liquid ventilation after ARDS; NPLV group, normal temperature PFC liquid ventilation after ARDS.

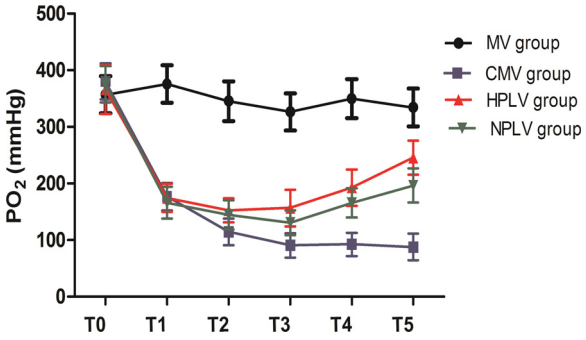

Fig. 2. Changes in the PaO_2 values (mmHg) among the 4 experimental groups ($\bar{x} \pm s$, $N = 9$).
Abbreviations: HPLV, hypothermic PFC liquid ventilation after ARDS; NPLV, normal temperature PFC liquid ventilation after ARDS; CMV, mechanical ventilation after ARDS; MV, mechanical ventilation after anesthesia and an injection of the same amount of normal saline.

3.3. Changes in hemodynamics between the experimental groups ($\bar{x} \pm s$, $N = 9$)

There was no significant difference in the heart rate (HR), mean arterial pressure (MAP) and cardiac output (CO) between the experimental groups at the time points of T0–T5 ($P > 0.05$) (Table 1)

The mechanical ventilation (MV) group was used as the control group. After anesthesia, the same amount of saline was injected through the jugular veins of all dogs. There was no significant difference in the HR, MAP and CO between the experimental groups at the time points of T0–T5 ($P > 0.05$).

3.4. Changes in the levels of IL-6 and TNF-α in peripheral blood samples

The serum concentrations of IL-6 and TNF-α in the CMV, NPLV and HPLV groups were increased at 2 (T3) and 4 h (T5) after ARDS, and their concentrations in the ARDS model groups were significantly ($P < 0.05$) higher than those in the MV group at the T3 and T5 timepoints (Fig. 3). The serum TNF-α concentration in the HPLV group at

Table 1
Effects of different temperatures of fluorocarbon PLV on hemodynamics in ARDS dogs ($\bar{x} \pm s$, N = 9).

Variables	Group	Before ARDS	ARDS	After ARDS(h)			
				1	2	3	4
HR (bpm)	HPLV group	156 ± 14	161 ± 15	156 ± 17	156 ± 15	154 ± 14	149 ± 17
	NPLV group	151 ± 17	162 ± 13	158 ± 16	154 ± 17	161 ± 16	151 ± 14
	CMV group	154 ± 15	160 ± 14	155 ± 15	152 ± 16	156 ± 14	154 ± 12
	MV group	153 ± 14	163 ± 13	157 ± 16	155 ± 16	155 ± 16	155 ± 13
MAP (mmHg)	HPLV group	107 ± 17	108 ± 15	101 ± 15	100 ± 13	101 ± 12	108 ± 15
	NPLV group	100 ± 16	99 ± 17	97 ± 18	105 ± 12	97 ± 18	104 ± 13
	CMV group	105 ± 15	101 ± 13	101 ± 14	99 ± 15	104 ± 14	100 ± 14
	MV group	103 ± 14	98 ± 15	102 ± 15	102 ± 14	101 ± 15	101 ± 15
CO ($L \cdot min^{-1}$)	HPLV group	3.7 ± 0.6	3.6 ± 0.7	3.6 ± 0.5	3.5 ± 0.8	3.5 ± 0.7	3.6 ± 0.5
	NPLV group	3.9 ± 0.4	3.7 ± 0.6	3.7 ± 0.4	3.6 ± 0.7	3.6 ± 0.6	3.7 ± 0.6
	CMV group	3.6 ± 0.6	3.5 ± 0.6	3.5 ± 0.6	3.5 ± 0.6	3.6 ± 0.5	3.6 ± 0.7
	MV group	3.8 ± 0.5	3.7 ± 0.5	3.6 ± 0.5	3.6 ± 0.6	3.7 ± 0.6	3.7 ± 0.5

Note: HR is the heart rate, MAP is the mean arterial pressure, and CO is the cardiac output; 1 mmHg = 0.133 kPa, 1 cmH_2O = 0.098 kPa.

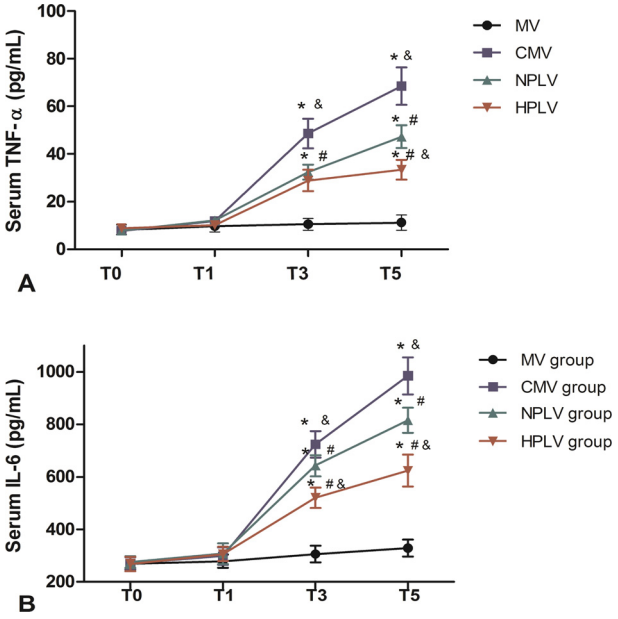

Fig. 3. Changes in the concentrations of IL-6 and TNF-α in peripheral blood samples.
The results are shown as the means ± SD (n = 9). $^{*}p < 0.05$ compared to the MV group; $^{\#}p < 0.05$ compared to the CMV group; $^{\&}p < 0.05$ compared to the NPLV group.

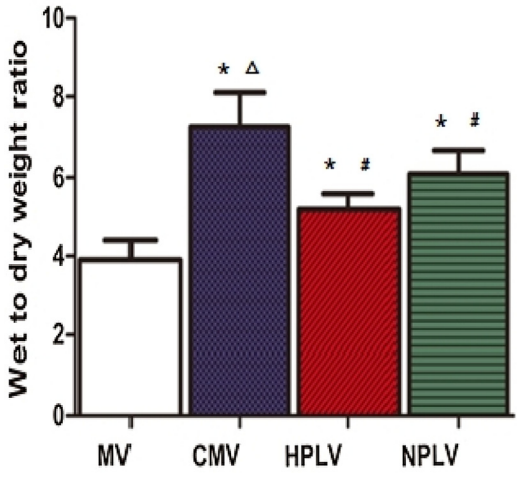

Fig. 4. W/D ratio in all groups.
The results are shown as the mean ± SD (n = 9). $^{*}p < 0.05$ compared to the MV group; $^{\#}p < 0.05$ compared to the CMV group; $^{\triangle}p < 0.05$ compared to the NPLV group.

the T5 time point was significantly lower ($P < 0.05$) than that in the NPLV group. The serum TNF-α concentrations in the HPLV and NPLV groups were significantly lower than those in the CMV group at the T3 and T5 time-points ($P < 0.05$). The serum TNF-α concentrations in the HPLV, NPLV and CMV groups at the T3 and T5 time-points were significantly higher than those in the MV group ($P < 0.05$) (Fig. 3A). Compared with the NPLV group, the serum IL-6 concentration was significantly decreased in the HPLV group at the T3 and T5 time-points ($P < 0.05$). The serum IL-6 concentrations in the HPLV and NPLV groups were significantly lower than those in the CMV group at the T3 and T5 time-points ($P < 0.05$) (Fig. 3B). At the T3 and T5 time-points, the serum IL-6 and TNF-α concentrations in the CMV, NPLV and HPLV groups were higher than their corresponding baseline values at T0 ($P < 0.05$).

3.5. Effects of PFC liquid ventilation-induced mild hypothermia on the lung W/D ratio

The lung wet-to-dry (W/D) weight ratio was used as an index of water accumulation in the lung, which is an indicator of the degree of lung edema. As shown in Fig. 4, the W/D ratio was significantly higher in the ARDS model groups than in the control group. Although the mean ± SD of the W/D ratio was lower in the HPLV group (5.2 ± 0.4) than in the NPLV group (5.9 ± 0.5), this difference was not significant ($p > 0.05$). However, the mean ± SD of the W/D ratio in the HPLV group (5.2 ± 0.4) was significantly lower than that in the CMV group (7.2 ± 0.6) ($P < 0.05$). Therefore, using PFC liquid ventilation-induced mild hypothermia appeared to significantly reduce the W/D ratio in the HPLV group.

3.6. Effect of PFC liquid ventilation-induced mild hypothermia on the concentrations of TNF-α, IL-6 and IL-10 in the BALF samples

The concentrations of TNF-α in BALF samples from the HPLV group were significantly lower ($P < 0.05$) than those in BALF samples from the NPLV and CMV groups (Fig. 5A). The BALF IL-6 concentrations in the HPLV and NPLV groups were significantly lower than those in the CMV group ($P < 0.05$) (Fig. 5B). The concentrations of TNF-α and IL-6 in BALF samples from the HPLV and NPLV groups were significantly lower than those in BALF samples from the CMV group ($P < 0.05$). The concentrations of TNF-α and IL-6 in BALF samples from the HPLV, NPLV and CMV groups were higher than those in BALF samples from the MV group ($P < 0.05$) (Fig. 5). The concentrations of IL-10 in BALF samples from the HPLV group were markedly higher than those in BALF

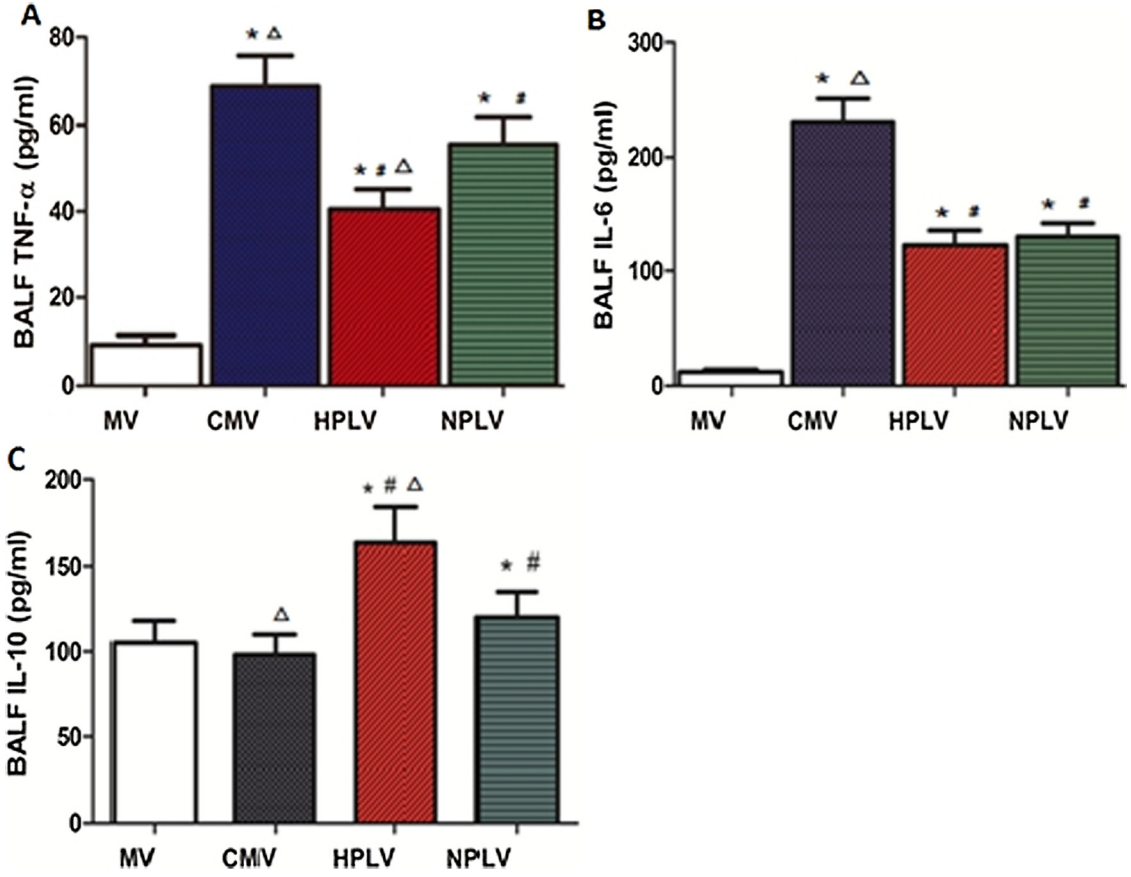

Fig. 5. The concentrations of TNF-α, IL-6, and IL-10 in BALF samples from dogs.
(A)The concentrations of TNF-α in BALF samples. (B) The concentrations of IL-6 in BALF samples. (C) The concentrations of IL-10 in BALF samples.
Values are expressed as the means ± SD (n = 9). $^{*}p < 0.05$ compared to the MV group; $^{\#}p < 0.05$ compared to the CMV group; $^{\triangle}p < 0.05$ compared to the NPLV group.

samples from the NPLV, MV, and CMV groups ($P < 0.05$) (Fig. 5C). The BALF IL-10 concentrations in the HPLV and NPLV groups were significantly higher than those in the MV and CMV groups ($P < 0.05$) (Fig. 5C).

3.7. Analysis of myeloperoxidase expression in lung tissue

The expression of myeloperoxidase (MPO) in the HPLV group was significantly ($p < 0.05$) lower than that in the CMV and NPLV groups (Fig. 6). The expression of MPO in the NPLV group was significantly ($p < 0.05$) lower than that in the CMV group (Fig. 6).

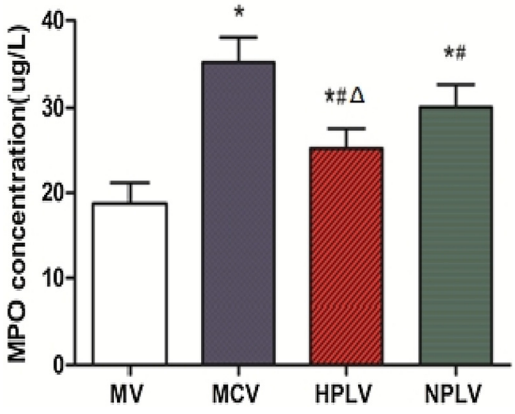

Fig. 6. The expression of myeloperoxidase (MPO) in all groups.
The results are shown as the mean ± SD (n = 9). $^{*}p < 0.05$ compared to the MV group. $^{\#}p < 0.05$ compared to the CMV group. $^{\triangle}p < 0.05$ compared to the NPLV group.

3.8. Analysis of pathological injuries to lung tissue in canines

The effects of PLV-induced mild hypothermia on pulmonary tissue were evaluated by examining histopathologic changes, including intra-alveolar hemorrhage, intra-alveolar edema, interstitial edema, pulmonary interstitial hyperemia, neutrophil infiltration and hyaline membrane thickness. Fields were scored as 0, 1, 2, 3 or 4, corresponding to normal (no injury), mild (injury < 25% field of vision), moderate (injury < 50% field of vision), severe (injury < 75% field of vision) and extremely severe (injury ≥ 75% field of vision), respectively. Each slice was observed for 10 fields, and the cumulative average lung pathological injury score was calculated. Dogs treated with HPLV showed reduced levels of pulmonary pathological injury. The mean cumulative score of lung injury in the HPLV group (7.1 ± 1.2) was significantly lower than those in the CMV (11.9 ± 1.3) and NPLV (9.1 ± 1.2) groups ($P < 0.05$). Compared with the MV group, the cumulative mean scores of lung injury in the CMV, NPLV and HPLV groups were significantly increased ($P < 0.05$), as shown in Fig. 7.

As shown in Fig. 8, slice A shows a representative image of lung tissue from canines in the MV group, which was generally normal, with occasional atelectasis and pulmonary edema (Fig. 8A). The pathological changes observed in the lung tissue slices were the most serious in the CMV group and included inflammation, hemorrhage and exudation. Focal hemorrhage and necrosis was accompanied by focal neutrophil infiltration and aggregation, extensive edema in alveoli, focal necrosis, infiltration and aggregation of neutrophils in the interstitium and

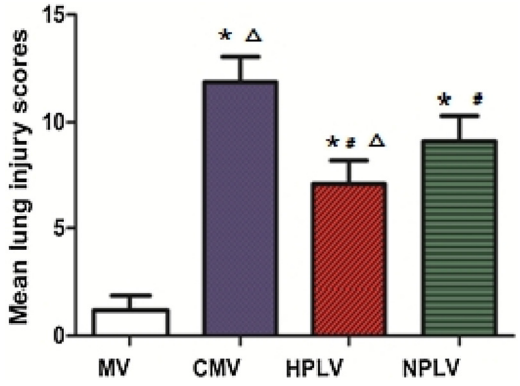

Fig. 7. Mean lung injury scores.
The cumulative mean lung injury scores for the HPLV group (7.1 ± 1.2) were significantly lower than those in the CMV (11.9 ± 1.3) and NPLV (9.1 ± 1.2) groups ($P < 0.05$). The results are shown as the mean ± SD ($n = 9$). $^{*}p < 0.05$ compared to the MV group. $^{\#}p < 0.05$ compared to the CMV group. $^{\triangle}p < 0.05$ compared to the NPLV group.

alveoli, and focal atelectasis (Fig. 8B). The pathological changes observed in the lung tissue slices from the NPLV group were serious and primarily showed necrosis and exudation. Focal hemorrhage and necrosis were observed beside the bronchus, accompanied by the infiltration and aggregation of focal neutrophils. The surrounding alveolar cavity was filled with reddish edema, which was less severe than that observed in the CMV group (Fig. 8D). In the HPLV group, pathological damage was significantly reduced compared to that in the NPLV and CMV groups and primarily included serous exudation, accompanied by small focal hemorrhage; in addition, the pulmonary alveolar cavity showed reddish edema with a few exuded red blood cells (Fig. 8C).

3.9. Effects of PLV-induced mild hypothermia on the expression level of NF-κB p65 in lung tissues

We characterized NF-κB p65 expression in lung tissue by immunohistochemistry, with brown granules indicating the presence of NF-κB p65 (Fig. 9). The MOD was calculated by Image-Pro software (as shown in Fig. 10). In the MV group, low expression levels of NF-κB p65 were observed on the surface of the vascular, bronchiolar and alveolar epithelia (Fig. 9A). In the CMV group, large amounts of NF-κB p65 protein were observed in the lung tissues, the bronchiolar and the alveolar epithelia (Fig. 9B). In the NPLV group, the distribution of NF-κB p65 protein in the bronchi and lung tissues was similar to that observed in the CMV group, but the expression level of NF-κB p65 protein was lower than that observed in the CMV group (Fig. 9D). The expression level of NF-κB p65 protein in the HPLV group was lower than that in the NPLV and CMV groups (Fig. 9C). The MOD of NF-κB p65 in the HPLV group was significantly lower than those in the NPLV and CMV groups ($P < 0.05$). The MODs in the HPLV and NPLV groups were significantly lower than those in the CMV group ($P < 0.05$). The MODs in the HPLV, NPLV and CMV groups were significantly higher than those

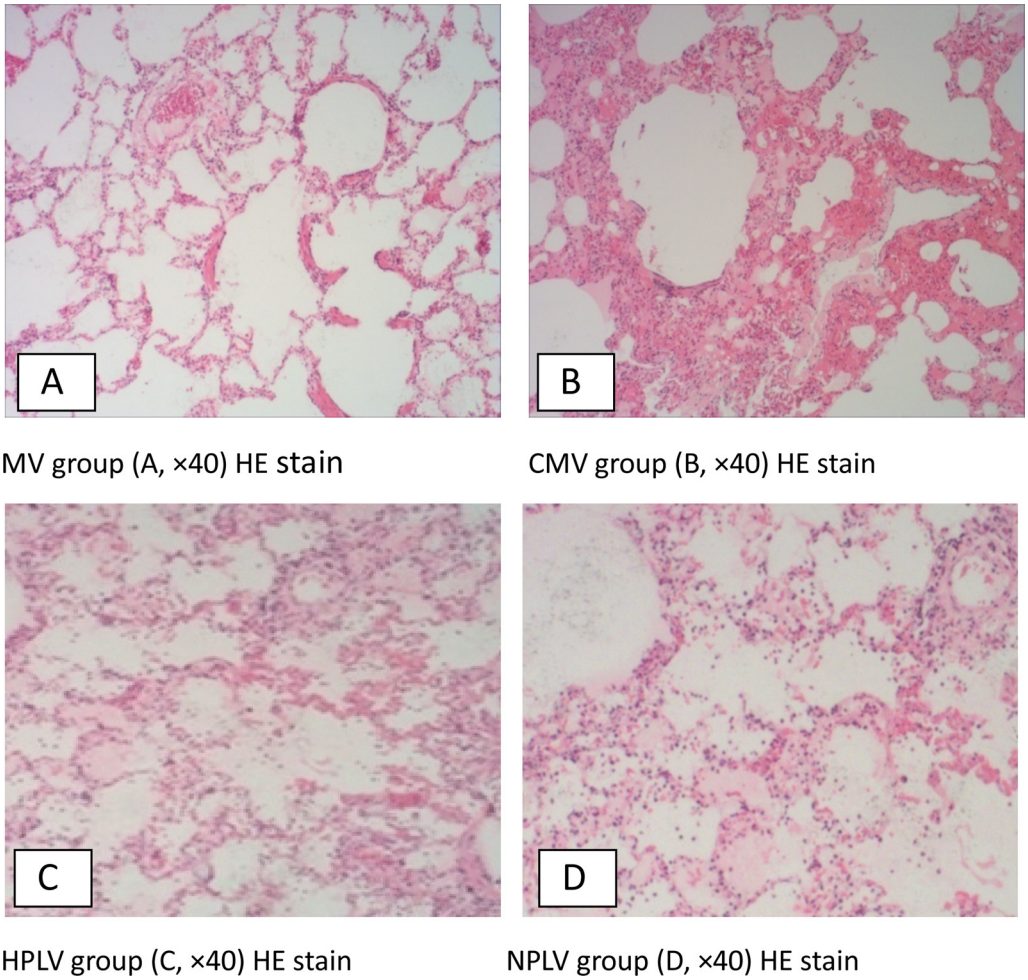

Fig. 8. Photomicrographs of lung tissue in each group (HE stain). A=MV group, B = CMV group, C = HPLV group, D = NPLV group. In the HPLV group, the pathological damage was significantly reduced compared to that in the NPLV and CMV groups.

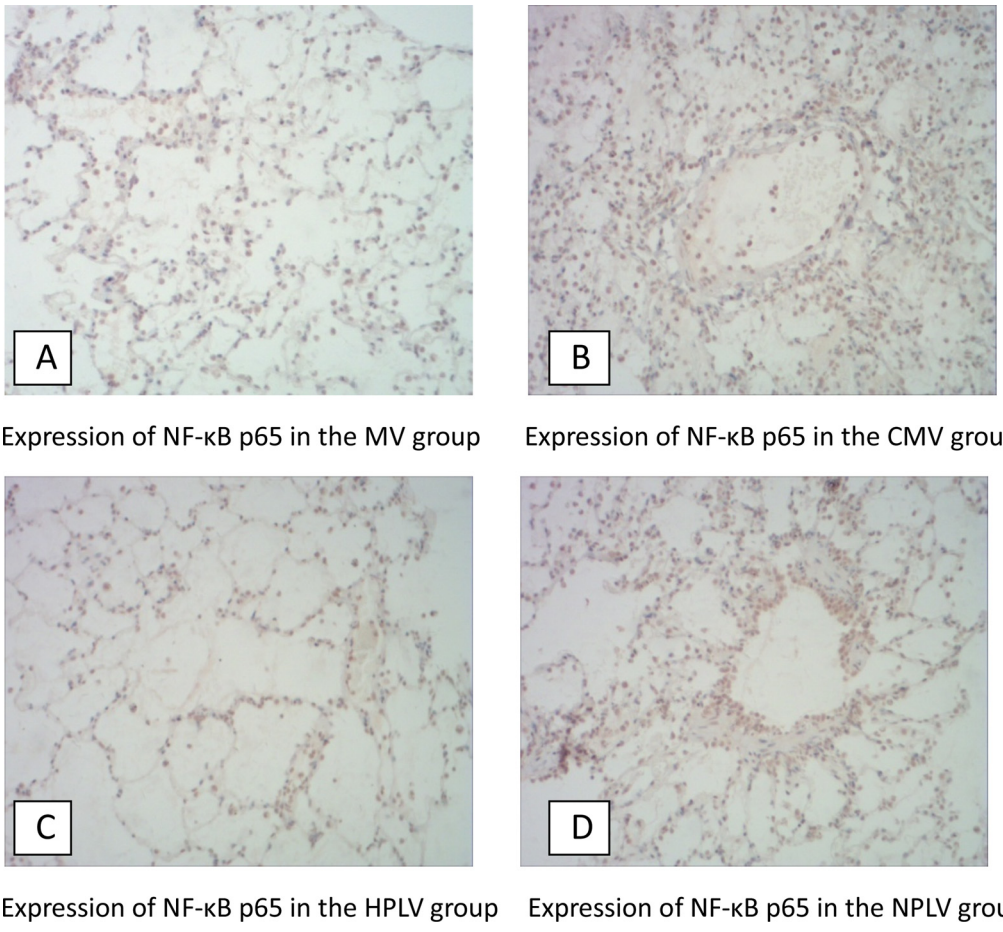

Fig. 9. NF-κB p65 immunohistochemistry in lung tissues from canines (immunohistochemical stain). A=MV group, B = CMV group, C = HPLV group, D = NPLV group. Magnification ×100. The expression level of NF-κB p65 protein in the HPLV group was lower than that in the NPLV and CMV groups.

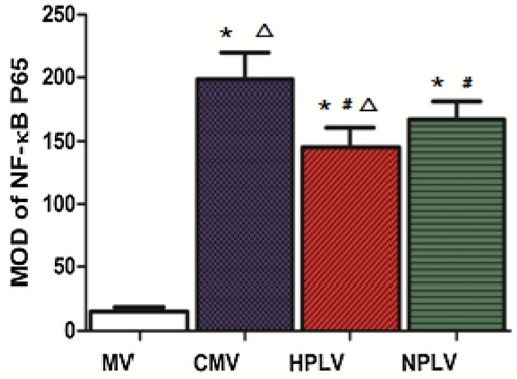

Fig. 10. The MOD of NF-κB p65 Values are expressed as the means ± SD. $^{*}p < 0.05$ compared to the MV group. $^{\#}p < 0.05$ compared to the CMV group. $^{\triangle}p < 0.05$ compared to the NPLV group.

in the MV group ($P < 0.05$) (Fig. 10).

4. Discussion

Here, we showed that in an oleic acid-induced ARDS canine model, PFC-mediated, PLV-induced mild hypothermia significantly improved lung functions, as indicated by increased arterial blood PaO_2 and decreased lung wet-dry weight ratios (Figs. 2, 4), and it reduced pulmonary injury, as indicated by preserved lung structures (Fig. 8). Moreover, PFC-mediated PLV-induced mild hypothermia significantly alleviated the inflammatory response, as indicated by minimized increases in the concentrations of IL-6 and TNF-α in peripheral blood and lung BALF, increased IL-10 levels in the BALF of the lung tissue, decreased MPO levels, and downregulated expression levels of NF-κB p65 in lung tissues (Fig. 9). We found that PLV-induced mild hypothermia provided protection against oleic acid-induced ARDS in dogs and that there were no adverse effects on hemodynamics. Thus, our study provides a theoretical basis for using PFC combined with mild hypothermia to treat ARDS in the clinic, especially when this syndrome is associated with fever.

Mild therapeutic hypothermia as a new approach to lung rest for the treatment of ARDS can reduce the overexpression and release of cytokines in lung tissue, inhibit neutrophil aggregation, and reduce oxygen consumption [12–14]. Additionally, PFC-mediated PLV is a novel ventilation method that can improve respiratory functions during ARDS and reduce lung injuries [15]. Here, we showed that PLV in conjunction with mild hypothermia has a better effect in improving lung function and attenuating pulmonary injury than does NPLV in treating oleic acid-induced ARDS in dogs. PLV-induced mild hypothermia may play a synergistic role in the treatment of dogs with ARDS. The mechanism underlying the protection afforded by PLV combined with mild hypothermia against ARDS remains controversial. Therefore, we attempted to elucidate the mechanisms of action by which PLV-induced-mild hypothermia influences inflammatory regulators and pulmonary histopathology.

An ARDS model in dogs was generated using oleic acid in our experiment. The animal model of oleic acid-induced lung injury better simulates the pathophysiological manifestations of patients with ARDS caused by severe trauma, multiple fractures, and fat embolism. As it is independent of inflammatory cells and their active products, this ARDS model helps to elucidate the role of inflammatory cells and their active products in the pathogenesis of ARDS. In this study, PLV- induced mild hypothermia significantly increased the expression of the anti-inflammatory factor IL-10 in BALF and attenuated the expression of

interleukin (IL-6) and tumor necrosis factor-α (TNF-α) in peripheral blood and in lung BALF. In recent years, some studies have shown that hypothermia treatment can reduce ventilator-induced lung injury in animal models of ALI by reducing the release of proinflammatory cytokines TNF-α and IL-6 in lung tissue and plasma [7,14], increasing the amount of anti-inflammatory cytokines (IL-10) in lung tissue [16,17], and improving pulmonary vascular manifestations and alveolar epithelial damage [18]. PLV combined with mild hypothermia may prevent polymorphonuclear neutrophils (PMNs) from infiltrating lung tissues and inhibit the activity of PMNs by PFCs [19,20]. More importantly, PLV-induced mild hypothermia may combine the advantages of mild hypothermia and PLV, and may have a synergistic role in the treatment of ARDS. Much evidence exists that ARDS represents an inflammatory injury of the lung [21], primarily by PMN infiltration [22]. The infiltration of PMNs may represent the core pathogenic mechanism underlying ARDS. To determine the degree of pulmonary retention and accumulation of PMN, we measured the expression of MPO, which is an enzyme mainly present in azurophil granules of PMNs, and used this expression level as an index of the number of PMNs in the lung. It also reflected the degree of pulmonary retention and accumulation of PMNs [23]. In this study, PLV-induced mild hypothermia markedly decreased the expression level of MPO in the HPLV group compared with the CMV and NPLV groups (Fig. 6). This result suggests that PLV combined with mild hypothermia treatment reduces the infiltration of PMNs into lung tissue, which may explain the reduced inflammation and lung injury. Previously, PFC has been shown to prevent PMN activation and to decrease the synthesis and release of inflammatory mediators from macrophages [20,24]. Enzymes, oxygen radicals and inflammatory mediators released from inflammatory cells have been found to be the primary factors associated with ARDS [25]. TNF-α and IL-6 are proinflammatory cytokines that mediate the activation of PMNs and their adhesion to vascular endothelial cells. Under the regulation of a chemotactic factor, a large number of aggregates at inflammatory sites can degranulate PMNs, causing the release of lysosomal enzymes, resulting in damage to alveolar epithelial cells or endothelial cells, an increased permeability of alveolar-capillary membranes and the further aggravation of lung injuries.

Since expressions levels of TNF-α and IL-6 are regulated at the transcriptional level by NF-κB, which is a posttranscriptionally regulated transcription factor, heterodimers of NF-κB components, which are mostly p50/p65, bind to the inhibitor IκB in the cytoplasm of resting cells, which masks the nuclear localization sequence of NF-κB. In response to diverse stimuli, including TNF-a, bacteria, or toxins, IκB is phosphorylated by IκB kinase (IKK) and detached from NF-κB, allowing NF-κB to translocate to the nucleus, where it binds to target genes and promotes transcription [26,27]. This action allows NF-κB to regulate the expression of many cell factors, such as IL-6 and IL-8 [28]. NF-κB plays a key role during the regulation of most of the cytokines involved in the pathogenesis of ARDS. The activation of NF-κB may be the most important step during the activation of the complex cytokine network [29]. Here, we showed that PLV combined with mild hypothermia decreased the expression of NF-κB p65 in lungs. The therapeutic effects of PLV-induced mild hypothermia on ARDS may be mediated through the inhibition of NF-κB expression and the reduced release of a variety of inflammatory mediators, such as TNF-α and IL-6 [30]. In addition, IL-10 is a pleiotropic cytokine that controls inflammatory processes by suppressing the production of proinflammatory cytokines, which are known to be transcriptionally controlled by NF-kB. Some studies found that IL-10 functions to block NF-kB activity at two levels: 1) through the suppression of IKK activity and 2) through the inhibition of NF-κB DNA binding activity [31], PLV-induced mild hypothermia may reduce the expression of NF-κB by increasing the expression of IL-10. The activation of phosphor IκB was not analyzed in this study. Thus, it cannot presently be concluded which factors are responsible for the effect. In our study, the total expression of NF-κB p65 in lung tissue was determined by immunohistochemistry, which could not reflect the protein content of NF-κB p65 in the nucleus. Such measurements could be better performed by immunofluorescence, confocal assays, and western blotting. We will conduct further research in the next step.

The histologic scores of acute lung injury were lower in the PLV-induced mild hypothermia group (HPLV group) than in the PLV normothermia group (NPLV group) (Fig. 7). We showed that during PLV in conjunction with mild hypothermia, oleic acid-induced lung injury might develop to a lesser degree, possibly through the mechanism of increasing IL-10 and inhibiting the expression of NF-κB, which regulates a variety of inflammatory mediators, such as TNF-α and IL-6. The synergistic effect of PFC-mediated PLV in conjunction with mild hypothermia could have contributed to this result. The results of this study in dogs can be extrapolated to the clinic, especially in case of ARDS associated with fever. In particular, the degree of hypothermia in the present study—34-36 °C, a near normal temperature—was higher than the mild hypothermia currently used at the bedside. However, complications such as platelet dysfunction and susceptibility to infection need to be paid more attention [32].

5. Conclusion

The present results demonstrated that PLV-induced mild hypothermia attenuated lung injury and improved lung function during ARDS in canines. The increase in IL-10, decrease in MPO levels, downregulation of NF-κB p65 expression and modulation of the inflammatory response may be involved in this protective effect.

There is still much work to be done to improve the study. The limitations of our study include the relatively short treatment time used for PLV-induced mild hypothermia. Prolonged treatment times may return different results. In addition, we chose a mongrel dog due to the experimental conditions, which may have resulted in larger standard errors. The samples of each group is 9, which is not very large and may cause some errors.

Funding

This work was supported by Science and Technology Planning Project of Jiangxi Science and Technology Department grants 20151BBG70209.

Declaration of Competing Interest

The authors declare no competing financial interests.

Acknowledgement

We acknowledge the technical support and the help during the study from Dr. Guanghua. Guo, Dr. W. Liu, and N. Zhao from the First Affiliated Hospital of Nanchang University.

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