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ORIGINAL ARTICLE

Beneficial Effects Of Inhaled Nitric Oxide On Lung Pathology And Energy Metabolism In A Canine Model Of Smoke Inhalation Injury

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ABSTRACT

Nitric oxide (NO) exhibits positive effects in the treatment of acute lung injury (ALI); angiotensin converting enzyme (ACE) has been indicated as a marker of pulmonary endothelium damage in ALI. We examined the effects of inhaled NO on ACE activity, pulmonary pathology and energy metabolism in a canine model of smoke inhalation injury. Following smoke exposure, 17 dogs were randomly assigned to receive a mixture of NO (45 ppm) and O₂ (FiO₂=45%) (Treatment group, n=9) or O₂ alone (FiO₂=45%) (Control group, n=8) for 12 hours. As compared to O₂ alone, NO therapy effectively limited the increase in ACE activity and preserved lung ATP level and energy charge. Moreover, NO exerted a protective effect on the extensivemorpho-structural changes which were observed in the dogs who received O₂ alone. The present study demonstrates that inhaled NO after smoke inhalation injury may exert beneficial effects that are likely to be due in part to a protective effect on pulmonary endothelium and pulmonary haemodynamics.

KEY WORDS - Nitric oxide, acute lung injury, pulmonary endothelium, angiotensin converting enzyme, inhalation therapy, ATP

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INTRODUCTION

Pulmonary vascular endothelial damage is a common pathological feature in the early phase of most forms of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [1]-[4]. This damage results in the loss of endothelium-derived vasodilators, with diffuse vasoconstriction and increase in pulmonary

vascular resistance, as well as in the microvascular hyperpermeability to both fluid and proteins, with consequent interstitial and intra-alveolar oedema [1]-[4]. Lung endothelial cells are the major sites of the production of the angiotensin converting enzyme (ACE) [5] and several studies have suggested a role for the evaluation of plasma and lung ACE activity as a

marker of pulmonary damage, both in experimental and clinical settings of inhalation injury [6], [7]. In the recent years, inhaled nitric oxide (NO) therapy has been extensively used to treat a wide variety of lung diseases (as reviewed in reference 8). Acting as a selective pulmonary vasodilator, inhaled NO has been demonstrated to improve ventilation-perfusion matching, thus ameliorating oxygenation and lowering pulmonary vascular resistance in patients suffering from ALI and ARDS [8], but it is not clear what the effects of inhaled NO on pulmonary ACE activity, energy metabolism and pathology are. Previous studies from our laboratory and others showed that inhaled NO may exert beneficial effects in animal models of smoke inhalation injury, thus improving oxygenation, pulmonary hypertension and microvascular resistance [9]-[12]. The objective of this study was to examine the effects of inhaled NO on plasma and lung ACE activity, energy metabolism and pathology, by using a canine model of smoke inhalation injury. We hypothesized that inhaled NO would decrease plasma and lung ACE activity, preserve pulmonary energy metabolism and reduce lung histological damages.

METHODS

Animal model

The canine model of smoke inhalation injury which was used in the present study was previously described in detail [9], [13]. In summary, 17 healthy age- and weight-matched male cross-bred dogs were surgically instrumented under general anaesthesia and sterile conditions. Anaesthesia was induced with the intravenous administration of 3% sodium pentobarbital (30 mg/kg) and was maintained with a continuous infusion of barbiturate in order for it to be effective during the whole procedure. A 9F tracheal tube was introduced through a tracheotomy, a 7F three-channel Swan-Ganz catheter was advanced into the pulmonary artery via the right femoral vein and a 4F multipurpose catheter was advanced into the abdominal aorta via the right femoral artery for blood-pressure monitoring and arterial blood sample collection. Inhalation injury was induced by introducing a dense smoke which was produced by burning 150 g of white pine wood sawdust, into the dogs' respiratory tract through the tracheal tube. The noxious chemicals and gases which were generated from the incomplete combustion of sawdust are formaldehyde,

acetaldehyde, methane, formic acid and carbon monoxide. The respiratory rates and volumes were controlled by using a ventilator and they consisted of 18 breaths per minute of 320-350 ml (adjusted per dog's weight) of smoke in each breath. The dogs underwent 4 minutes of continuous smoke inhalation, followed by 5 minutes of room air ventilation and an additional 90 seconds of smoke inhalation. From our experience, we found that such exposition to smoke is sufficient to cause a severe inhalation injury. The dogs were then ventilated (Newport, E-200 ventilator) after receiving an intravenous succinylcholine loading dose of 1.5 mg/kg, followed by continuous infusion (30 drops/h), with a tidal volume of 13 ml/kg, respiratory rate of 18 breaths/min, an inspiration/expiration ratio of 1:1.5 and a constant PCO₂ level of 30 mmHg (with the aid of a CO₂ sensor – CO₂ monitor HP 14361A). Thirty minutes after the last smoke inhalation, the dogs were randomly assigned to be ventilated with a mixture of NO (45 ppm) and O₂ (FiO₂=45%) (Treatment group, n=9) or with O₂ alone (FiO₂=45%) (Control group, n=8); the temperature of the inhaled gas did not exceed 40° C. The concentrations of NO and NO₂ in the inspired gas were continuously monitored by using a gas analyzer (42C-NO-NO₂-Nox, USA). Twelve hours after the last smoke inhalation, the dogs were euthanized and the lungs were rapidly removed for biochemical and histopathological examination. Assuming the severe inhalation injury to be equivalent to 30% of the total body surface area (TBSA) which was burned, during the experiment all the dogs were hydrated according to the Parckland formula (4 ml/kg/% TBSA burned/24h). The lung tissue samples were obtained from 4 non-injured, non-treated dogs (Sham group, n=4) for comparisons. The experimental protocol was approved by the Experiment Animal Committee of the Bethune International Peace Hospital. The animals were handled in adherence to the Guidelines of the Chinese Association for Physiological Sciences.

Measured variables

Hemodynamic measurements. The mean pulmonary artery pressure (mPAP) and aortic pressure (AoP) were recorded invasively at each study time-point.

Biochemical analysis. Arterial blood samples were collected at the baseline (prior to any smoke inhalation) and 0.5, 2, 5, 8 and 12 hours after the last smoke inhalation for the

assessment of blood gases, carboxyhaemoglobin (HbCO) and nitrate content. Plasma was extracted as a supernatant after centrifugation of 2 ml of heparinized blood at 3000 rpm for 10 minutes and was then stored at a temperature of -20°C . In order to obtain bronchoalveolar lavage (BAL), the superior lobe of the left lung was irrigated with 30 ml of 0.9% saline solution via a principal bronchus, followed by aspiration of the liquid after 1 minute. This procedure was repeated 3 times with a total of 90 ml of saline solution; the overall amount of lavage liquid which was obtained, was then centrifuged at 1000 rpm for 5 minutes and the supernatant was collected and stored at -20°C . A sample of the left lung tissue, weighing approximately 150 mg, was homogenized in a solution of 0.9% NaCl at 4°C and was centrifuged at 3000 rpm for 20 minutes. The supernatant was then extracted and preserved at -20°C . ACE activity in plasma, BAL and lung tissue was determined by utilizing the synthetic ACE-specific substrate hippuryl histidyl leucine (HHL); ACE activity was quantified from the moles of hippuric acid (HA) formed, in time-fixed assays, by using high performance liquid chromatography (HPLC) separation of HA from HHL and UV-spectrophotometry for the quantitation of HA as in the standard Cushman and Cheung assay [14]. Colloid osmotic pressure (COP) in plasma and BAL was assessed by using a colloid osmometer. The total protein content in BAL was determined after Coomassie blue staining with an ultraviolet spectrophotometer (model DU-7 Beckman) with a reading wave-length of 595 nm; cattle serum protein was used to draw a standard comparison curve. The energy metabolism of the pulmonary tissue was estimated by reverse phase HPLC [15]. The actual content of ATP, ADP and AMP in the tissue samples was calculated as the ratio of the sample spike square and the standardized spike square (corresponding to $25\ \mu\text{g/ml}$ concentration of ATP, ADP and AMP). Energy charge (EC) was calculated according to the formula:

$$\text{EC} = (1/2\text{ADP} + \text{ATP}) / (\text{ATP} + \text{ADP} + \text{AMP}).$$

Histopathology and morphometry. We measured the lung water content according to the lung wet-to-dry weight ratio (W/D). For this purpose, the excised right lung was first weighed in a dry plate (wet weight – W). It was then weighed again after being dried at 80°C for 72 hours (dry weight – D). The W/D ratio was calculated as follows: $\text{W/D} \% = [(\text{W} - \text{D})/\text{W}] \times 100$. The diaphragmatic lobe of the left lung was

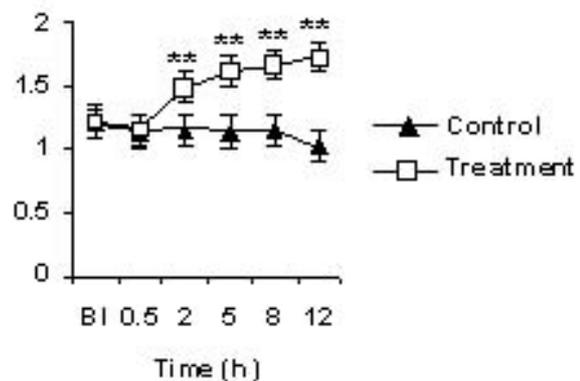
processed for histopathological examination. The tissue samples were fixed in 10% formalin, were embedded with paraffin wax, sectioned and stained with Hematoxyline-Eosine (HE) for light microscopy, or were fixed in 2.5% buffered glutaraldehyde and prepared routinely for transmission electron microscopy (TEM).

Statistical analysis

We adopted ANOVA and the Student's t test for comparisons as and when appropriate. The data are reported as mean \pm SEM; a p value < 0.05 was considered to be statistically significant.

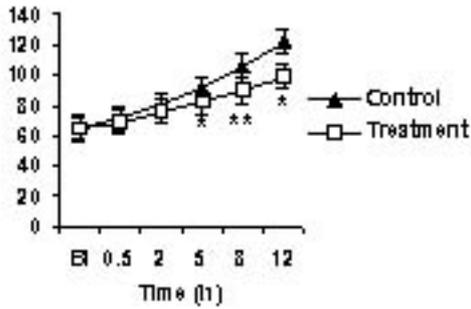
RESULTS

All animals survived the observation period. NO inhalation had no effects on mAP, but had effects on mPAP which decreased after 2 hours. The dogs exhibited the usual modifications in gas exchanges and HbCO levels which were associated with smoke inhalation injury; arterial blood methaemoglobin concentration resulted in $<2\%$ during the whole experiment in both study groups (data not shown). As expected, NO inhalation translated into higher arterial blood nitrate concentrations at each study time-point [Table/Fig. 1].



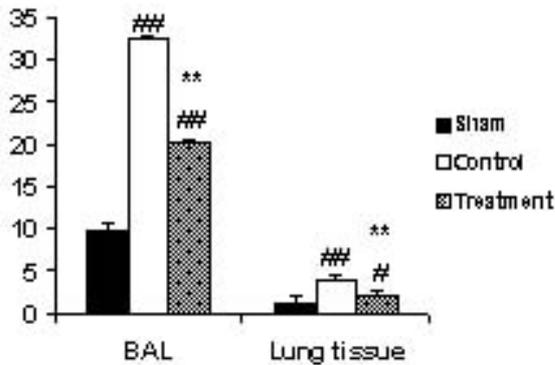
[Table/Fig. 1]: Plasma nitrate levels. BI, before injury. ** $p < 0.01$ Vs Control group. (Control, $n=8$; Treatment, $n=9$).

ACE activity. Comparisons of plasma ACE activity between the study groups are shown in [Table/Fig 2]. Early after injury, there was an increase in the ACE activity in the overall study population. In the Control group, the increase in ACE activity reached statistical significance after 5 hours ($p < 0.05$), resulting in nearly doubled values after 12 hours as compared to the baseline values. The NO treatment induced a significant decrease in the plasma ACE activity as compared to the controls.



[Table/Fig 2]: Plasma ACE activity. Plasma ACE activity increased in both groups after inhalation injury; NO inhalation significantly lowered the extent of the increase after 5 hours. BI, before injury. * $p < 0.05$ Vs Control; ** $p < 0.01$ Vs Control. (Control, n=8; Treatment, n=9).

ACE activities in BAL and lung tissue were dramatically increased in dogs who were exposed to inhalation injury as compared to the Sham dogs ($p < 0.01$, [Table/Fig 3]). However, NO inhalation was effective in significantly reducing the ACE activity in BAL (20.13 ± 0.23 Vs 32.46 ± 0.39 nmol/mg_{prot}/min, $p < 0.01$) and lung tissue (2.16 ± 0.25 Vs 3.96 ± 0.42 nmol/mg_{prot}/min, $p < 0.01$) as compared to O₂ inhalation alone.



[Table/Fig 3]: ACE activities in BAL and lung tissue. We observed a significant increase in ACE activities in BAL and lung tissue associated with inhalation injury effectively limited by inhaled NO. # $p < 0.05$ Vs Sham; ## $p < 0.01$ Vs Sham; ** $p < 0.01$ Vs Control. (Sham, n=4; Control, n=8; Treatment, n=9).

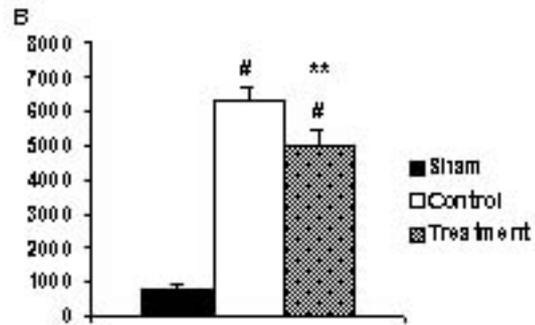
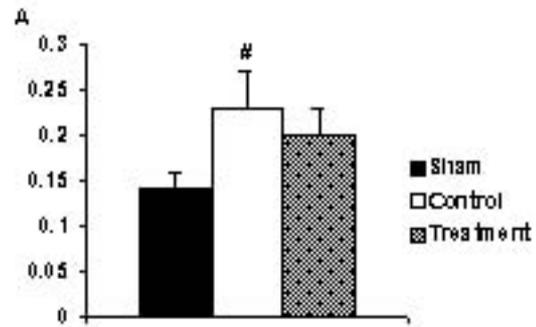
COP. Following inhalation injury, plasma COP was decreased in the Control group (significantly decreased after 8 hours, $p < 0.05$) and was substantially unchanged in the Treatment group, which was suggestive of a preserved COP in dogs who were randomized to NO inhalation [Table/Fig 4].

[Table/Fig 4]: Plasma colloid osmotic pressure (kPa) levels at each study time-point.

Group	Before	After injury					
	injury	0.5h	2h	5h	8h	12h	
Control	2.1 ± 0.19	1.98 ± 0.17	1.75 ± 0.16	1.67 ± 0.16	1.49 ± 0.14	1.50 ± 0.15	
Treatment	2.0 ± 0.20	1.97 ± 0.18	1.84 ± 0.17	1.79 ± 0.15	1.66 ± 0.16*	1.65 ± 0.14*	

mean ± SEM; * $p < 0.05$ Vs Control. (Control, n=8; Treatment, n=9)

Smoke exposure was associated with an impressive increase in COP and total protein content in BAL [Table/Fig 5]; NO inhalation exhibited a significant protective effect with regards to these variables as compared to O₂ inhalation alone (total protein content: 5000 ± 470 Vs 6290 ± 450 g/L, $p < 0.01$) [Table/Fig 5].



[Table/Fig 5]: COP and protein content in BAL. COP and protein content in BAL resulted significantly increased in injured dogs; NO exhibited a positive effect on these variables. # $p < 0.05$ Vs Sham; ** $p < 0.01$ Vs Control. (Sham, n=4; Control, n=8; Treatment, n=9).

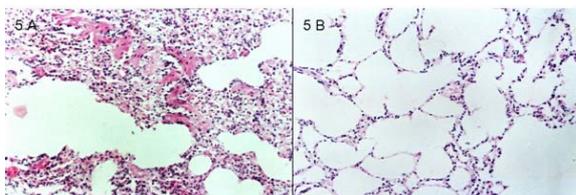
Energy metabolism. The dogs who were exposed to smoke inhalation injury exhibited a severe impairment of lung tissue energy metabolism [Table/Fig 6]. However, in the Treatment group, we observed a less severe depletion of ATP (11.53 ± 1.03 Vs 6.79 ± 0.78 μmol/mg_{prot}, $p < 0.01$), ADP (3.05 ± 0.36 Vs 2.73 ± 0.31 μmol/mg_{prot}, $p < 0.05$), and EC (0.77 ± 0.04 Vs 0.67 ± 0.06 , $p < 0.01$) than in the controls.

[Table/Fig 6]: Pulmonary energy metabolism

	Sham (n=4)	Control (n=8)	Treatment (n=9)
ATP ($\mu\text{mol}/\text{mg}_{\text{protein}}$)	20.41 \pm 0.97	6.79 \pm 0.78 ^{###}	11.53 \pm 1.03 ^{###**}
ADP ($\mu\text{mol}/\text{mg}_{\text{protein}}$)	4.17 \pm 0.41	2.73 \pm 0.31 [#]	3.05 \pm 0.36 [*]
AMP ($\mu\text{mol}/\text{mg}_{\text{protein}}$)	3.07 \pm 0.68	2.45 \pm 0.29 [#]	2.37 \pm 0.35
EC	0.81 \pm 0.07	0.67 \pm 0.06 ^{###}	0.77 \pm 0.04 ^{**}

mean \pm SEM; # $p < 0.05$ Vs Sham; ## $p < 0.01$ Vs Sham; * $p < 0.05$ Vs Control; ** $p < 0.01$ Vs Control. ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; EC, energy charge.

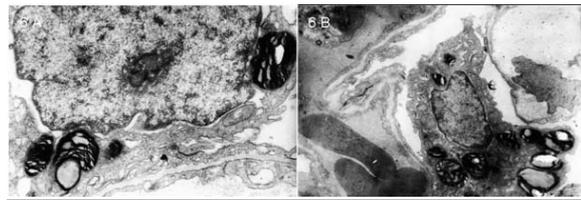
Histopathology. The W/D ratio was significantly increased ($p < 0.05$) in the dogs who were exposed to inhalation injury as compared to the Sham dogs (75.58 \pm 6.07%); however, no substantial difference was observed between the Control and the Treatment groups (W/D: 84.32 \pm 4.01 Vs 83.32 \pm 6.08%, $p = \text{NS}$). Light microscopic examination of the lung tissue samples from the Control dogs [Table/Fig. 7 A] revealed marked tissue haemorrhage and congestion, thickening of the alveolar wall with alveolar oedema and an intra-alveolar collection of inflammatory cells, deciduous epithelial cells and erythrocytes; some alveoli appeared to be ruptured and syncretized. Analysis of the samples from the Treatment group [Table/Fig. 7 B] revealed an apparently less severe degree of morpho-structural damage, because the damage was displayed with mild tissue haemorrhage and congestion, preserved alveolar anatomy and a modest infiltration of inflammatory cells and erythrocytes.



[Table/Fig. 7]: Light microscopy photographs. A, tissue sample from Control group; B, tissue sample from Treatment group (HE X 400)

TEM analysis showed interstitial oedema, derangement and the rupture of the fibrous structures, the casting of type II alveolar cells in the alveolar cavity with oedema and incrustation and the loss of integrity of the basal membrane in the samples which were collected from the dogs in the Control group [Table/Fig. 8 A]. The integrity of the alveolar-capillary membrane appeared instead to be maintained and the cellular damages appeared milder, with the conservation of the organization of the lamellar

bodies in the lung tissue samples from the Treatment group [Table/Fig 8 B].



[Table/Fig 8]: Transmission electron microscopy photographs. A, tissue sample from Control group; B, tissue sample from Treatment group (TEM X 21000).

DISCUSSION

In patients with ALI or ARDS, NO inhalation has been shown to improve oxygenation without affecting the outcome [8]. However, evidence from experimental studies has suggested that NO which is given after inhalation injury, may exert several additional positive properties beyond the sole improvement of oxygenation. Ogura et al. reported that inhaled NO significantly attenuated pulmonary vasoconstriction and pulmonary arterial hypertension, thus moderately improving the ventilation-perfusion mismatch in an ovine model of smoke inhalation injury [10], [11]. Recently, Enkhabaatar et al. showed that inhaled NO which was given 22 hours after injury, along with reducing pulmonary vascular resistance and pulmonary artery hypertension, was effective in reducing microvascular hyperpermeability and lung water content [12]. No change in aortic pressure was found to be related with NO inhalation in these studies.

The results of the present study confirm the effects of inhaled NO on the improvement of oxygenation and pulmonary arterial hypertension which were observed in these previous studies; furthermore, our data provides the first evidence that NO inhalation therapy exhibits beneficial effects on lung pathology and energy metabolism, thus limiting the severe damages which are associated with smoke inhalation injury. These beneficial effects may in part be explained by a potential protective effect which is exerted by inhaled NO on pulmonary vascular endothelial cells [6], [7], as suggested by lower ACE activities which were observed in the treatment group. ACE is a non-specific dipeptidylcarboxy-peptidase that removes COOH-terminal dipeptides from a variety of substrates, including bradykinines and it also catalyzes the conversion of Angiotensin I

into Angiotensin II [5]. Although it is present in many tissues, this enzyme is mainly expressed on the plasma membrane of the pulmonary vascular endothelial cells (5). Numerous studies indicated a role for ACE activity as a specific index of pulmonary endothelial damage of different aetiologies [16]-[19]. Specifically, a significant increase in ACE activity has been observed in plasma, BAL and lung tissue in patients and animals after smoke inhalation injury [6], [7]. Our observations showed that plasma ACE activity was increased early after injury and the further increase which was detected in the course of the experiment, might have potentially paralleled progressive endothelial cell damage. Along with a significant reduction in the plasma levels, which was observed as compared to the controls, the protective effect of NO inhalation was suggested by a lower expression of ACE activity, both in lung tissue and BAL.

Pulmonary microvasculature hyperpermeability to fluid and protein is observed early after inhalation injury and it is a direct expression of endothelial cell damage [1]-[4]. Endogenous tachykinins and hydroxyl radicals are likely to represent the mediators of increased permeability [20]-[22], which in turn, lead to the extravasation of plasma and macromolecules into the interstitial space, with perialveolar and submucosal peribronchial and peritracheal oedema and into the alveolar cavity through a loss of integrity of the alveolar-capillary barrier. In the present study, inhaled NO was effective in significantly reducing BAL COP and total protein content when compared to O₂ therapy alone; these results appear to be likely to be achieved by limiting endothelial cell dysfunction, thus halting the tendency to increase the permeability.

Although we observed a positive effect of inhaled NO COP increase and protein accumulation, both in BAL and lung tissue, which was suggestive of limited vascular hyperpermeability, the lung W/D ratio appeared to be substantially unchanged in the control and the treatment groups, even if it was sensibly increased as compared to the non-injured sham dogs. A potential explanation for this apparent lack of effect of NO inhalation on the lung water balance might be the limitation of our observation period to 12 hours, which was intended by design to investigate the acute

therapeutic effect of NO in the setting of smoke inhalation injury. An early increase (i.e. < 12 hours following smoke exposure) in extravascular lung water content is very uncommon in patients suffering from inhalation injury and it is a result of the direct chemical toxicity of inhaled gases [23]-[25]. A significant increase in extravascular lung water is usually first seen in the first 24 hours, with persistently elevated values for more than 48 hours [23]-[25]. However, the available data from the literature, which is relative to the effects of inhaled NO on the pulmonary water content in inhalation injury, it still appears to be controversial [26]-[28].

Microvascular damage with oedema and increased pulmonary vascular resistance, along with airway obstruction significantly impairs oxygenation and lung tissue blood supply, thus negatively affecting the cellular energy metabolism, ATP synthesis and survival [21], [29]. Mammalian cells depend on ATP to maintain their cellular activities and structural integrity; ATP depletion translates into cellular dysfunctions and ultimately, death. Compared to the Sham group, there was a severe depletion of ATP, ADP and EC in the Control group. However, in the Treatment group, we observed a less severe depletion of ATP, ADP and EC than that which was seen in the controls. Dogs who were exposed to smoke inhalation injury exhibited a severe impairment of lung tissue energy metabolism. Inhaled NO preserved the lung tissue production of ATP, thus contributing to the maintenance of a normal EC, potentially through improvement in pulmonary haemodynamics and oxygenation [9]-[12].

The pathological correlates of smoke inhalation injury are acute cell membrane damage with oedema and hyperaemia of the tracheo-bronchial mucosa (which progressively leads to necrotic tracheo-bronchitis with pseudo-membranes formation and airways obstruction), interstitial and intra-alveolar congestion and oedema due to damages of the alveolar epithelial barrier and lung endothelium [30]-[32]. In our study, we focused mostly on the latter aspects. The morphological changes occurring in the alveolar epithelium classically included intracellular oedema with focal bleb and vesicle formation in the type-I alveolar cells and alterations in the membrane-bound vacuoles of the type-II cells; endothelial cells showed similar changes,

although they were less severe [30]-[32]. This pattern of lesions was observed in dogs who were treated with O₂ alone after smoke inhalation injury; rather, NO inhalation therapy resulted in a significant limitation of microscopic damages of the alveolar and the endothelial cells. We might thus hypothesize that this positive effect is obtained through NO-mediated improvement in oxygenation and pulmonary energy metabolism [33], [34]. Further investigations are needed, however, to clarify as to which mechanisms underlie the mitigation of the histological damages which were observed in NO-treated dogs.

In conclusion, the present study demonstrates that inhaled NO, when administered early after smoke exposure, can significantly decrease plasma and lung ACE activity, preserve pulmonary energy metabolism and reduce lung histological damages in a canine model of inhalation injury. These beneficial effects are likely to be due, in part to a protective effect which is exerted by NO on the pulmonary vascular endothelial cells and pulmonary haemodynamics.

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