Meconium aspiration increases iNOS expression and nitrotyrosine formation in the rat lung

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Background: **Despite it is recognized that high level of nitrotyrosine formation may play a role in acute lung injury (ALI), few studies have assessed protein nitration in ALI after meconium aspiration. This study was undertaken to observe the expression of inducible nitric oxide synthase (iNOS) and formation of nitrotyrosine in ALI after meconium aspiration and evaluate the contribution of iNOS and nitrotyrosine to tissue injury.**

Methods: **Sixteen healthy male Sprage-Dawley rats were divided randomly into control group and meconium group, which were administrated intratracheally 1 ml/kg saline or 20% human newborn meconium suspension respectively. The animals were sacrificed 24 hours after treatment. Bronchoalveolar lavage fluid (BALF) cell count, BALF protein, pulmonary myeloperoxidase (MPO) activity, malondialdehide (MDA), and nitrate/nitrite levels were measured. Western blot was used to determine the expression of pulmonary nitrotyrosine, a specific "footprint" of peroxynitrite and iNOS. Lung injury score was also evaluated.**

Results: **Compared with the control group, the rats in the meconium group showed an increase in cell count (mean±SD 4.04±1.01 vs 0.53±0.19×10^6/ml, *P*<0.01), BALF protein (mean±SD 2.54±0.74 vs 0.67±0.26 mg/L, *P*<0.01), pulmonary MPO activity (mean±SD 1.49±0.22 vs 0.62±0.16 U/g wet lung, *P*<0.01), MDA level (mean±SD 3.30±0.85 vs 1.40±0.35 nmol/mg protein, *P*<0.01), nitrate/nitrite level (mean±SD 12.77±5.00 vs 4.89±1.32 μmol/mg protein, *P*<0.01), and lung injury score (9.88±1.36 vs 2.25±1.04, *P*<0.01). Western blot examination demonstrated increased expression of nitrotyrosine and iNOS in the meconium group (mean±SD 0.46±0.19 vs 0.11±0.08 and 1.49±0.60 vs 0.13±0.11, respectively, *P*<0.01).**

Conclusions: **Meconium causes increased expression of pulmonary iNOS, leading to over production of NO and nitrotyrosine, which may be of pathogenic importance in ALI after meconium aspiration.**

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Key words: **meconium;**
lung injury;
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nitric oxide synthase

Introduction

Meconium aspiration syndrome (MAS) remains one of the principal causes of neonatal respiratory distress, frequently leading to respiratory failure, persistent pulmonary hypertension of the newborn (PPHN) or death.[1] Meconium-induced lung injury is associated with many pulmonary changes that contribute to respiratory failure, including airway obstruction, inflammatory cell infiltration with the release of bioactive mediators and surfactant dysfunction. It is firmly established that pulmonary interstitial inflammation and inactivation of surfactant are the underlying causes of acute respiratory distress syndrome (ARDS) and pulmonary hypertension.[2-4] Many animal and human studies focused on the efficiency of exogenous surfactant in treating meconium aspiration[5-7] and pulmonary inflammation with meconium,[8-10] but the mechanism of surfactant inactivity in meconium-induced respiratory failure is not clear.

Previous study *in vitro* has shown that meconium is able to induce mRNA expression of inducible nitric oxide synthase (iNOS) followed by a high level of NO production.[10] Reaction of NO with superoxide anions produces peroxynitrite ($ONOO^-$), which is a highly oxidative species capable of nitrating tyrosine residues of numerous proteins, leading to the formation of nitrotyrosine. Studies have suggested that an increased endogenous NO production can potentiate lung injury by promoting oxidative or nitrosative stress, stimulating

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inflammation, and inactivating surfactant.[11-13] The relationship of $ONOO^-$ with acute lung injury (ALI) has been the focus of numerous animal or human studies. Despite several studies suggested that the high level of nitrotyrosine formation plays a role in ALI, few studies have assessed protein nitration in ALI after meconium aspiration.

Furthermore, pulmonary inflammation with meconium aspiration is peaked during 16-24 hours.[8] Mechanical support with oxygen may also induce lung inflammation and injury. To better understand the pathogenesis of acute lung injury caused by meconium, we established a rat model of meconium-induced ALI with neither O_2 supplement nor mechanical support and observed the changes of pulmonary inflammatory response, formation of nitrotyrosine and expression of iNOS in 24 hours after meconium aspiration.

In this study, we hypothesized that meconium would increase pulmonary NO and nitrotyrosine by inducing the expression of pulmonary iNOS.

Methods

Meconium preparation

The first-passed meconium taken from urine-free diapers of normal term infants was placed in sterile jar and frozen for less than 7 days and then lyophilized, pooled, and diluted with sterile saline solution to a final concentration of 20% suspension. This meconium slurry was stored in 2 ml aliquots and frozen at -80℃ until use.

Animal management

Sprague-Dawley rats were obtained from the Animal Center of the Zhejiang Medical Institute, Hangzhou, China. Healthy male rats of 130-170 g (30-45 days old) were utilized for the present experiments. Meconium was administered to the respiratory tract of rats as the described previously.[8] Briefly, the rats were slightly anesthetized after intraperitoneal injection of 40 mg/kg pentobarbital. A small midline incision was made on the ventral aspect of the neck to expose the trachea, while an endotracheal tube was placed through the incision. The tube was then stabilized. Prior to meconium instillation, the rats were injected intraperitoneally 2 mg/kg vecuronium bromide. After 15 minutes, the rats were divided randomly into control group and meconium group (n=8, each). Afterwards, 1 ml/kg sterile saline or 20% meconium was instilled through the endotracheal tube, followed by a 3 ml bolus of air to disperse the meconium into the lung. Half the dose was given with the rat lying on one side and half with the rat lying on the other side. Then, skin and tracheal incisions were closed with 4-0-nylon suture and all the rats were allowed to breathe spontaneously in room air.

Sample collection and storage

Twenty-four hours after surgery, the rats were killed. The chest of the animals was opened by a midline incision, the lungs were isolated, and the lavage of the left lung was performed. Tissue samples from the right lung were obtained.

Total cell count and protein concentration of bronchoalveolar lavage fluid (BALF)

Through tracheostomy, the left lung was lavaged using three 1-ml aliquots of cold sterile saline, each of which was instilled and withdrawn 3 times, and then mixed. The recovered total BALF was always more than 90%. A total cell count of mixed BALF was obtained within 4 hours. Protein concentration of BALF was determined by the Bradford protein assay.[14]

Pulmonary myeloperoxidase (MPO) activity

As a measure of pulmonary neutrophil influx and activity, the MPO activity was assessed in duplicate on homogenized lung samples according to the manufacture's manual. One unit of the MPO activity was expressed as units per gram wet lung tissue.

Pulmonary malondialdehide (MDA) assay

Pulmonary MDA levels were assayed in duplicate on homogenized lung samples. The results were standardized for tissue protein concentration determined by the Bradford protein assay.[14]

Pulmonary nitrate/nitrite assay

Pulmonary nitrate/nitrite levels were assayed in duplicate on homogenized lung samples according to the manufacture's manual. The results were standardized for tissue protein concentration determined by the Bradford protein assay.[14]

Western blot analysis of nitrotyrosine formation and iNOS protein expression

Lung tissues were homogenized with 5 volumes of lysis buffer containing 1% (vol/vol) NP-40, 0.5% (wt/vol) sodium deoxycholate, and 0.1% (wt/vol) sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS), to which a protein inhibitor, phenylmethylsulfonyl fluoride

100 μg/ml, was added. The homogenate was left on ice for 30 minutes before centrifugation at 10000 × g. Protein content was estimated, and aliquots of 50 μg of homogenate protein were mixed a ratio of 1:1 with 2×sample buffer containing 125 mmol Tris, 20% (wt/vol) glycerol, and 4% (wt/vol) SDS. Separated on 10% (wt/vol) PAGE gel the mixture was transferred to nitrocellulose-ECL membrane, and stained with Coommassie blue to verify the equivalent transfer of samples. The membrane was blocked by incubation with 3% (wt/vol) fat dry milk in PBS for 1 hour at room temperature in shaking. Thereafter, the membrane was incubated overnight at 4℃ with either mouse monoclonal anti-nitrotyrosine (Calbiochem-Novabiochem Co., San Diego, USA) or rabbit polyclonal anti-iNOS or anti-actin (Santa cluz, USA). The primary antibodies were used at dilutions of 1:500, 1:1000 or 1:1000. The blots were washed four times (10 minutes each time) with Tris-buffer saline Tween-20 (TBST), comprising 50 mmol Tris-HCl (pH 7.6), 150 mmol NaCl and 0.1% Triton X-100, and incubated subsequently for 2 hours at room temperature in Tris-buffered saline with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (1:5000 dilution; Pierce). Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) system (Pierce) and quantified by densitometric analysis, using Scion image software. The results were calculated as a relative ratio of specific band to the actin one.

Histology

For histological analysis of the lung, pulmonary tissues taken from the right lobe were fixed in buffered formalin. The tissues were dehydrated, embeded in paraffin and selected. The sections in 5 μm thickness were stained with hematoxylin and eosin for light microscopic analysis. To determine the extent and severity of lung tissue injury, the samples were assessed by a pathologist blinded to the grouping of rats. A score from 0 to 4 was assigned for each of three different characteristics:[15] (i) the extension of leukocyte infiltration: 0=0%, 1=0-25%, 2=25%-50%, 3=50%-75%, and 4=75%-100% of the section areas occupied by leukocytes; (ii) the amount of intra-alveolar leukocytes: 0=no, 1=occasional, 2=several leukocytes in the alveoli; 3=alveoli almost full of leukocytes, and 4=alveoli distended by tightly packed leukocytes; and (iii) the amount of exudative debris, including fibrin, hyaline membranes, edema fluid and meconium: 0=alveoli open, 1=exudate scarcely seen, 2=exudate clearly visible, 3=alveoli almost full of exudate, and 4=alveoli distended by exudate. The calculated total injury score was considered the sum of these scores.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Student's *t* test was used to detect the difference between the groups. The Mann-Whitney μ test was used for the analysis of lung injury score. A *P* value less than 0.05 was considered statistically significant.

Results

Histological findings

The control lungs were normal and essentially well aerated (Fig. 1A). Meconium instillation induced a marked inflammatory reaction. The alveoli contained various amounts of leukocytes, fibrin and occasionally epithelial cells of instilled meconium. The lungs from the meconium group appeared to be edematous and had patchy areas of hemorrhage (Fig. 1B). Lung injury score increased more significantly in the meconium group than in the control group (mean±SD 9.88±1.36 vs 2.25±1.04, $P<0.01$, Fig. 1C).

Pulmonary MPO activity and BALF cell count

Pulmonary MPO activity, a marker of leukocyte infiltration, was increased more significantly in the meconium group than in the control group (mean ±SD 1.49±0.22 vs 0.62±0.16 U/g wet lung, $P<0.01$). At the same time, the total cell count of BALF was significantly increased in the meconium group (mean ±SD 4.04±1.04 vs 0.53±0.19×10^6 cells/ml, $P<0.01$) (Table).

BALF protein concentration

Compared with the control group, the rats in the meconium group were characterized by significantly increased BALF protein concentration (mean±SD 2.54 ±0.74 vs 0.67±0.26 mg/L, $P<0.01$) (Table).

Pulmonary MDA and nitrate/nitrite level

Compared with the control group, the rats in the meconium group showed a marked increase in pulmonary MDA level (mean±SD 3.30±0.85 vs 1.40± 0.35 nmol/mg protein, $P<0.01$) and nitrate/nitrite level (mean±SD 12.77±5.00 vs 4.89 ±1.32 μmol/mg protein, $P<0.01$) (Table).

Pulmonary nitrotyrosine and expression of iNOS

Western blot analysis of nitrotyrosine demonstrated a prominent band of nitrotyrosine and iNOS at approximately 60 kD and 115 kD, respectively. The intensity of both nitrotyrosine and iNOS bands was greater in the meconium group than in the control group, indicating that meconium-induced ALI increases the formation of pulmonary nitrotyrosine (0.46±0.19 vs 0.11±0.08, $P<0.01$) and the expression of pulmonary iNOS (mean±SD 1.49±0.60 vs 0.13±0.11, $P<0.01$) (Fig. 2).

Discussion

The results of the present study have shown that meconium aspiration is characterized by an increase in pulmonary MDA level (a marker of oxidative injury) and leakage alveolocapillary protein, and that meconium also induces pulmonary iNOS protein expression and overproduction of NO. For the first time, we found that nitrotyrosine, a marker of peroxynitrite production, is increased in patients with ALI due to meconium aspiration.

Meconium contains such noxious agents as cholesterol, free fatty acids and bilirubin, which may initiate inflammatory reaction in the lungs and interfere with surfactant function. Previous *in vitro* studies demonstrated that surfactant was inactivated by both water and choloroform-soluble fractions of meconium;[16,17] but the pathogenic mechanisms of this alteration are not elucidated.

The mechanism of activated neutrophils in patients with ALI caused by meconium aspiration has been well investigated. Influx of neutrophils into the airspaces is a consistent feature of ALI after various direct and indirect insults. Besides neutrophils, pulmonary macrophages including intra-alveolar and pulmonary vascular macrophages were shown to be involved in the pathogenesis of ALI.[4,18,19] It was reported that meconium could induce iNOS gene expression and lead to a high output of NO from alveolar macrophages in a dose-dependent manner. Moreover, it has a direct effect, not via cytokine production, on the NO

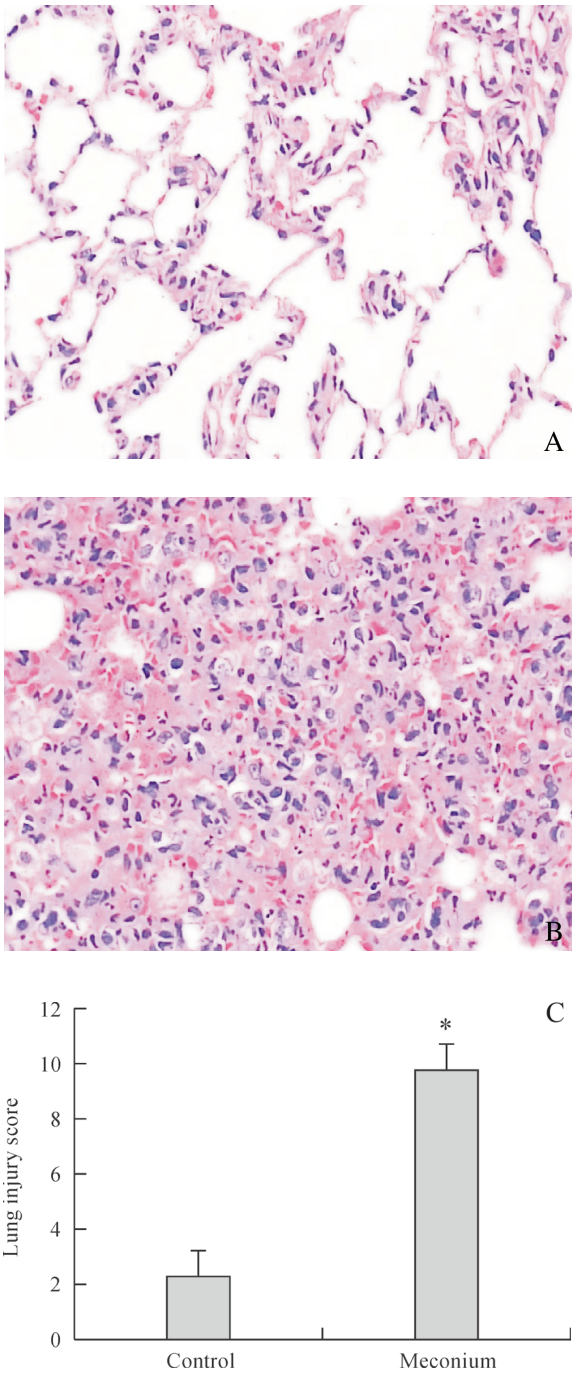

Fig. 1. Histological findings. **A**: In the control group, the lungs were normal and essentially well aerated. **B**: In the lungs of the meconium group, alveoli spaces filled with neutrophils. **C**: Lung injury score increased in the meconium vs control group ($P<0.01$).

Table. Comparisons of cell count of BALF and protein, pulmonary MPO activity, MDA and nitrate/nitrite level in the rats of the 2 groups (means ±SD, $n=8$)

Group	Cell count (×10^6/ml)	Protein (mg/L)	MPO (U/g wet lung)	MDA (nmol/mg prot)	Nitrate/nitrite (μmol/mg prot)
Meconium	4.04±1.01*	2.54±0.74*	1.49±0.22*	3.30±0.85*	12.77 ±5.00*
Control	0.53±0.19	0.67±0.26	0.62±0.16	1.40±0.35	4.89 ±1.32

*: vs the control group, $P<0.01$.

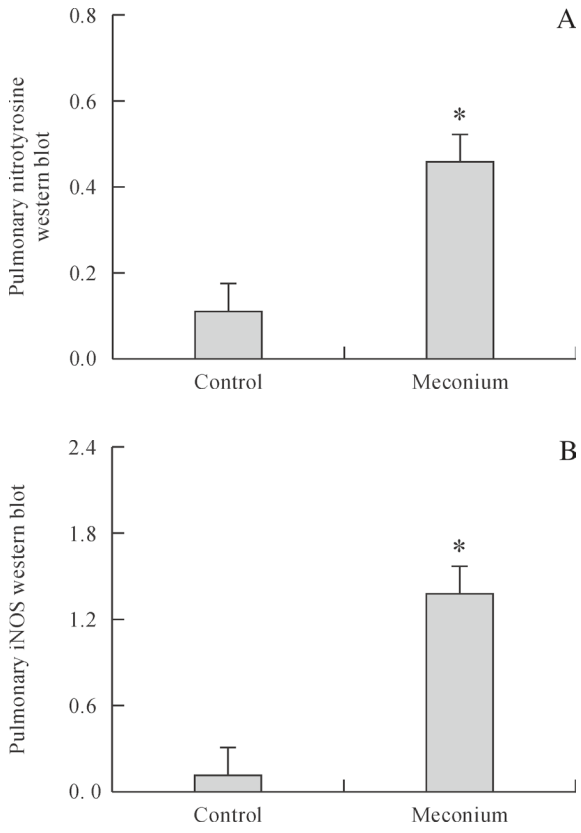

Fig. 2. A prominent band of nitrotyrosine protein at approximately 60 kD shown by western blot analysis of nitrotyrosine. **A**: The intensity of the band is greater in the meconium vs control group (P<0.01). **B**: A prominent band of iNOS protein at approximately 115 kD shown by western blot analysis of iNOS. The intensity of the band is greater in the meconium vs control group (P<0.01).

production. Several mechanisms through which high levels of NO are produced by iNOS, can mediate lung injury. Over production of endogenous NO may cause an increased permeability of vascular endothelial cell interspaces, inhibit leukocyte adhesion, degrade carbohydrates, and induce lipid peroxidation.[20] The results of this study have shown that meconium could induce iNOS protein expression and overproduction of NO and MDA.

The generation of oxidative and nitrosative species is a principal cause of inflammatory injury. The term of nitrosative species refers to NO, peroxynitrite ($ONOO^-$), and nitrogen dioxide. As a free radical, NO is oxided, reduced, or complexed with other biomolecules, depending on the microenvironment. Reaction of NO with superoxide anions produces peroxynitrite, which is a highly oxidative species that is capable of nitrating tyrosine residues of numerous proteins, leading to the formation of nitrotyrosine and finally protein inactivation.[11,21] Nitrotyrosine is a stable end-product of reactive nitrogen species (RNS)-mediated reactions. Therefore, it serves as footprints of RNS action. High levels of nitrotyrosine formation have been shown to be involved in ALI in humans and LPS-injected animals. It can diminish the function of a variety of crucial proteins, including pulmonary surfactant protein A (SP-A) and manganese superoxide dismutase.[22] The results of previous *in vitro* studies indicated that the nitration of human SP-A by $ONOO^-$ inhibits its lipid aggregation and mannose binding activity. This may be one of the factors responsible for the increased susceptibility of patients with ARDS to nosocomial infections.[21,23]

In vitro studies have indicated that the neutrophils and plasma proteins accumulating in the alveoli as a result of the inflammatory response are potential inhibitors of surfactant.[4,6] On the other hand, it is speculated that the nitration of surfactant protein may be another cause of surfactant inactivation from the results of this study.

In conclusion, instillation of human meconium into the animal lung induces up-regulation of lung iNOS protein expression, thus leading to the overproduction of NO and the formation of nitrotyrosine. Nitration of surfactant protein may be another cause of surfactant inactivation that is related to ARDS and pulmonary hypertension caused by meconium aspiration. The consequence of protein nitration in patients with ALI due to meconium aspiration remains to be determined.

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References

1 Finer NN. Surfactant use for neonatal lung injury: beyond respiratory distress syndrome. Pediatr Respir Rev 2004; 5(Suppl A):S289-297.
2 Vidyasagar D, Lukkarinen H, Kaapa P, Zagariya A. Inflammatory response and apoptosis in newborn lungs after meconium aspiration. Biotechnol Prog 2005;21:192-197.

3 Oh MH, Bae CW. Inhibitory effect of meconium on pulmonary surfactant function tested *in vitro* using the stable microbubble test. Eur J Pediatr 2000;159:770-774.
4 Fuloria M, Wu Y, Brandt ML, Rubin BK. Effect of meconium on the surface properties of perflubron. Pediatr Crit Care Med 2004;5:167-171.
5 Hu XW, Zhu YR, Lu Y, Lam LK, Zhang LE, Shao XM, et al. Effects of combined surfactant and inhaled nitric oxide in ventilated rabbits with meconium aspiration-induced acute lung injury. Chin J Pediatr 2003;41:761-765.
6 Lu KW, Taeusch HW, Robertson B, Goerke J, Clements JA. Polymer-surfactant treatment of meconium-induced acute lung injury. Am J Respir Crit Care Med 2000;162:623-628.
7 Wiswell TE, Knight GR, Finer NN, Donn SM, Desai H, Walsh WF, et al. A multicenter, randomized, controlled trial comparing Surfaxin (Lucinactant) lavage with standard care for treatment of meconium aspiration syndrome. Pediatrics 2002;109:1081-1087.
8 Zagariya A, Bhat R, Uhal B, Navale S, Freidine M, Vidyasagar D. Cell death and lung cell histology in meconium aspirated newborn rabbit lung. Eur J Pediatr 2000;159:819-826.
9 Korhonen K, Soukka H, Halkola L, Peuravuori H, Aho H, Pulkki K, et al. Meconium induces only local inflammatory lung injury in piglets. Pediatr Res 2003;54:192-197.
10 Li YH, Yan ZQ, Brauner A, Tullus K. Meconium induces expression of inducible NO synthesis and activation of NF-kB in rat alveolar macrophages. Pediatr Res 2001;49:820-825.
11 Kooy NW, Royall JA, Ye YZ, Kelly DR, Beckman JS. Evidence for *in vivo* peroxynitrite production in human acute lung injury. Am J Respir Crit Care Med 1995;151:1250-1254.
12 Zhu S, Ware LB, Geiser T, Matthay MA, Matalon S. Increased levels of nitrite and surfactant protein a nitration in the pulmonary edema fluid in patients with acute lung injury. Am J Respir Crit Care Med 2001;163:166-172.
13 Zsengeller ZK, Ross GF, Trapnell BC, Szabo C, Whitsett JA. Adenovirus infection increases iNOS and peroxynitrite production in the lung. Am J Physiol Lung Cell Mol Physiol 2001;280:L503-511.
14 Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-254.
15 Holopainen R, Aho H, Laine J, Halkola L, Kaapa P. Nitric oxide inhalation inhibits pulmonary apoptosis but not inflammatory injury in porcine meconium aspiration. Acta Paediatr 1999;88: 1147-1155.
16 Meister J, Balaraman V, Ramirez M, Uyehara CF, Killeen J, Ku T, et al. Lavage administration of dilute surfactant in a piglet model of meconium aspiration. Lung 2004;182:227-240.
17 Lu KW, Goerke J, Clements JA, Taeusch HW. Hyaluronan reduces surfactant inhibition and improves rat lung function after meconium injury. Pediatr Res 2005;58:206-210.
18 Berdeli A, Akisu M, Dagci T, Akisu C, Yalaz M, Kultursay N. Meconium enhances platelet-activating factor and tumor necrosis factor production by rat alveolar macrophages. Prostaglandins Leukot Essent Fatty Acids 2004;71:227-232.
19 Long XY, Du LZ, Yang LF, Shen HQ. Effects of inhaled nitric oxide on rabbits with meconium aspiration pneumonia. Chin J Pediatr 2003;41:570-573.
20 Kristof AS, Goldberg P, Laubach V, Hussain SN. Role of inducible nitric oxide synthase in endotoxin-induced acute lung injury. Am J Respir Crit Care Med 1998;158:1883-1889.
21 Zhu S, Kachel DL, Martin WJ 2nd, Matalon S. Nitrated SP-A does not enhance adherence of Pneumocystis carinii to alveolar macrophages. Am J Physiol 1998;275(6 Pt 1): L1031-1039.
22 MacMillan-Crow LA, Crow JP, Kerby JD, Beckman JS, Thompson JA. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. Proc Natl Acad Sci USA 1996;93:11853-11858.
23 Gole MD, Souza JM, Choi I, Hertkorn C, Malcolm S, Foust RF 3rd, et al. Plasma protein modified by tyrosine nitration in acute respiratory distress syndrome. Am J Physiol Lung Cell Mol Physiol 2000;278:L961-967.

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