

N-Acetylcysteine Advancement of Surfactant Therapy in Experimental Meconium Aspiration Syndrome: Possible Mechanisms

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Summary

Meconium aspiration syndrome (MAS) is meconium-induced respiratory failure of newborns associated with activation of inflammatory and oxidative pathways. For severe MAS, exogenous surfactant treatment is used which improves respiratory functions but does not treat the inflammation. Oxidative process can lead to later surfactant inactivation; hence, surfactant combination with antioxidative agent may enhance the therapeutic effect. Young New Zealand rabbits were instilled by meconium suspension and treated by surfactant alone, N-acetylcysteine (NAC) alone or by their combination and oxygen-ventilated for 5 h. Blood samples were taken before and 30 min after meconium application and 30 min, 1, 3 and 5 h after the treatment for evaluating of oxidative damage, total leukocyte count, leukocyte differential count and respiratory parameters. Leukocyte differential was assessed also in bronchoalveolar lavage fluid. NAC alone had only mild therapeutic effect on MAS. However, the combination of NAC and surfactant facilitated rapid onset of therapeutic effect in respiratory parameters (oxygenation index, PaO₂/FIO₂) compared to surfactant alone and was the only treatment which prevented neutrophil migration into the lungs, oxidative damage and lung edema. Moreover, NAC suppressed IL-8 and IL-β formation and thus seems to be favorable agent for improving surfactant therapy in MAS.

Key words

Meconium aspiration • Surfactant inactivation • N-acetylcysteine • Oxidative damage • Neutrophils

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Introduction

Meconium is the first newborn's stool containing huge number of substances including triglycerides, cholesterol and fatty free acids, bile acids, bilirubin, dietary fiber, hemoglobin, proteins and proinflammatory cytokines interleukin (IL) 1β, IL-6, IL-8 or tumor necrosis factor (TNF) α (Yamada *et al.* 2000, de Beaufort *et al.* 2003), all having great potential to inactivate pulmonary surfactant and start up inflammation if aspirated.

Aspiration of meconium occurs in the term and post-term newborns intrauterinely or perinatally mostly under conditions of hypoxia and leads to serious disease called meconium aspiration syndrome (MAS) combining mechanical obstruction of airways, pulmonary vasoconstriction, lung edema, surfactant dysfunction and oxidative and inflammatory changes associated with chemical pneumonitis. If airway suctioning, oxygen delivery and ventilatory support are not sufficient for overcoming of severe respiratory insufficiency of newborn, exogenous surfactant may be administered being the last step before extracorporeal membrane oxygenation (Findlay *et al.* 1996).

Exogenous surfactant replaces the function of inactivated endogenous surfactant which lies in lowering of surface tension of small airways and alveoli (Sun *et al.* 1993). Such surfactant-mediated increase in lung compliance reduces the demand of newborn's respiratory muscle work and improves the gas exchange, thereby lowering oxygen requirements. Moreover, if administered diluted in a way of bronchoalveolar (or lung) lavage, surfactant removes at least partially meconium from lower airways and thus prevents both the mechanical

obstruction and proinflammatory stimuli. Enhancement of surfactant therapy can be reached by administration of undiluted surfactant bolus following previous lung lavage (Kaneko *et al.* 2001).

Even within the highest possible effectiveness of surfactant therapy, the meconium-induced inflammatory response persists in the lungs with miscellaneous intensity. Meconium had been shown to activate toll-like receptor 4 (TLR4) complex CD14/TLR4/MD-2 (Salvesen *et al.* 2010) which had been for long time thought to be associated only with bacterial lipopolysaccharides (LPS; Latz and Golenbock 2003) and which is present on different types of cells including macrophages, endothelial and epithelial cells (Andonegui *et al.* 2003, Skerrett *et al.* 2004, Xiang *et al.* 2010, Poledne 2013). Subsequent nuclear factor kappa B (NF- κ B) activation triggers additive cytokine production which potentiates the effect of cytokines inherent in the meconium itself (Yamada *et al.* 2000). Chemoattracted leukocytes infiltrate the lung tissue and react by degranulation and release of proteolytic enzymes on the one hand and by production of free radicals on the other one.

Both these actions may damage tight junctions and cells themselves and what is more, inactivate exogenous surfactant lipids and proteins (Bouhafs and Jarstrand 2002, Grommes and Soehnlein 2011). Such inactivation occurs in hours or days according to seriousness of inflammation and in clinical practice has been named “postsurfactant slump” (Donn and Dalton 2009). Infants, who initially responded positively to exogenous surfactant, subsequently deteriorated and developed increasing oxygen requirements and the need of repeated surfactant doses (Katz and Klein 2006).

According to European Consensus Guidelines 2013, non-invasive methods of respiratory support such as continuous positive airway pressure (CPAP) are largely recommended in newborns with respiratory distress prior to mechanical ventilation whenever it is possible. If needed, intubation for mechanical ventilation should be accompanied by surfactant administration every time and replaced by non-invasive support as soon as possible (Sweet *et al.* 2013). Despite intensive investigation of new techniques of surfactant administration, use of endotracheal tube remains still the gold standard (Lopez *et al.* 2013). In this context, repeated need of exogenous surfactant during postsurfactant slump means also repeated intubation of the newborn and all of the accompanying risks (Sweet *et al.* 2013). For this reason, it may bring benefit to reduce

oxidative/inflammatory processes and keep surfactant preparations active as long as possible by preventing them from inactivation.

In our previous study, we have found that combination of exogenous surfactant and budesonide managed to prevent inflammation-mediated surfactant inactivation in experimental model of MAS (Mikolka *et al.* 2013). Inhaled budesonide has been associated with lower adverse effects compared to systematic glucocorticoid treatment (Halliday *et al.* 2001), however, early postnatal glucocorticoid application generally is thought to have long-term consequences (Yeh *et al.* 2004, Fok 2009).

N-acetylcysteine (NAC) is a thiol, a mucolytic agent and a precursor of L-cysteine and reduced glutathione (GSH), serving with high efficiency as a substrate for synthesis of intracellular GSH prior to direct scavenging of ROS. On molecular level, NAC was found to inhibit p38 MAP kinase, activating protein AP-1 and NF- κ B, to reduce expression of adhesion molecules, IL-8, TNF- α and IL-1 β levels and prevent neutrophil migration and/or activation into the lung tissue (Davreux *et al.* 1997, Zafarullah *et al.* 2003, Cuzzocrea *et al.* 2001, Patterson *et al.* 2003, Rushworth and Megson 2014) thereby exerting both anti-oxidative and anti-inflammatory abilities. Moreover, NAC is considered to be very safe and without adverse effects if the dosage does not exceed about 3 grams/day (Dodd *et al.* 2008). In lower doses it had been administered even to newborns (Walls *et al.* 2007) and successfully used in the treatment of acute lung injury/acute respiratory distress syndrome – the group where inflammation plays role and where MAS also belongs to (Soltan-Sharifi *et al.* 2007). The NAC capability to reduce the viscosity of meconium by breaking disulphide bonds between protein molecules provides further possible advantage in MAS management (Ivanov 2006). Although NAC was found to prevent surfactant inactivation in MAS (Mokrá *et al.* 2013), the clinical outcome on respiratory parameters and possible anti-inflammatory action of NAC combined with surfactant has not been proven yet.

Having previous experience with surfactant lung lavage and administration of combined therapy in experimental rabbit model of MAS (Ševcová-Mokrá *et al.* 2004, Mikolka *et al.* 2013), we supposed that exogenous surfactant together with NAC may improve lung functions with higher efficiency compared to either treatment alone. In this pilot study, we have evaluated lung function parameters, the dynamic onset of oxidative

damage and neutrophil migration into the lung tissue and cytokine formation between meconium-instilled animals and groups treated with surfactant or NAC monotherapy and combined therapy.

Methods

General design of experiments

Design of experiments was approved by the local Ethics Committee of Jessenius Faculty of Medicine and National Veterinary Board. Meconium collected from healthy term neonates had been lyophilized and stored at -20°C . Immediately before use, meconium was resuspended in 0.9 % NaCl at a concentration of 25 mg/ml. Young New Zealand white rabbits (Velaz s. r. o., ČR) of 2.5 ± 0.4 kg body weight (b.w.) were anesthetized with intramuscular ketamine (20 mg/kg b.w.; Narketan, Vétouinol, UK) and xylazine (5 mg/kg b.w.; Xylarium, Riemser, Germany), followed by infusion of ketamine (20 mg/kg/h). Tracheotomy was performed and catheters were inserted into a femoral artery and right atrium for the blood sampling and blood pressure measuring, and into a femoral vein for drug and anesthetics administration. Animals were then paralyzed with pipecuronium bromide (0.3 mg/kg b.w./30 min; Arduan, Gedeon Richter, Hungary) to avoid spontaneous breathing and subjected to a pressure-controlled ventilator (Beat-2, Chirana, Slovakia). All animals were then ventilated with a frequency of 30/min, fraction of inspired oxygen (FiO_2) of 0.21, inspiration time T_i 50 %, peak inspiratory pressure (PIP) to keep a tidal volume (V_T) between 7-9 ml/kg b.w. and no positive end-expiratory pressure (PEEP) at this stage of experiment. After 15 min of stabilization, lung function parameters (PIP, PEEP) were recorded, blood gases (PaO_2 and PaCO_2) were analyzed (RapidLab 348, Siemens, Germany) and arterial blood sample was taken for biochemical analysis to obtain basal values (BV).

Meconium instillation

Consequently, the rabbits were intratracheally administered 4 ml/kg b.w. of meconium suspension (25 mg/ml) divided into two equal portions with animal positioned to the right and left. From this moment on, animals were ventilated with FiO_2 1.0 and PEEP 0.3 kPa. In the meconium-instilled animals, respiratory failure developed within 30 min, defined as $>30\%$ decrease in dynamic lung-thorax compliance and $\text{PaO}_2 < 10$ kPa at FiO_2 1.0. In 30 min period of meconium action, all

above-mentioned parameters were recorded.

Administration of treatment

After 30 min of meconium administration, the animals were randomly divided into four groups according to the treatment: 1) meconium without treatment (Mec group, $n=6$); 2) meconium with surfactant treatment (Surf group, $n=6$); 3) meconium with N-acetylcysteine treatment (NAC group, $n=6$); 4) meconium with combined surfactant and N-acetylcysteine treatment (Surf+NAC group, $n=6$).

In all surfactant-treated animals, the procedure of surfactant administration proceeded in two steps described in details elsewhere (Mikolka *et al.* 2013). Briefly, modified porcine surfactant (Curosurf, Chiesi Farmaceutici, Italy; 80 mg phospholipids (PL)/ml) was used. At first, two lung lavages had been performed using Curosurf diluted by saline at PL concentration of 5 mg/ml and volume of 10 ml/kg b.w. After the second lavage, undiluted Curosurf was given at a dose of 100 mg PL/kg, 1.25 ml/kg b.w. as a supplementation dose.

In NAC-treated groups, intravenous N-acetylcysteine (10 mg/kg b.w.; ACC Injekt, Salutas Pharma GmbH, Germany) was given. All animals were oxygen-ventilated for additional 5 h after the treatment. Blood gases and respiratory parameters were recorded at 30 min, 1, 2, 3, 4, and 5 h after the treatment. At the end of experiments, animals were sacrificed by an overdose of anesthetics.

Measurement of lung functions parameters

Tracheal airflow was measured by a heated Fleisch head connected to a pneumotachograph. Airway pressure was registered *via* a pneumatic catheter placed in the tracheal tube and connected to electromanometer. Mean airway pressure (MAP) was calculated as: $\text{MAP}=(\text{PIP}+\text{PEEP})/2$; $\text{PaO}_2/\text{FiO}_2$ parameter as ratio between PaO_2 and FiO_2 and oxygenation index (OI) was calculated as: $\text{OI}=(\text{MAP}\times\text{FiO}_2)/\text{PaO}_2$. PaO_2 and PaCO_2 were analyzed periodically.

Biochemical analyses of oxidative stress in arterial blood samples

Samples of arterial blood were collected before meconium instillation and 30 min after meconium instillation, then 30 min, 1, 3, and 5 h after the treatment. All blood samples were immediately centrifuged at $1000g/4^{\circ}\text{C}$ for 15 min. Plasma had been taken and stored at -80°C for further biochemical analyses. Products of

oxidative stress were evaluated by commercially available kits: BlueGene Rabbit Nitrotyrosine ELISA (E04N0005; Shanghai BlueGene Biotech CO., LTD) for nitrotyrosine formation (product of protein nitrosylation) and OxiSelect TBARS Assay Kit (STA-330; Cell Biolabs Inc.) for malondialdehyde/thiobarbituric reactive substances (TBARS) evaluation (product of lipid peroxidation) according to the manufacturer's instructions. The values were expressed as percentual changes of BV calculated for each animal.

Counting of cells in the arterial blood samples

In collected samples of arterial blood, total leukocyte count was determined microscopically in a counting chamber after staining by Türk. Differential leukocyte count was estimated microscopically after staining by Pappenheim. The values of total leukocyte count were expressed as a percentage of BV counted for each animal to get more precise information about the individual changes. The values of differential leukocyte count were expressed as percentual portion of each cell type.

Bronchoalveolar lavage fluid cells evaluation

After sacrificing the animal, lungs and trachea were excised. Left lungs were lavaged by saline (0.9 % NaCl, 37 °C) 3x10 ml/kg, bronchoalveolar lavage fluid (BALF) was centrifuged at 1500 rpm for 10 min. Total number of cells in the BALF was determined microscopically in a counting chamber. Differential count of cells in the BALF sediment was evaluated microscopically after staining by Pappenheim.

Wet/dry lung weight ratio evaluation

Strips of the right lung tissue were cut, weighed and dried at 60 °C for 24 h to determine wet/dry (W/D) lung weight ratio, expressing the rate of lung edema formation.

Biochemical analyses of cytokines in lung tissue homogenates

For cytokine evaluation, strips of the right lung tissue were homogenized in ice-cold PBS (0.02 mol/l, pH 7.2) for final concentration 10 %, subjected to two freeze-thaw cycles and centrifuged for 15 min at 1500g according to manufacturer's instructions. Supernatants were removed and analyzed by commercial BlueGene Rabbit ELISA Kits for IL-1 β , IL-8 and TNF- α (E04I0010, E04I0056 and E04T0008; Shanghai

BlueGene Biotech CO., LTD). Data were expressed as pg/ml of homogenate.

Statistical analysis of results

Statistical analyses were performed by STATISTICA (StatSoft, Inc.; ČR). Two-way analysis of variance (ANOVA) with Duncan post-hoc test was used for the parameters with dynamic changes (grouping factors "group" and "time"): PaCO₂, PaO₂/FiO₂; OI; nitrotyrosine, TBARS, leukocyte count, leukocyte differential). Non-parametric analysis (Kruskal-Wallis ANOVA test) was used for comparison of total and differential leukocyte count in BALF, cytokine level in homogenates and edema of lung tissue. A value of p<0.05 was considered to be statistically significant. Numeric values are expressed as mean \pm standard error of mean (SEM).

Results

Lung function parameters before meconium instillation

24 rabbits of both genders were used in this experiment. There were no differences in entry parameters (body weight, gender) and lung function parameters did not differ before meconium instillation between groups.

Recovery of the therapeutic bronchoalveolar lavage (BAL) fluid

In both surfactant-treated groups (Surf and Surf+NAC), 10 ml/kg of diluted exogenous surfactant were used for therapeutic bronchoalveolar lavage. The recovery of BAL was similar between both groups (67.4 \pm 3.4 % for Surf group and 74.8 \pm 2.7 % for Surf+NAC group, p=0.14, Mann-Whitney U test).

Effect of therapy on the lung function parameters

Meconium instillation led to significant worsening in respiratory parameters (PaCO₂, PaO₂/FiO₂, OI) and before therapeutic intervention (30 min after meconium), this deterioration had been similar in all groups (p<0.001 vs. BV, non-significant among groups).

The administration of NAC alone (NAC group) did not bring any effect on PaO₂/FiO₂ (Fig. 1A) and PaCO₂ remained at very high levels all the time; however, there was a significant improvement in OI from the third hour of therapy (p<0.05 vs. Mec; Fig. 1B, 2).

Surfactant treatment improved the lung functions more effectively. Surfactant raised PaO₂/FiO₂

significantly in the second and the third hour after administration compared to both untreated animals and NAC-treated group ($p < 0.01$ and $p < 0.05$ vs. both (Mec and NAC) group in the 2nd and the 3rd h, respectively). OI was enhanced after surfactant administration significantly

from the first hour and this improvement lasted until the end of experiment ($p < 0.01$ to 0.001 vs. Mec; $p < 0.05$ to 0.001 vs. NAC; Fig. 1A,B) with PaCO₂ reaching lower values in the fifth hour ($p < 0.01$ vs. Mec, Fig. 2).

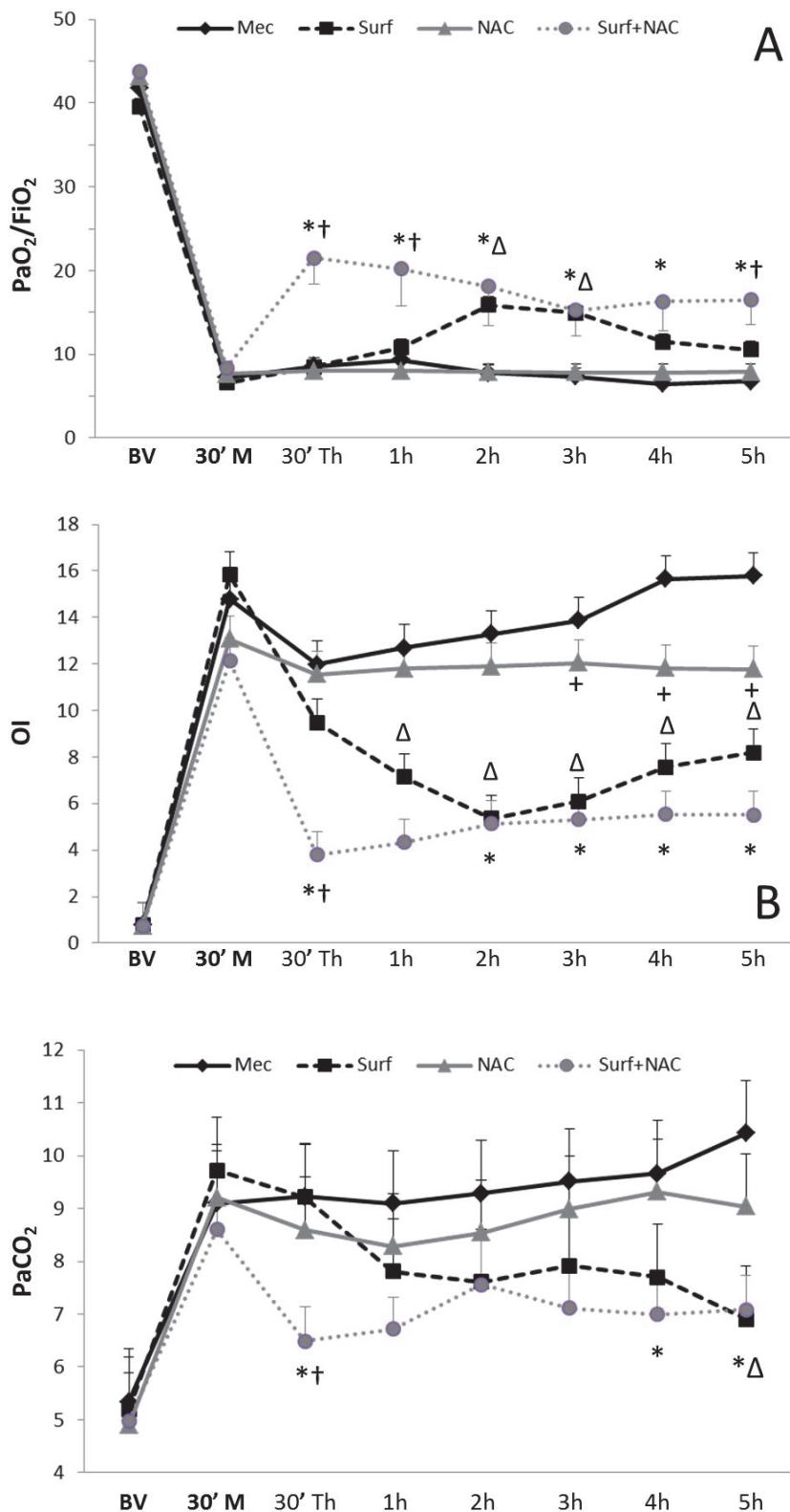


Fig. 1. PaO₂/FiO₂ (A) and oxygenation index (B) before meconium administration (BV), 30 min (30') after meconium (M) and therapy (Th) administration during 5 h of experiment. Mec – untreated group, Surf – Surfactant-only treated group, NAC – N-acetylcysteine-only treated group, Surf+NAC – Surfactant and N-acetylcysteine treated group; + $p < 0.05$ for NAC vs. Mec, $\Delta p < 0.05$ to 0.001 for Surf vs. Mec and NAC, * $p < 0.01$ to 0.00001 for Surf+NAC vs. Mec and NAC, † $p < 0.05$ to 0.001 for Surf+NAC vs. Surf

Fig. 2. PaCO₂ before meconium administration (BV), 30 min (30') after meconium (M) and therapy (Th) administration during 5 h of experiment. Mec – untreated group, Surf – Surfactant-only treated group, NAC – N-acetylcysteine-only treated group, Surf+NAC – Surfactant and N-acetylcysteine treated group; $\Delta p < 0.05$ for Surf vs. Mec, * $p < 0.05$ for Surf+NAC vs. Mec, † $p < 0.05$ for Surf+NAC vs. Surf

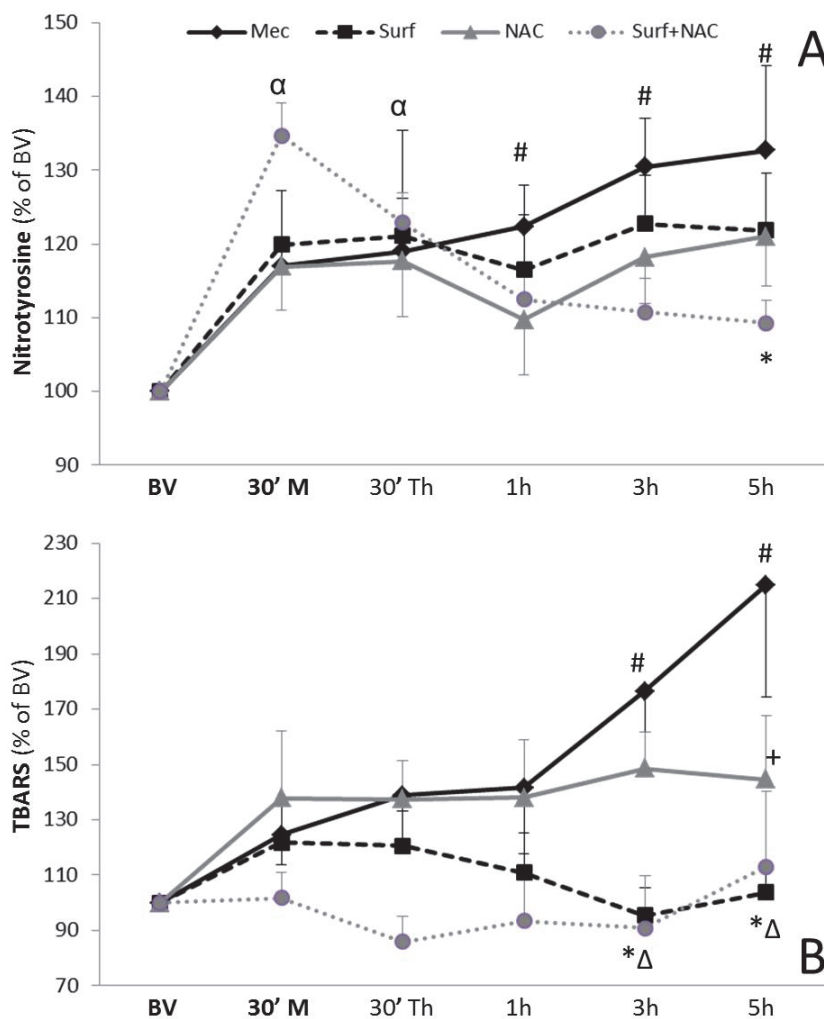


Fig. 3. Nitrotyrosine (**A**) and TBARS (**B**) formation before meconium administration (BV), 30 min (30') after meconium (M) and therapy (Th) administration during 5 h of experiment. Mec – untreated group, Surf – Surfactant-only treated group, NAC – N-acetylcysteine-only treated group, Surf+NAC – Surfactant and N-acetylcysteine treated group; # $p < 0.05$ for Mec vs. BV, α $p < 0.05$ to 0.01 for Surf+NAC vs. BV, Δ $p < 0.05$ to 0.001 for Surf vs. Mec, * $p < 0.05$ to 0.001 for Surf+NAC vs. Mec

Combined therapy of surfactant and NAC revealed quick onset of the benefit. The improvement reached very high significance already in the first 30 min after administration of the therapy on both parameters $\text{PaO}_2/\text{FiO}_2$ and OI ($p < 0.01$ to 0.001 vs. Mec for $\text{PaO}_2/\text{FiO}_2$; $p < 0.00001$ vs. Mec for OI; Fig. 1A,B) with lower PaCO_2 values ($p < 0.05$ vs. Surf and Mec in 30 min, and vs. Mec in the 4th and the 5th h of therapy, Fig. 2). In $\text{PaO}_2/\text{FiO}_2$, combined therapy had been more effective compared to NAC alone ($p < 0.01$ to 0.001 vs. NAC all the time). Moreover, the onset of the effect was more rapid compared to surfactant alone ($p < 0.001$ vs. Surf in the first 30 min and 1 h of therapy) and the duration of effect remained until the end of experiment, with significant difference at $p < 0.05$ vs. Surf at 5 h after the treatment (Fig. 1A). In OI and PaCO_2 evaluation, the quick onset of the combined therapy compared to surfactant alone remained significant in the first 30 min ($p < 0.001$ vs. Surf; Figs 1B, 2).

Effect of therapy on oxidative parameters

Nitrotyrosine formation

After untreated meconium administration, the increase in nitrotyrosine formation became evident in 1.5 h ($p < 0.05$ vs. BV, with borderline significance of $p = 0.08$ in the first hour after meconium). In the Surf and NAC treated groups, the level increase varied whole the time on borderline values ($p = 0.051$ to 0.07 vs. BV) with the onset 30 min after meconium administration and the only difference reached in the 3rd h in Surf group ($p < 0.05$ vs. BV, in the 5th h $p = 0.051$). In combined Surf+NAC group, the increase in nitrotyrosine formation became significant during the first 30 min after meconium administration ($p < 0.01$ vs. BV) and this elevation lasted even 30 min after treatment administration ($p < 0.05$ vs. BV), but then the treatment managed to decrease nitrotyrosine levels (Fig. 3A).

The inter-group statistical comparison revealed

significant improvement in nitrotyrosine production only in Surf+NAC group in the time of 5 h after the treatment ($p < 0.05$ vs. Mec), with borderline tendency to improvement seen at 3 h ($p = 0.08$ vs. Mec).

TBARS formation

The increase in TBARS concentration appeared in untreated group 3.5 h after meconium administration

($p < 0.05$ and $p < 0.0001$ vs. BV in the 3rd and the 5th h). None of the treated groups showed significant increase in TBARS production during experiment (Fig. 3B).

The administration of both surfactant alone and surfactant combined with NAC ameliorated oxidative status in the 3rd and the 5th h after the treatment ($p < 0.05$ and $p < 0.001$ vs. Mec), NAC alone had effect only at the time of 5 h ($p < 0.05$ vs. Mec).

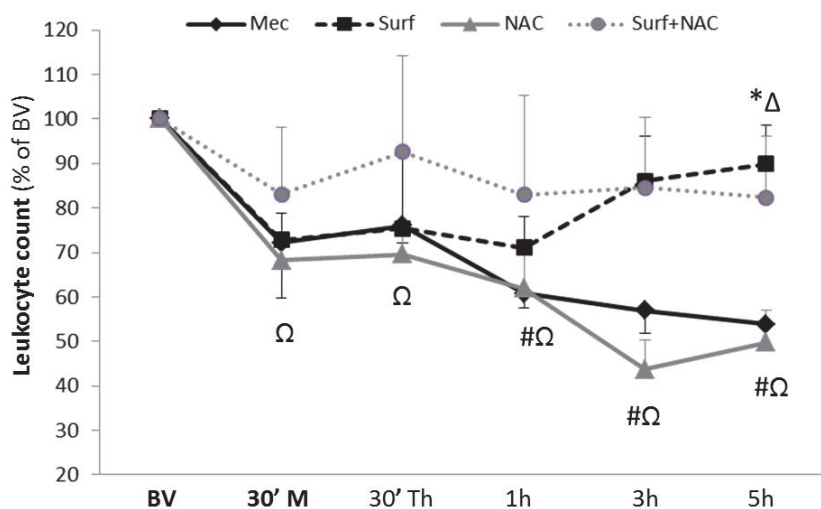


Fig. 4. Total leukocyte count in peripheral blood before meconium administration (BV), 30 min (30') after meconium (M) and therapy (Th) administration during 5 h of experiment. Mec – untreated group, Surf – Surfactant-only treated group, NAC – N-acetylcysteine-only treated group, Surf+NAC – Surfactant and N-acetylcysteine treated group; # $p < 0.01$ for Mec vs. BV, Ω $p < 0.05$ to 0.001 for NAC vs. BV, Δ $p < 0.05$ for Surf vs. Mec, * $p < 0.05$ Surf+NAC vs. Mec

Effect of therapy on leukocyte count and migration

Total leukocyte count in peripheral blood

Administration of meconium led to significant decrease in the total count of leukocytes in peripheral blood. In untreated group, the fall reached significance 1.5 h after meconium ($p < 0.01$ vs. BV), whereas in the NAC-treated group the fall was more pronounced and significant 30 min after meconium administration with no effect of therapy ($p < 0.05$ to 0.001 vs. BV). In both surfactant groups (Surf and Surf+NAC), the fall of leukocytes was borderline before the therapy ($p = 0.07$ and $p = 0.09$ 30 min after meconium administration) and the therapy managed to prevent additional fall (Fig. 4). Comparison of groups showed significant enhancement in leukocyte count in both surfactant-treated groups in the 5th h of the therapy with the tendency seen already in the 3rd h ($p < 0.05$ vs. Mec in the 5th h for Surf and Surf+NAC; in the 3rd h $p = 0.06$ vs. Mec for Surf and $p = 0.07$ vs. Mec for Surf+NAC).

Leukocyte differential count in peripheral blood

Meconium shifted percentual proportions in

leukocyte types (Table 1). In Mec group, neutrophil demargination and consequently relative lymphocyte fall appeared in the 5th h of the experiment ($p < 0.001$ vs. BV for both types); this neutrophil increase (and lymphocyte decrease) reached also significant difference in comparison to NAC and Surf+NAC groups ($p < 0.05$ vs. both groups).

Similar but more pronounced proportional shift was found in Surf group, with significant neutrophil abundance and lymphocyte fall in the 3rd and the 5th h compared to basal value ($p < 0.01$ vs. BV for both types and both times) and to other groups ($p < 0.05$ in 3 h vs. NAC group and $p < 0.001$ in 5 h vs. NAC and Surf+NAC groups).

Neutrophil and lymphocyte changes in NAC and Surf+NAC groups were of the same kind with significance reached at 5 h (both $p < 0.05$ vs. BV).

There were no significant changes in monocyte, eosinophil and basophil percentage during experiment within or between experimental groups (Table 1; for basophils data not shown, as their occurrence is very rare and for all groups and all times the number was 0.0 to 0.1).

Table 1. Differential leukocyte count in peripheral blood before meconium administration (BV), 30 min (30') after meconium (Mec) and therapy (Th) administration during 5 h of experiment.

	BV	30' Mec	30' Th	1 h Th	3 h Th	5 h Th
<i>Neutrophils (%)</i>						
<i>Mec</i>	4.3±1.9	13.5±5.9	12.6±5.2	10.3±2.0	17.7±4.7	38.3±7.7 ^{aef}
<i>Surf</i>	2.7±1.1	7.4±3.1	6.8±0.6	8.1±1.7	25.6±4.5 ^{be}	46.1±4.9 ^{bef}
<i>NAC</i>	2.8±1.3	3.4±1.9	4.9±2.5	7.8±0.5	9.1±3.1	21.8±6.6 ^c
<i>Surf+NAC</i>	2.8±0.9	1.9±0.2	5.0±1.5	9.7±4.3	17.2±4.3	23.1±6.8 ^d
<i>Lymphocytes (%)</i>						
<i>Mec</i>	92.3±1.8	85.6±6.1	84.8±4.5	87.4±2.3	80.7±4.8	59.6±7.7 ^{aef}
<i>Surf</i>	95.6±1.4	90.7±3.6	92.0±0.9	90.3±1.3	72.2±5.0 ^{be}	51.2±4.9 ^{bef}
<i>NAC</i>	95.2±1.6	94.4±1.7	93.5±2.5	90.6±0.5	88.7±3.1	76.7±6.7 ^c
<i>Surf+NAC</i>	94.8±0.5	96.1±0.2	93.9±1.5	87.7±4.5	81.5±4.7	75.7±6.6 ^d
<i>Monocytes (%)</i>						
<i>Mec</i>	1.6±0.6	1.5±0.6	1.4±0.5	1.3±0.8	1.2±0.3	1.3±0.4
<i>Surf</i>	1.1±0.4	0.9±0.2	0.8±0.3	0.7±0.4	0.8±0.2	1.2±0.2
<i>NAC</i>	1.3±0.2	1.3±0.3	1.2±0.4	1.2±0.4	1.3±0.2	1.2±0.4
<i>Surf+NAC</i>	1.2±0.4	0.9±0.2	0.8±0.4	1.1±0.5	0.9±0.5	0.8±0.6
<i>Eosinophils (%)</i>						
<i>Mec</i>	1.2±0.6	0.9±0.6	0.9±0.3	0.8±0.3	0.4±0.5	0.9±0.4
<i>Surf</i>	0.6±0.3	1.1±0.7	0.8±0.4	0.8±0.3	1.1±0.4	1.5±0.6
<i>NAC</i>	0.8±0.3	0.6±0.3	0.9±0.4	0.5±0.3	0.5±0.4	0.6±0.4
<i>Surf+NAC</i>	1.1±0.3	0.8±0.3	0.5±0.4	0.7±0.3	0.5±0.3	0.5±0.2

Mec – untreated group, Surf – Surfactant-only treated group, NAC – N-acetylcysteine-only treated group, Surf+NAC – Surfactant and N-acetylcysteine treated group; ^a p<0.001 for Mec vs. BV, ^b p<0.01 for Surf vs. BV, ^c p<0.05 for NAC vs. BV, ^d p<0.05 for Surf+NAC vs. BV, ^e p<0.05 for Mec and Surf vs. NAC, ^f p<0.05 for Mec and Surf vs. Surf+NAC

Bronchoalveolar lavage fluid leukocytes

At the end of experiment, left lungs were lavaged by 3 x 10 ml/kg of saline. Recovery of BALF was similar in all groups (76.2±3.2 % for Mec; 74.6±6.6 % for Surf; 77.4±4.3 % for NAC and 82.6±2.1 % for Surf+NAC group, all p>0.05).

The number of leukocytes in BALF after meconium administration was relatively high (102±39 x 10⁷ in ml of BALF). Therapeutic intervention decreased the number of leukocytes, however, the significant effect was seen only in combined therapy (11±2 x 10⁷ in ml of BALF; p<0.01 vs. Mec) with borderline significance for NAC treatment (18±2 x 10⁷ in ml of BALF; p=0.08 vs. Mec; Table 2).

Differential leukocyte count in BALF had been also affected. Compared to Mec group, both Surf and Surf+NAC groups showed lower portion of neutrophils and higher portion of monocytes/macrophages (p<0.05) with no changes in lymphocytes and eosinophils. NAC-

treatment did not influence differential leukocyte count.

Effect of therapy on lung tissue inflammation

Cytokine levels

Comparison of cytokine levels in lung tissue homogenates showed decrease in IL-1β (p<0.01) and IL-8 (p<0.05) after NAC treatment and IL-1β (p<0.05) after Surf+NAC treatment compared to Mec group (Table 3). IL-8 after Surf+NAC treatment was at p=0.09 and TNF-α remain unchanged in all treated groups compared to Mec.

Lung edema

Lung edema was evaluated by calculating of wet tissue weight and dry tissue weight ratio (Table 3). From all the administered therapies, only the combination of surfactant and NAC managed to prevent lung edema formation (p<0.01 vs. Mec).

Table 2. Total and differential leukocyte count in bronchoalveolar lavage fluid.

	Total count ($\times 10^7/\text{ml}$)	Neutrophils (%)	Monocytes/ Macrophages (%)	Lymphocytes (%)	Eosinophils (%)
<i>Mec</i>	102.4 \pm 39.1	69.0 \pm 5.1	26.4 \pm 4.5	1.7 \pm 0.4	2.9 \pm 1.2
<i>Surf</i>	44.4 \pm 10.4	39.8 \pm 7.2 ^b	57.1 \pm 7.3 ^b	1.3 \pm 0.3	1.8 \pm 0.6
<i>NAC</i>	18.1 \pm 2.4	58.9 \pm 9.0	37.0 \pm 8.7	2.5 \pm 0.9	1.6 \pm 0.6
<i>Surf+NAC</i>	11.6 \pm 2.1 ^a	40.1 \pm 9.5 ^b	56.4 \pm 10.3 ^b	1.3 \pm 0.2	1.2 \pm 0.7

Mec – untreated group, Surf – Surfactant-only treated group, NAC – N-acetylcysteine-only treated group, Surf+NAC – Surfactant and N-acetylcysteine treated group; ^a p<0.01 for Surf+NAC vs. Mec, ^b p<0.05 for Surf and Surf+NAC vs. Mec

Table 3. Wet to dry (W/D) lung weight ratio and levels of cytokines IL-1 β , IL-8 and TNF- α in lung tissue homogenates.

	W/D ratio	IL-1 β (pg/ml)	IL-8 (pg/ml)	TNF- α (pg/ml)
<i>Mec</i>	6.0 \pm 0.2	142.0 \pm 13.0	48.3 \pm 6.5	35.3 \pm 2.1
<i>Surf</i>	5.4 \pm 0.2	121.0 \pm 16.6	42.6 \pm 4.5	33.0 \pm 2.4
<i>NAC</i>	6.1 \pm 0.3	74.2 \pm 4.1 ^a	29.0 \pm 6.4 ^b	37.6 \pm 2.5
<i>Surf+NAC</i>	5.0 \pm 0.1 ^a	95.1 \pm 15.8	33.8 \pm 5.3 ^b	33.8 \pm 1.8

Mec – untreated group, Surf – Surfactant-only treated group, NAC – N-acetylcysteine-only treated group, Surf+NAC – Surfactant and N-acetylcysteine treated group; ^a p<0.01 for NAC and Surf+NAC vs. Mec, ^b p<0.05 for NAC and Surf+NAC vs. Mec

Discussion

As it was expected, meconium instillation led to significant deterioration in respiratory parameters (PaO₂/FiO₂, OI, PaCO₂ and also in O₂ saturation and pH – data not shown) in few minutes after administration. This was associated with progressive formation of oxidative products of ongoing protein nitrosylation and lipid peroxidation (nitrotyrosine and TBARS). Here we need to note that we have expected faster onset of oxidative damage in untreated Mec group, especially for TBARS formation which became evidential up to 3.5 h after instillation (compare Imai *et al.* 2008); however, the levels of oxidative products right in the lungs could be different in comparison to levels in systemic circulation. Meconium administration also eventuated in leukocyte abundance in lungs, with high portion of neutrophils supposedly attracted by higher cytokine levels, all resulting in lung edema formation, typical for MAS (Pratesi and Dani 2012).

As we supposed, the effect of NAC alone in the treatment of MAS was rather low. There was no difference between NAC group and non-treated group in

PaO₂/FiO₂, and higher values of PaCO₂ were observed due to impossibility of further increase in ventilatory pressures, to which the animals tended to respond by hypotension (data not shown). However, OI was improved from the third hour of the therapy, probably *via* inhibition of inflammatory processes in the lungs and at least partial preservation of endogenous surfactant function which we have previously confirmed (Mokrá *et al.* 2013). Administration of NAC prevented oxidative damage to both lipids and proteins (as seen from reduced TBARS and nitrotyrosine production) and led to mild enhancement in lipid peroxidation when compared to Mec group. This effect had been estimated as NAC is considered to have powerful antioxidant potential *via* replenishing GSH levels (Rushworth and Megson 2014). Even though NAC prevented neutrophil demarginalization, it managed to hinder neutrophil sequestration into the lungs just partially, which manifested by mild modification of leukocyte count in BAL and unchanged neutrophil percentage. There is evidence that neutrophil sequestration into the lungs can be inhibited by NAC due to reduced expression of adhesion molecules ICAM-1 and VCAM-1 (Zafarullah *et*

al. 2003, Cuzzocrea *et al.* 2001). Decrease in neutrophil recruitment and cytokine-induced neutrophil chemoattractant (CINC) suppression was also seen in rats when repeated doses of NAC were used (Blackwell *et al.* 1997, Cuzzocrea *et al.* 2001), but not after single dose (Fan *et al.* 1998). In cardiac surgery patients, pre-treatment by NAC diminished IL-8 levels and elastase activity in BALF and decreased the percentage of harvested neutrophils from 16.5 % to 2.3 % – BALF neutrophil count correlated negatively with enhanced PaO₂/FiO₂ level at borderline significance ($p=0.054$, De Backer *et al.* 1996). However, there is also theory that NAC does not prevent neutrophil lung sequestration but do prevent neutrophil activation (Davreux *et al.* 1997) and in rats instilled intratracheally with LPS (which activates the same receptor complex as meconium), neither pre-treatment with the same dose of NAC as ours (10 mg/kg) managed to lower total leukocyte count and neutrophil fraction in BALF (Jansson *et al.* 2005). Similarly to our results, these authors did not find any improvement in lung edema formation and TNF- α in BALF was decreased only in cases where low LPS dose had been given. By contrast, they did not find any NAC-mediated improvement in BALF levels of IL-1 β , whereas the levels of IL-1 β in lung tissue homogenates were decreased after NAC in our experiment. Taken together, the efficacy of NAC against inflammation and lung injury strongly differs not only according to dose and time management of the treatment, but also to the seriousness of induced inflammation. In our model, the presence of meconium with the content of inflammatory substances worked as strong inflammatory inductor and given dose of NAC was not sufficient to improve therapeutic results by itself.

However, enhancement was reached if the meconium had been at least partly removed from lungs by lung lavage. The procedure of diluted surfactant lavage therapy followed by bolus dose of concentrated surfactant, which was performed in our experiment, had been discussed in details elsewhere (Mikolka *et al.* 2013). Briefly, integration of surfactant lavage and bolus administration facilitates taking advantages of both approaches – removal of meconium which reduces mechanical obstruction and action of meconium-contained cytokines together with sufficient replacement of inactivated endogenous surfactant. Surfactant was successfully used in the treatment of MAS in newborns almost 20 years ago (Findlay *et al.* 1996, Soll and Dargaville 2007). In agreement with other authors, we

saw enhancement in lung function parameters (PaO₂/FiO₂ and OI) after administration of surfactant and also prevention from oxidative damage to lungs. However, in the course of the time, we saw disappearance of significance in PaO₂/FiO₂ when compared to Mec group in the fourth hour and later. This could mildly predicate the start-up of the process of surfactant inactivation due to inflammatory processes which normally can be seen in clinical practice (Katz and Klein 2006) and which can be result of protein nitrosylation noticed in the 3rd h after surfactant treatment.

Surfactant did not prevent leukocyte migration into the lungs, although the portion of BALF neutrophils was lower compared to untreated animals. These results are consistent with previous findings that exogenous surfactant can reduce macrophage-mediated neutrophil chemotaxis (Finck *et al.* 1998). Moreover, there was no improvement of inflammatory cytokines and lung edema formation at the end of experiment after surfactant-only treatment. Exogenous surfactant is considered to have also anti-inflammatory properties (Thomassen *et al.* 1992, Borron *et al.* 2000), however, there are also works which – similarly to ours – showed no effect of surfactant on cytokine production under different types of lung injury if administered as monotherapy (Wu *et al.* 2010, Dani *et al.* 2011). We may speculate about the mechanisms of cytokine suppression mediated by surfactant (see also Thomassen *et al.* 1994), but generally it seems that factors such as the time of surfactant administration and also the type and composition of used surfactant (especially the presence of surfactant proteins SP-A and SP-D) play role in this inconsistency between different studies.

Lower surfactant activity against inflammation had been overcome by combination of surfactant and NAC treatment in our experiments. At first, we supposed that NAC would replenish GSH levels and thus prevent oxidative impairment of surfactant proteins and lipids in the period of time. However, we found significant and very rapid onset of improvement in PaO₂/FiO₂, PaCO₂ and OI parameters in the first 30 min after administration of the combined therapy. The significance was reached also in comparison with surfactant-only treatment which indicates that this enhancement cannot be attributed just to bronchoalveolar lavage and meconium removal. There are several possible mechanisms of such quick onset of efficacy. At first, NAC is able to cleave disulphide bonds in the mucoprotein molecules. This action that made NAC to be wide-used mucolytic agent, is also responsible

for NAC ability to decrease the tenacity of the meconium (Pusterla *et al.* 2004) which had been previously used in newborns with meconium ileus as a non-surgical strategy (Emil *et al.* 2004). Moreover, NAC is able to cross cell membranes in relatively easy way resulting in quick tissue distribution after intravenous application (Rushworth and Megson 2014). Taken together, we can speculate that reduction of meconium viscosity combined with surfactant supplementation and GSH replenishment provided rapid enhancement which we have seen already in 30 min after administration of the therapy. Moreover, there is a molecular effect of NAC, which was found to inhibit cyclooxygenase-2 (COX2) induction in vascular smooth muscle cells through suppression of p38 mitogen-activated protein kinase pathway which is redox-sensitive (Zafarullah *et al.* 2003, Bernatova 2014). COX2 expression is known to be meconium-induced (Kytölä *et al.* 2003) and COX2-derived product thromboxane is directly responsible for LPS-induced bronchoconstriction *via* several cooperating mechanisms (Uhlir *et al.* 1996, Ermert *et al.* 2003). Inhibition of this signalisation may result in lower airway resistance and thus increase in lung function parameters.

Combination of surfactant and NAC managed to prevent oxidative damage to lungs and were the only treatment which brought significant improvement in nitrotyrosine levels when compared to Mec group. Nitrotyrosine production is a consequence of inducible nitric oxide synthase (iNOS) induction which is NF- κ B-mediated and induced in MAS (Kytölä *et al.* 2003, Kopincová *et al.* 2011, 2012). On one hand, NAC suppress NF- κ B activation *via* changes in redox state (Haddad 2002). On the other hand, NF- κ B can be induced also by the presence of IL-1 and TNF- α which are contained in meconium (Yamada *et al.* 2000, Haddad 2002, de Beaufort *et al.* 2003). In this context, meconium

removal by surfactant lavage prevents cytokine-induced NF- κ B actions. Altogether, this improvement is probably a result of surfactant and NAC co-action, which was not so evident when these agents were administered separately.

Similar co-action was seen regarding leukocyte count and neutrophil influx into the lungs. As NAC alone moderately decreased number of cells in BALF and surfactant alone decreased neutrophil portion, working together this combination managed to keep BALF cells count of treated animals in significantly lower values with declined percentage of neutrophils, although lung tissue levels of IL-8 were not significantly reduced ($p=0.09$). Prevention of different inflammatory factors led to reduced lung edema formation which helped to maintain respiratory parameters improved until the end of experiment, implicating that combination of NAC and exogenous surfactant in experimental MAS may bring at least partial therapeutic benefit when compared to monotherapy. Further studies are needed to clarify detailed mechanisms of action of this combination. However, there is a promise of new approach to MAS treatment which perhaps will reach clinical use.

Conflict of Interest

There is no conflict of interest.

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