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EXPERIMENTAL

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Mitigation of endotoxin-induced acute lung injury in ventilated rabbits by surfactant and inhaled nitric oxide

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Abstract *Objective*: To evaluate the efficacy of surfactant and inhaled nitric oxide (iNO) in endotoxin-induced acute lung injury (ALI).
Design: Prospective, randomised, controlled experimental study.
Setting: A medical university hospital research laboratory.
Intervention: Twenty-nine adult rabbits (2.4–3.4 kg) were given two doses of intravenous endotoxin (*Escherichia coli*) (0.01 mg/kg and, 12 h later, 0.1 mg/kg), and then subjected to mechanical ventilation. After 8 h these animals were allocated to four treatment groups: (1) control, (2) iNO at 20 ppm (NO), (3) surfactant at 100 mg/kg (Surf) and (4) both surfactant and iNO as in groups 2 and 3 (SNO), and ventilated for a further 6 h followed by broncho-alveolar lavage (BAL), analysis of surfactant contents in BAL fluid and histological examination of the lungs.
Measurements and results: All the animals had developed ALI with respiratory failure 8 h after the second dose of endotoxin as evidenced by a decrease of PaO_2/FIO_2 from 520 ± 30 to 395 ± 19 mmHg and dynamic compliance (Cdyn) from 1.20 ± 0.11 to 0.73 ± 0.05 ml/cmH_2O × kg, and an increase of intrapulmonary shunting (Qs/Qt) from 7.5 ± 0.8 % to 12.9 ± 1.0 % (all measurements $p < 0.01$ versus baseline). In the SNO group, values for PaO_2/FIO_2, Cdyn and Qs/Qt after 6 h were 301 ± 15 mmHg, 0.67 ± 0.05 ml/cmH_2O × kg and 16.5 ± 0.8 %, compared to 224 ± 26 mmHg, 0.53 ± 0.04 ml/cmH_2O × kg and 24.1 ± 2.0 %, respectively, in the control group (all measurements $p < 0.01$). Both Surf and NO groups showed intermediate levels of these parameters. In both Surf and SNO groups, the minimum surface tension of BAL fluid was lower, and the content of disaturated phosphatidylcholine/total protein higher, than in the control and NO groups ($p < 0.01$). Histological features of lung injury were less prominent and wet/dry lung weight ratio lower in the NO, Surf and SNO groups. Decreased surfactant protein A (SP-A) and its mRNA expression were found in all endotoxin-exposed groups, but the SP-A content of the SNO group was moderately improved in comparison to the control group. Surfactant aggregate size was not affected.
Conclusion: Early application of surfactant and iNO moderately mitigated ALI as reflected by improvement of lung mechanics, pulmonary perfusion and morphology.

Key words
Sepsis · Endotoxin · Nitric oxide · Pulmonary surfactant · Acute respiratory distress syndrome · Respiratory therapy

Introduction

Sepsis or systemic inflammation-associated acute lung injury (ALI), often caused by Gram-negative bacteria infection, is one of the leading causes of morbidity and mortality in patients of all ages requiring mechanical ventilation. Septic ALI may lead to acute respiratory distress syndrome (ARDS) associated with pulmonary hypertension and hypoxic respiratory failure. Traditional therapies with mechanical ventilation, antibiotics and vasoactive drugs may have limited effects. In septic ALI/ARDS, inhaled nitric oxide (iNO) selectively dilates intrapulmonary vasculature and improves pulmonary perfusion without causing systemic hypotension [1, 2]. Clinical trials have also shown that exogenous surfactant or iNO tends to be effective in restoring lung function in patients with septic ALI/ARDS [3, 4]. Other studies have suggested that the pathophysiology of ALI and ARDS includes dysfunction of both surfactant and NO synthesis [5, 6, 7], and that ALI could be ameliorated if appropriate intervention is applied early enough, reducing the risk of pulmonary hypertension and hypoxic respiratory failure [8]. A recent clinical case report [9] and preliminary experimental studies [10, 11] using various animal ALI models have revealed immediate therapeutic effects of a combined exogenous surfactant therapy and iNO. However, our knowledge of the efficacy of iNO and surfactant in septic ALI/ARDS is limited to each therapy alone. In the present study, we tested both surfactant and iNO in a rabbit model of endotoxin-induced ALI and hypothesised that early application of a combined therapy with surfactant and iNO would attenuate inflammatory ALI as effectively as previously shown for non-inflammatory ALI [10, 11, 12]. As endotoxin may mediate damage to the type II alveolar epithelial cells, which are the site of pulmonary surfactant synthesis and metabolism, we also studied surfactant phospholipid aggregate size and contents of surfactant protein A (SP-A) and its mRNA in experimental animals subjected to different treatment protocols.

Materials and methods

Surfactant

Porcine lung surfactant phospholipid extract was prepared as reported elsewhere [12]. Briefly, surfactant phospholipids were isolated from fresh pig lungs by broncho-alveolar lavage (BAL), multiple steps of centrifugation, chloroform/methanol (2/1, v/v) extraction, cold acetone precipitation, filtration and evaporation of chloroform and acetone under nitrogen gas. The final product was resuspended in 0.9 % NaCl at a final concentration of 40 mg/ml and stored at –20 °C. A single batch of the surfactant preparation was used for the study, and total phospholipids (TPL) and disaturated phosphatidylcholine (DSPC) of surfactant were determined as described by Bartlett [13] and Mason et al. [14], respectively.

Nitric oxide

Nitric oxide gas (Shanghai BOC, Shanghai, China) at 1000 ppm in pure nitrogen was supplied to the ventilator circuit about 20 cm proximally to the endotracheal tubing connector, with a mass flow controller (0–200 ml/min, Shengye Technology and Development, Beijing, China) for regulation of NO gas flow. Concentrations of NO and NO_2 were measured using an electrochemical NO/NO_2 analyser, NOxBOX I (Bedfont Scientific, Kent, England).

Animal management and priming dose of endotoxin

Protocols for animal care and experimental management were approved by the Children's Hospital Scientific Committee, Shanghai Medical University. Thirty-four healthy adult New Zealand White rabbits, both male and female, with a mean body weight of 2.6 ± 0.2 kg (mean ± SD, range 2.4–3.4 kg) were used in this study. Five of these animals did not receive endotoxin and served as normal controls (Normal group), and 29 were subjected to endotoxin administration. During the day prior to the experiment, a first dose of endotoxin (*Escherichia coli*, serotype 055/B5, Cat. No. L2637, Sigma Chemical, St. Louis, Mo.) was given to the 29 animals at 0.01 mg/kg through a marginal ear vein, and the animals then had food and water *ad libitum* up to the time of the experiment. The role of this priming dose was to increase tolerance to the subsequent large dose of endotoxin (see below), thereby providing a less dramatic and more uniform toxic effect in the experimental animals [15]. Twelve hours later the animals were sedated with i.m. 2 mg/kg of diazepam (5 mg/ml), and anaesthetised with i.v. pentobarbital sodium (10 mg/ml) at a dose of 2 ml/kg. Additional i.v. pentobarbital sodium was provided at 0.5 ml/kg · h for maintenance of anaesthesia. The animals were tracheotomised, intubated and ventilated mechanically with a pressure-controlled Wave ventilator E-200 (Newport Medical Instrument, Newport Beach, Calif.), initially set at a peak inspiratory pressure (PIP) of 10–15 cmH_2O to provide a tidal volume (V_T) of 8–10 ml/kg body weight, a frequency of 30/min, an inspiration to expiration time ratio (I:E) of 1:1.5 and a fraction of inspired oxygen (FIO_2) of 0.21. An 18G cannula was inserted in the right femoral artery for collection of blood samples and measurement of mean systemic arterial pressure (SAP) with a pressure transducer and a monitor (Department of Physiology, Shanghai Medical University, Shanghai). Another thin catheter was inserted in the right external jugular vein for infusion of endotoxin into, and collection of mixed venous blood from, the right ventricle of the heart. When the animals had been stabilised, blood samples were taken for measurement of baseline values of pH, PaO_2, $PaCO_2$, SaO_2, PvO_2, $PvCO_2$, SvO_2 with a Ciba-Corning 170 blood gas analyser and determination of nitrite/nitrate and methaemoglobin (MetHb) (see below). Urine samples were also collected for measurement of nitrite/nitrate. Baseline values for dynamic compliance (Cdyn) and respiratory resistance (Rrs) were measured from 10 consecutive breaths with an orifice pneumotachograph GM 250 Navigator (Newport Medical Instrument, Newport Beach, Calif.), using an infant-type differential pressure transducer placed in the Y-piece of the ventilator circuit. Cdyn was expressed as ml/cmH_2O × kg and Rrs as cmH_2O/l × s.

Second administration of endotoxin

After the baseline measurements, the 29 experimental animals, previously primed with 0.01 mg/kg of endotoxin, were given endotoxin directly into the right ventricle of the heart (the position of

the catheter tip was verified by pressure waveform recording) at a dose of 0.10 mg/kg, diluted in 10 ml of sterilised saline and infused by a microtransfusion pump for 30 min. The animals were ventilated with FIO_2 gradually increasing to 0.3–0.6 (to keep PaO_2 at 60 mmHg or above), a frequency of 30/min, a positive end-expiratory pressure (PEEP) of 4 cmH_2O and variable PIP to provide a V_T of 8–10 ml/kg, keeping $PaCO_2$ at around 35–40 mmHg. Ringer's lactate (pH 6.0–7.5) and 2.5 % sodium bicarbonate were infused intravenously at a rate of 8–10 ml/kg · h to counterbalance septic shock and correct base excess below normal (–5 mmol/l) due to endotoxaemia. Physiological intrapulmonary shunting (Qs/Qt) was calculated using the standard shunt equation [16] by measuring oxygen content in mixed venous blood (when FIO_2 was temporarily raised to 1.0 for 15 min), taken from the right ventricle of the heart and expressed as a percentage (%) of the total pulmonary blood flow.

Experimental protocols

From pilot experiments, we found that ALI would be established 12 h after the second endotoxin infusion in the current ventilation mode, as evidenced by $PaO_2/FIO_2 \leq 2$ of 300 mmHg or less [17], Qs/Qt > 15 %, decrease of Cdyn of more than 30 % from baseline [18] and significantly increased white blood cells in broncho-alveolar lavage fluid [19]. In the present study we chose to treat the animals at an earlier stage of the disease. Eight hours after administration of the major dose of endotoxin, the animals were randomly allocated to four treatment groups, receiving: (1) mechanical ventilation only (Control), (2) inhalation of 20 ppm NO (NO), (3) intratracheal instillation of a bolus of surfactant at 100 mg phospholipids/kg body weight (Surf) and (4) combined treatment with 100 mg surfactant phospholipids/kg body weight and inhalation of 20 ppm NO (SNO). Surfactant was instilled into the lungs via a thin endotracheal tube at 2.5 ml/kg followed by manual ventilation for 1 min to facilitate even distribution in the lungs. This moment was regarded as treatment time 0 h. All animals (including the five animals of the normal group) were subsequently ventilated for another 6 h and values for Cdyn, Rrs and arterial pH, PaO_2 and $PaCO_2$ measured every hour. The animals were ventilated with variable PIP and a PEEP of 4 cmH_2O to maintain V_T at 8–10 ml/kg, a frequency of 40/min, and an I:E of 1:1. Our goals were to achieve values of blood pH, $PaCO_2$ and PaO_2 in the ranges of 7.30–7.50, 30–40 mmHg and 80–100 mmHg, respectively. Qs/Qt was determined at 0,2 and 6 h. At the end of treatment, animals were killed by an overdose of intravenous pentobarbital sodium (50 mg/ml) and the lungs were processed (see below). The five animals in the normal group not receiving any endotoxin were treated with mechanical ventilation and subjected to the same measurements.

Wet-to-dry lung weight ratio and broncho-alveolar lavage

When an animal was killed, the chest wall was opened and the left main bronchus was ligated to allow selective lavage of the right lung via a tracheal cannula. A piece of lung tissue (about 1 g) from the posterior part of the left lower lobe was cut and its wet weight determined in an automatic electric balance (AP250D, Ohaus, Florham, N.J.). The piece of lung tissue was then dried in an oven at 80 °C for 48 h and weighed again to obtain its dry weight for calculation of the wet-to-dry weight ratio (W/D). Broncho-alveolar lavage (BAL) was performed by intratracheal instillation of 20 ml/kg body weight of 0.9 % NaCl at 37 °C. After three washes, this volume was collected. The washing procedure was performed three times (corresponding to three volumes of saline) and more than 80 % of the instilled BAL fluid (BALF) was recovered from each animal. This material was pooled, immediately centrifuged for 10 min at 200 *g* and 4 °C for cell count (see below). The supernatant volume was recorded and this fluid was stored at –20 °C for chemical analysis (see below). An aliquot of BALF from each animal was centrifuged with an ultraspeed Hitachi 70P-72 centrifuge at 40,000 *g* and 4 °C for 15 min. The resulting supernatant and sediment pellet were used for analysis of small and large aggregate forms of surfactant phospholipids (see below) according to Veldhuizen et al. [20].

Chemical and cytological analysis of broncho-alveolar lavage fluid

Aliquots of BALF were extracted with threefold volumes of chloroform/methanol (2:1, v/v) to isolate phospholipids in the chloroform phase. DSPC, the major surface-tension lowering component of lung surfactant, was separated from unsaturated phospholipids as described by Mason et al. [14] using osmium tetroxide and neutral aluminium column chromatography. Amounts of DSPC and TPL were determined according to the method described by Bartlett [13] and corrected by the total volume of BALF and body weight. Values for TPL are presented as mg/kg, and those for DSPC as percentage of TPL (DSPC/TPL). Total proteins (TP) in BALF were measured according to the method of Lowry and associates [21] using bovine serum albumin as standard, corrected by total volume of BALF and body weight, and presented as mg/kg. DSPC/TP ratio was expressed as μg/mg. This ratio largely reflects the balance between alveolar surfactant and plasma proteins leaking into the airspaces as a consequence of ALI. Phospholipid contents of large and small aggregates in BALF were determined in the same manner. White blood cells in sediment pellets of BALF after low speed centrifugation were counted and expressed as the number × 10^6/l.

Surface tension measurements

These measurements were performed with a modified Wilhelmy balance (Biegler, Vienna, Austria) [22]. Twenty millilitres of BALF was poured into a Teflon trough of the balance and kept at 37 °C. The surface tension of the fluid was recorded continuously during 50 % cyclic area compression at a rate of 1 cycle/min, for a total of 60 cycles (i.e. 60 min.). Values for minimum and maximum surface tension (γ_{min}, γ_{max}, respectively) were obtained at minimum and maximum surface area, respectively.

Determination of surfactant protein A and its mRNA

Surfactant protein A in BALF was determined with an enzyme-linked immunosorbent assay using a mice monoclonal antibody against rat SP-A (PE-10, Dako Japan, Kyoto, Japan) cross-reacting with rabbit SP-A. We applied this technique according to a modified method as reported by McCormac et al. [23]. Rabbit SP-A mRNA in lung tissue samples was determined with the Northern blot technique using a plasmid vector PGEM3Z (kindly provided by Dr. J.A Whitsett, University of Cincinnati, Ohio), and followed the protocols described by Ohashi et al. [24].

Measurements of nitrite/nitrate and methaemoglobin

Blood samples representing baseline, 0 and 4 h were taken for measurement of nitrite/nitrate using a modified Griess method

with which nitrate was reduced to nitrite by cadmium as described by Shi et al. [25], and nitrite/nitrate in urine was also measured with the same method. Nitrite/nitrate was expressed as μmol/l. MetHb was determined according to the method described by Hegesh et al. [26], and expressed as a percentage of total haemoglobin (Hb).

Histological examination of lungs

After macroscopical examination of the appearance of the lungs, tissue blocks from the left lung of 4–6 animals in each group were fixed by immersion in 4% formaldehyde and embedded in paraffin. Sections stained with haematoxylin and eosin were examined by light microscopy for evidence of atelectasis, interstitial and intra-alveolar oedema, hyaline membranes, haemorrhage and recruitment of inflammatory cells to the alveolar spaces.

Statistics

Data are presented as means and standard deviations (SD). Analysis of variance (ANOVA) was performed for physiological data and other parametric data, and between-group differences were further examined by Student-Newman-Keuls post hoc test. Within-group differences between different time points were determined with Wilcoxon signed-rank test. The Pearson method was applied to test correlation of physiological and biochemical data. A p-value ≤ 0.05 or less was regarded as statistically significant.

Results

General condition of animals

Mean values for body weight in the various groups were 2.6 ± 0.2–2.7 ± 0.3 kg, and baseline values for SAP were 83 ± 4–86 ± 14 mmHg and heart rate 231 ± 30–272 ± 18/min (only the heart rate of the control group was lower than that of the normal group, $p < 0.05$). Baseline values of PaO_2/FIO_2, $PaCO_2$, pH, Cdyn, Rrs and Qs/Qt are shown in Fig. 1 (A-F), and no significant difference was found across the groups except for Qs/Qt, which was significantly lower in the normal, than in the control, group. Eight hours after administration of the major endotoxin dose (corresponding to treatment time 0 h in Fig. 1) the general condition of all the endotoxin-exposed animals deteriorated. Mean values of SAP were 66 ± 21–78 ± 8 mmHg and those for heart rate were variable (177 ± 14–246 ± 13, control vs normal, $p < 0.01$; Surf vs NO and normal, $p < 0.05$). pH values were lower than 7.30 during the whole treatment period, although intravenous Ringer's lactate and sodium bicarbonate were given according to the protocols (mainly adjusted to the urine output and deficiency of base excess). No attempt was made to overcome acidosis by hyperventilation.

Lung function measurements

Values for PaO_2/FIO_2, $PaCO_2$, pH, Cdyn, Rrs and Qs/Qt at 0, 2 and 6 h are shown in Fig. 1 (A–F). During the experiment mean values of V_T were, in all the groups, generally kept at 8–10 ml/kg when PIP was adjusted between 15 ± 1 and 18 ± 3 cmH_2O across the groups over time (not statistically significant between the groups), to maintain $PaCO_2$ within the normal range (Fig. 1B). At 0 h (i.e., 8 h after major dose of endotoxin), the endotoxin-exposed animals showed a significant reduction of PaO_2/FIO_2 from 520 ± 30 to 395 ± 19 mmHg and of dynamic compliance (Cdyn) by more than 40% (from 1.20 ± 0.11 to 0.73 ± 0.05 ml/cmH_2O × kg), and an increase of Rrs by 34% (from 26.0 ± 4.8 to 35.5 ± 5.5 cmH_2O/l × s) and Qs/Qt from 7.5 ± 0.8% to 12.9 ± 1.0% from corresponding baseline levels (all measurements $p < 0.01$ versus baseline). At 6 h of treatment, in the SNO group, PaO_2/FIO_2, Cdyn, Rrs and Qs/Qt were 301 ± 15 mmHg, 0.67 ± 0.05 ml/cmH_2O × kg, 35.9 ± 4.7 cmH_2O/l × s and 16.5 ± 0.8%, compared to 224 ± 26 mmHg, 0.53 ± 0.04 ml/cmH_2O × kg, 42.4 ± 2.3 cmH_2O/l × s and 24.1 ± 2.0% in the control group, respectively (all measurements $p < 0.01$). However, the improvement did not reach the levels comparable to those of the normal group (494 ± 5 mmHg, 1.02 ± 0.05 ml/cmH_2O × kg, 29.8 ± 3.3 cmH_2O/l × s and 9.43 ± 1.3%, respectively, $p < 0.01$ vs SNO group). Both Surf and NO groups showed intermediate levels of these parameters. For the material as a whole, PaO_2/FIO_2 at 6 h was closely correlated to simultaneous recordings of Qs/Qt and Cdyn (correlation coefficient, $r = -0.80$ and $r = 0.83$, respectively, $p < 0.001$), and Qs/Qt was also inversely correlated to Cdyn ($r = -0.79$, $p < 0.001$).

Chemical and cytological analysis of broncho-alveolar lavage fluid

Values for TPL, DSPC/TPL, TP and DSPC/TP in BALF, and for TPL and DSPC/TPL in the large and small aggregates, are shown in Table 1. TPL, DSPC/TPL and DSPC/TP were higher in both the Surf and SNO groups than in the control and NO groups ($p < 0.01$). TP levels in the NO, Surf and SNO groups were lower than in the control ($p < 0.01$). In both Surf and SNO groups administration of surfactant restored DSPC/TPL and DSPC/TP to levels comparable to, or higher than, those in the normal group. Both DSPC/TPL and DSPC/TP were correlated to PaO_2/FIO_2 ($r = 0.53$ and 0.58, respectively), Cdyn ($r = 0.55$ and 0.58), Rrs ($r = -0.47$, -0.48), Qs/Qt ($r = -0.59$, -0.65) and W/D ($r = -0.57$, -0.67) at 6 h of treatment (all measurements $p < 0.01$). W/D was inversely correlated to PaO_2/FIO_2 and Cdyn ($r = -0.84$ and -0.79, $p < 0.001$) and directly correlated to Qs/Qt and Rrs ($r = 0.94$,

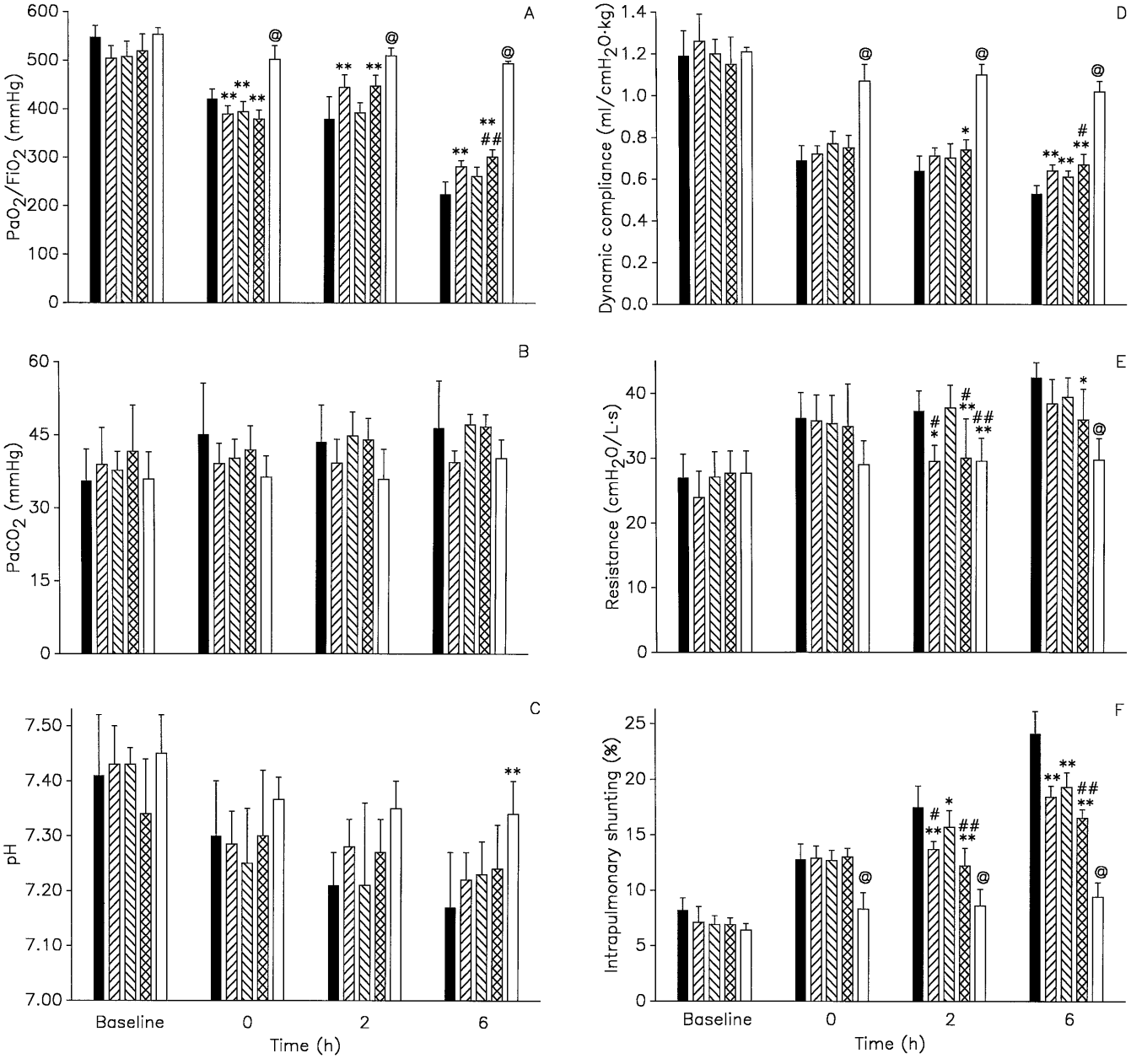

Fig. 1 (**A**) PaO_2/FIO_2, (**B**) $PaCO_2$, (**C**) pH, (**D**) Cdyn, (**E**) Rrs and (**F**) Qs/Qt at baseline and treatment time (*Time*) 0, 2 and 6 h, corresponding to 8, 10 and 14 h after the second (major) dose endotoxin administration. (*filled bar* Control (mechanical ventilation only), *right diagonally hatched bar* Inhaled nitric oxide (NO), *left diagonally hatched bar* Surfactant (Surf), *cross-hatched bar* Surfactant and inhaled NO (SNO), and *blank bar* Normal (not receiving endotoxin)). Bars and ticks are mean values and SD, $n = 5$–8. Values of PaO_2/FIO_2, Cdyn, Rrs and Qs/Qt for groups of Control, NO, Surf and SNO at time 0 h were significantly different from the corresponding baseline values ($p < 0.01$, by within-group Wilcoxon signed-rank test) * $p < 0.05$, ** $p < 0.01$ versus control, # $p < 0.05$, ## $p < 0.01$ versus Surf, @ $p < 0.01$ versus all other groups

$p < 0.001$ and $r = 0.48$, $p < 0.01$, respectively). White blood cell counts are also shown in Table 1. In all groups receiving endotoxin there were significant increases of white blood cells in BALF. Microscopically, these cells were macrophages, monocytes and polymorphonuclear leukocytes. Red blood cells also significantly increased in BALF from all the endotoxin-exposed groups (data not shown).

Table 1 Biochemical and cytological analysis of bronchoalveolar lavage fluid (BALF)

Group	n	TPL (mg/kg)	DSPC/TPL (%)	TP (mg/kg)	DSPC/TP (μg/mg)
Control	8	7.8 ± 0.5	29 ± 2	103 ± 5	21 ± 3
NO	7	8.3 ± 0.4	30 ± 2	81 ± 3**	33 ± 4
Surf	7	27 ± 2**##	42 ± 3**##	94 ± 5**##	124 ± 12**##
SNO	7	30 ± 2**++	46 ± 3**++	71 ± 3**++	184 ± 11**++
Normal	5	12 ± 1@	36 ± 2@	50 ± 2@	94 ± 17@

Group	Large aggregate		Small aggregate		White cell counts in BALF ($\times 10^6$/L)
	TPL (mg/kg)	DSPC/TPL (%)	TPL (mg/kg)	DSPC/TPL (%)	
Control	3.7 ± 0.4	52 ± 3	4.1 ± 0.4	8.0 ± 2.4	19.8 ± 1.3
NO	4.3 ± 0.4	53 ± 3	3.9 ± 0.3	8.8 ± 2.5	13.8 ± 1.1**
Surf	14 ± 1**	65 ± 4**##	12 ± 1.4**##	18 ± 3**##	18.6 ± 1.2**##
SNO	18 ± 2**##++	68 ± 4**##	12 ± 0.6**##	15 ± 4**##	13.5 ± 0.8**++
Normal	7.9 ± 1.2@	54 ± 2++	4.2 ± 0.3++	14 ± 1**##	0.6 ± 0.1@

Definition of abbreviations: TPL = total phospholipids, DSPC = disaturatedphosphatidyl-choline, TP = total proteins, Control = mechanical ventilation only, NO = inhaled NO, Surf = surfactant, SNO = surfactant and inhaled NO, and Normal = not receiving endotoxin. Values are means and SD.

** $p < 0.01$ versus Control;
$p < 0.01$ versus NO;
++ $p < 0.01$ versus Surf;
@ $p < 0.01$ versus all other groups

Table 2 Minimum and maximum surface tension (γ_{min}, γ_{max}, respectively) of bronchoalveolar lavage fluid (n = 4) at 60th cycle, and wet-to-dry lung weight ratio (W/D)

Group	γ_{min} (mN/m)	γ_{max} (mN/m)	W/D
Control	21 ± 1	41 ± 1	4.37 ± 0.11
NO	26 ± 3	42 ± 3	3.83 ± 0.13**
Surf	3 ± 2**##	32 ± 1**##	4.02 ± 0.10**##
SNO	14 ± 5##+	35 ± 1**##	3.59 ± 0.11**##++
Normal	10 ± 9*##	36 ± 3	2.13 ± 0.08@

Control = mechanical ventilation only, NO = inhaled NO, Surf = surfactant, SNO = surfactant and inhaled NO, and Normal = not receiving endotoxin. Values are means and SD (n = 4 for surface tension, n = 5–8 for W/D).
* $p < 0.05$, ** $p < 0.01$ versus Control;
$p < 0.01$ versus NO;
+ $p < 0.05$, ++ $p < 0.01$ versus Surf;
@ $p < 0.01$ versus all other groups

Surface tension of broncho-alveolar lavage fluid and wet-to-dry lung weight ratio measurements

Values for surface tension of BALF and W/D are shown in Table 2. The lowest values for γ_{min} and γ_{max}, were seen in the Surf group. The SNO group also had lower surface tension than in the control and NO groups. Values for W/D in the NO, Surf and SNO groups were lower than in the control group, and the lowest average W/D value was found in the SNO group.

Measurements of methaemoglobin and nitrite/nitrate

Methaemoglobin in each group was less than 1 % of the Hb at baseline, and less than 3 % throughout inhalation of NO in both NO and SNO groups. Values for serum and urinary nitrite/nitrate are presented in Table 3. Baseline levels of serum nitrite/nitrate were about the same in all groups, and increased at 0 h by approximately 50 % compared to the respective baseline values ($p < 0.01$). During treatment there was a further increase of serum nitrite/nitrate in all groups exposed to endotoxin. In contrast, at baseline, mean levels of urinary nitrite/nitrate in the four groups of endotoxin (first and low dose) exposed animals were about 2–3 times as high as in the normal group, with larger variations. In the NO and SNO groups inhalation of NO was associated with moderately increased nitrite/nitrate in serum and urine. A moderate increment of urinary, but not of serum, nitrite/nitrate was found in the normal group subjected to neither endotoxin nor iNO.

Measurement of surfactant protein A and its mRNA in the lungs

Contents of SP-A in BALF and SP-A mRNA in lung tissue are shown in Table 4. Values for both SP-A and SP-A mRNA were significantly lower in all animals receiving endotoxin than in the normal group (all measurements, $p < 0.01$). SP-A in BALF was higher in the SNO group than in the control group ($p < 0.01$). Levels of SP-A mRNA in the NO and

Table 3 Nitrite/nitrate in serum and urine samples

Group	Serum nitrite/nitrate (μmol/L)			Urinary nitrite/nitrate (μmol/L)		
	Baseline	0 h	4 h	Baseline	0 h	4 h
Control	52 ± 7	73 ± 6	93 ± 6	531 ± 431	763 ± 531	978 ± 568
NO	51 ± 8	74 ± 6	114 ± 11**	499 ± 417	670 ± 389	982 ± 579
Surf	48 ± 8	74 ± 7	96 ± 9##	481 ± 257	593 ± 316	879 ± 450
SNO	51 ± 7	75 ± 7	118 ± 9**++	637 ± 343	723 ± 202	1028 ± 337
Normal	50 ± 8	52 ± 7@	53 ± 8@	180 ± 59	234 ± 83	352 ± 147

Definition of abbreviations: Control = mechanical ventilation only, NO = inhaled NO, Surf = surfactant, SNO = surfactant and inhaled NO, and Normal = not receiving endotoxin. Values are means and SD (n = 5–8).

** $p < 0.01$ versus Control;
$p < 0.01$ versus NO;
+ $p < 0.05$, ++ $p < 0.01$ versus Surf;
@ $p < 0.01$ versus all other groups

Table 4 Contents of surfactant protein A (SP-A) and expression of SP-A mRNA in the lungs

Group	SP-A (μg/kg)	SP-A mRNA
Control	80.8 ± 18.2	50.3 ± 8.9
NO	92.6 ± 11.5	49.8 ± 9.4
Surf	91.8 ± 18.0	49.7 ± 7.1
SNO	108.9 ± 18.8**	47.6 ± 6.1
Normal	173.7 ± 15.5@	82.1 ± 12.9@

Control = mechanical ventilation only, NO = inhaled NO, Surf = surfactant, SNO = surfactant and inhaled NO, and Normal = not receiving endotoxin. Values are means and SD (n = 5–8).
** $p < 0.01$ versus Control; @ $p < 0.01$ versus other 4 groups

SNO groups were not different from those in the control and Surf groups.

Histological examination of the lungs

Macroscopically, the lungs of animals in the control, NO, Surf and SNO groups were atelectatic, congested and oedematous with haemorrhagic spots on the pleural surface. In the normal animals no such changes were found and the lungs were generally well aerated. Microscopically, atelectasis, intra-alveolar and interstitial oedema, patchy alveolar haemorrhage, hyaline membranes and recruitment of leukocytes to the alveolar spaces were found in all animals receiving endotoxin, but not in the normal group. The intra-alveolar inflammatory cells were identified as predominantly macrophages and polymorphonuclear leukocytes. The inflammatory changes tended to be less severe and alveolar aeration much improved in the Surf and SNO groups in comparison with the control and NO groups. Representative photomicrographs of the lungs from each group are presented in Fig. 2.

Discussion

Evaluating different treatment protocols in a rabbit model of endotoxin induced ALI, we found that early intervention with surfactant plus iNO mitigated deterioration of lung function and attenuated lung injury without causing adverse effects. Animals receiving this combined treatment showed a moderate improvement of several parameters reflecting gas exchange, pulmonary mechanics, surfactant composition and function, and lung morphology. However, there is no substantial advantage of combined treatment compared to surfactant or iNO alone. In general, surfactant facilitates recruitment of gas exchange units, increases endogenous NO production [27] and inhibits inflammatory cell-mediated injury to the lungs [28]. Furthermore, recent studies have revealed that iNO causes down-regulation of the inflammatory process in the lungs [29] and improvement of gas exchange in severe hypoxic respiratory failure [30]. In view of these potential advantages, the combination of these two therapies for prevention or treatment of inflammatory lung injury should be considered.

The animal ALI model used in this study was well characterised by physiological, biochemical and histopathological criteria. In the pilot study we found accumulation of neutrophils in the lungs 12 h after a low dose of endotoxin priming and evidence of severe ALI within 12 h after the major endotoxin infusion. Since we wanted to administer surfactant and/or iNO before ALI was fully established, we initiated treatment 8 h after the major endotoxin infusion. This can be considered an "early" intervention aimed at preventing further deterioration of lung function. In contrast to other ALI models [31, 32, 33, 34, 35] with either endotoxin priming or sustained endotoxin infusion for many hours or days, or intratracheal instillation of endotoxin, the method we applied in the present study (a low dose of endotoxin for priming and a major dose for challenge 12 h apart), consistently induced ALI with respiratory failure in the subsequent 12–14 h, facilitating comparison of the various therapies. The low priming dose of endotoxin potentiat-

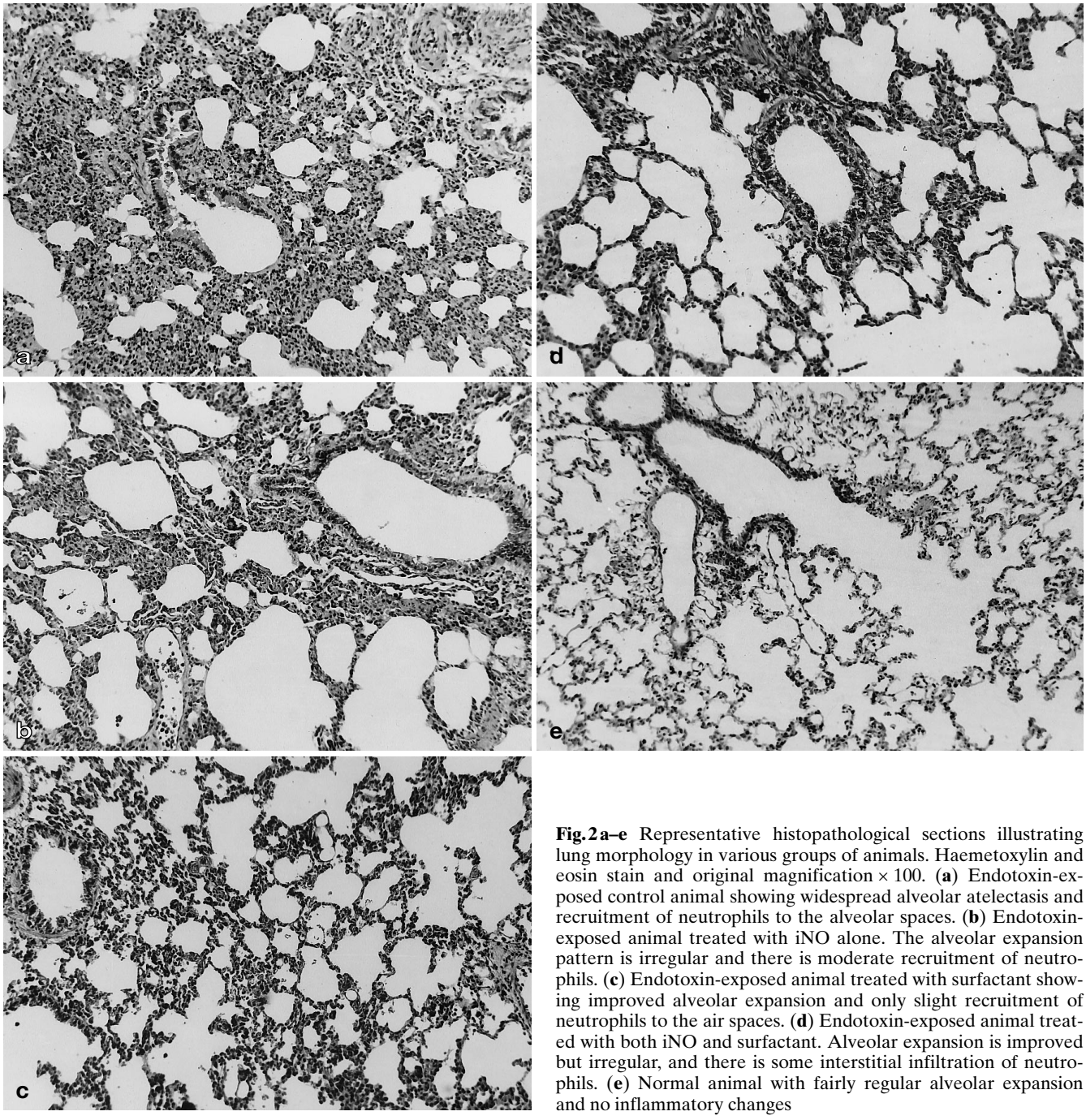

Fig. 2 a–e Representative histopathological sections illustrating lung morphology in various groups of animals. Haemetoxylin and eosin stain and original magnification × 100. (**a**) Endotoxin-exposed control animal showing widespread alveolar atelectasis and recruitment of neutrophils to the alveolar spaces. (**b**) Endotoxin-exposed animal treated with iNO alone. The alveolar expansion pattern is irregular and there is moderate recruitment of neutrophils. (**c**) Endotoxin-exposed animal treated with surfactant showing improved alveolar expansion and only slight recruitment of neutrophils to the air spaces. (**d**) Endotoxin-exposed animal treated with both iNO and surfactant. Alveolar expansion is improved but irregular, and there is some interstitial infiltration of neutrophils. (**e**) Normal animal with fairly regular alveolar expansion and no inflammatory changes

ed endogenous NO production, as reflected by elevated urinary nitrite/nitrate levels, without causing septic shock. The development of ALI due to this low dose endotoxin priming and high dose endotoxin challenging may be attributed to an increase of prostacyclin in the lungs and damage of pulmonary endothelium [15].

The role of exogenous surfactant was investigated in the present study. We analysed surfactant phospholipids in BALF and tested surface activity of the lavage fluid. In general, exogenous surfactant elevated TPL by more than 1–2 times compared to normal and endotoxin-exposed animals not receiving surfactant, increased DSPC/TPL and DSPC/TP, and reduced dynamic surface tension significantly. We also separated the large aggregates from the small ones in BALF, and found that most of the DSPC fraction remained in the large aggre-

gation form in both endotoxin-exposed and normal animals. Exogenous surfactant had no effects on the white cell counts of BALF, but endotoxin provoked accumulation of white blood cells in the alveolar compartment. Endotoxin may inhibit surfactant phospholipid production [6, 7] as illustrated in our study by reduced TPL, DSPC/TPL, DSPC/TP and SP-A in BALF, and attenuated expression of surfactant protein mRNA in the endotoxin-exposed lungs. NO also inhibits DSPC synthesis by cultured type II alveolar epithelial cells [36]. These defects should, to some extent, be compensated for by exogenous surfactant. However, surfactant had limited effects, possibly due to inhibition by both endotoxin and iNO in the lungs. A recent study by Lutz et al. [37] has shown that exogenous surfactant, delivered as an aerosol, attenuates intrapulmonary leukocyte infiltration and pulmonary arterial pressure elevation and prevents hypoxaemia and intrapulmonary shunting induced by endotoxin. The surfactant-mediated modulation of pulmonary haemodynamics is, at least in part, related to endogenous NO production, as proposed by Yu et al. [27]. As levels of SP-A mRNA in the NO and SNO groups were not significantly lower than those in the control and Surf groups, and the DSPC level in the NO groups was not significantly lower than that in the control group, there is no direct evidence that synthesis of SP-A and DSPC is inhibited by NO under the present experimental conditions.

Increased intra-alveolar permeability and exudative damage to the lung are important factors in the pathophysiology of ALI induced by endotoxin. The role of NO in the maintenance of the pulmonary microvascular barrier function and permeability is controversial [38, 39, 40]. In acute inflammatory lung disease increased release of cytokines and proteases by neutrophils and alveolar macrophages aggravate endogenous surfactant dysfunction. Upgrading the surfactant pool may be helpful in these conditions. Thus, increased surfactant phospholipids-to-TP level in the present study correlated with lung mechanics and gas exchange, decreased intrapulmonary shunting and vascular-to-alveolar permeability, and enhanced lung fluid clearance, probably predicting a better outcome. Whether the use of exogenous surfactant has any protective role against the potentially adverse effects of iNO remains unclear. Our data from direct measurement of the surface activity of phospholipids from BALF revealed that γmin was lowest in the Surf group, higher in the SNO group and definitely not improved in the NO group, suggesting that exogenous surfactant altered in vivo surface activity in the lungs, and that iNO alone does not have any effects on surfactant function.

In summary, low dose priming followed by intravenous challenge with endotoxin resulted in ALI and respiratory failure in adult rabbits. Early administration of iNO together with surfactant mitigated the neutrophil-mediated inflammatory process, reduced pulmonary ventilation-perfusion mismatching, surfactant dysfunction and permeability and improved alveolar air expansion and histological sections. More work is required to evaluate the long-term efficacy of this combined treatment for prevention of sepsis-induced inflammatory lung injury.

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