

# Lung Inflammatory and Oxidative Alterations After Exogenous Surfactant Therapy Fortified With Budesonide in Rabbit Model of Meconium Aspiration Syndrome

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## Summary

Meconium aspiration syndrome (MAS) triggers inflammatory and oxidative pathways which can inactivate both pulmonary surfactant and therapeutically given exogenous surfactant. Glucocorticoid budesonide added to exogenous surfactant can inhibit inflammation and thereby enhance treatment efficacy. Neonatal meconium (25 mg/ml, 4 ml/kg) was administered intratracheally (i.t.) to rabbits. When the MAS model was prepared, animals were treated with budesonide i.t. (Pulmicort, 0.25 mg/kg, M+B); with surfactant lung lavage (Curosurf<sup>®</sup>, 10 ml/kg, 5 mg phospholipids/ml, M+S) followed by undiluted Curosurf<sup>®</sup> i.t. (100 mg phospholipids/kg); with combination of budesonide and surfactant (M+S+B); or were untreated (M); or served as controls with saline i.t. instead of meconium (C). Animals were oxygen-ventilated for additional 5 h. Cell counts in the blood and bronchoalveolar lavage fluid (BAL), lung edema formation (wet/dry weight ratio), oxidative damage of lipids/proteins and inflammatory expression profiles (IL-2, IL-6, IL-13, TNF- $\alpha$ ) in the lung homogenate and plasma were determined. Combined surfactant+budesonide therapy was the most effective in reduction of neutrophil counts in BAL, oxidative damage, levels and mRNA expression of cytokines in the lung, and lung edema formation compared to untreated animals. Curosurf fortified with budesonide mitigated lung inflammation and oxidative modifications what indicate the perspectives of this treatment combination for MAS therapy.

## Key words

Meconium aspiration • Exogenous surfactant • Budesonide • Inflammation • Oxidation • Lung injury

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## Introduction

Meconium aspiration syndrome (MAS) is a serious disease of the neonates. The pathophysiology of MAS involves airway obstruction by meconium, dysfunction of pulmonary surfactant, inflammation, ventilation-perfusion mismatch, pulmonary vasoconstriction, lung edema, and airway hyperreactivity.

Aspirated meconium triggers the inflammation via TLR4/MD-2 CD14 signaling complex leading to increase in transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B) (Salvesen *et al.* 2010). NF- $\kappa$ B translocation initiates simultaneously both pro-oxidative and inflammatory cascades. Meconium itself and meconium-stained amniotic fluid are rich sources of cytokines IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  (de Beaufort *et al.* 2003). Damage to the alveolar-capillary membrane and action of chemotactic IL-8 present in meconium provoke intrapulmonary sequestration of neutrophils. Excessive activation of cells leads to oxidative damage of the tissue by release of cytotoxic and immune cell-activating agents such as proteinases, cationic polypeptides, cytokines, and reactive oxygen and nitrogen species (RONS) through their

oxidant-generating systems. Vice versa, oxidative stress can increase production of pro-inflammatory cytokines (Grommes and Soehnlein 2011).

The complex action of oxidative processes and inflammation, components of aspirated meconium and plasma proteins leaking through the alveolar-capillary membrane finally deteriorates the alveolar type II cells. This results in decreased production and function of surfactant, and alveolar collapse. Dysfunction of endogenous surfactant can be mitigated by administration of exogenous surfactant. However, the above mentioned processes may also affect exogenous surfactant delivered as a therapy (Nkadi *et al.* 2009). From this reason, suppression of oxidation and inflammation appears to be advantageous, as it may diminish inactivation of given surfactant and thereby enhance efficacy of the surfactant therapy.

For this purpose, synthetic glucocorticoid budesonide was chosen for inhibition of inflammation and inflammation-induced oxidation modification. Glucocorticoids (GCs) suppress NF- $\kappa$ B and consequently the expression of NF- $\kappa$ B-dependent pro-inflammatory gene. Thus, they inhibit the transcription of cytokines relevant for acute lung injury, e.g. IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, TNF- $\alpha$ , and granulocyte-macrophage colony-stimulating factor. GCs also act on the antagonist of IL-1 receptor and on anti-inflammatory cytokines IL-4, IL-10, and IL-13 with synergy to control the host-defense response. GCs stimulate apoptosis of T-cells, eosinophils, and monocytes, inhibit neutrophil activation, and maintain endothelial integrity and vascular permeability (Fernandes *et al.* 2005, Newton *et al.* 2010). Previous experimental (Kirimi *et al.* 2003, Mokra *et al.* 2007) and clinical studies (da Costa *et al.* 2001, Basu *et al.* 2007) showed that systemically given GCs improve the lung function in MAS, however, can be accompanied with adverse effects (Mokra *et al.* 2008).

Therefore, we have assumed that addition of glucocorticoid directly into exogenous surfactant for intratracheal (i.t.) delivery can affect local injury and reduce the secondary surfactant inactivation. This study determined whether budesonide can mitigate inflammation and oxidation in the meconium-injured lung and whether its administration coupled with surfactant may enhance the efficacy in comparison with monotherapies (surfactant or budesonide). For this purpose, young rabbits were used for creation of MAS model as body weight and size of the airways are comparable to neonates what is particularly important in

studies with application of different ventilation techniques or lung lavage with diluted surfactant (Ohama and Ogawa 1999, Lam *et al.* 2000, Calkovska *et al.* 2008).

## Methods

### *Animals*

For experiments, eight-week-old New Zealand white rabbits (supplied by Department of Toxicology and Laboratory Animal Breeding Station, Dobra Voda, Slovakia) of both genders with a body weight of  $1.9 \pm 0.2$  kg,  $n=33$  in total were used. Animals were divided into 5 groups according to the treatment: meconium-instilled untreated group (M,  $n=7$ ), meconium-instilled and surfactant-treated group (M+S,  $n=7$ ), meconium-instilled and budesonide-treated group (M+B,  $n=5$ ), meconium-instilled and surfactant+budesonide treated group (M+S+B,  $n=7$ ), and controls with i.t. saline instilled instead of meconium (C,  $n=7$ ).

### *Meconium, exogenous surfactant, budesonide*

First-pass meconium was collected from diapers of 25 healthy term neonates born in University Hospital in Martin, Slovakia. Meconium was pooled, lyophilized and stored at  $-20$  °C. Before i.t. instillation, meconium was suspended in 0.9 % NaCl at a concentration of 25 mg/ml.

Modified porcine surfactant (Curosurf®, Chiesi Farmaceutici, Italy; 80 mg phospholipids (PL) /ml) was used for surfactant therapy. For therapeutic lung lavage (i.e. for washout of meconium), Curosurf was diluted in saline to a PL concentration of 5 mg/ml. After the lung lavage, a supplementation dose of Curosurf in undiluted form was administered i.t. at a dose of 100 mg/kg to recover loss of endogenous surfactant by the lavage procedure.

Budesonide (Pulmicort susp inh, AstraZeneca, 0.5 mg/ml) at a dose of 0.25 mg/kg was used for i.t. administration as a monotherapy (M+B) or added to a supplementation dose of Curosurf (M+S+B).

### *General protocol of experiments*

Experiments were carried out in accordance with the European Guidelines on Laboratory Animal Care, and were authorized by the local Ethics Committee of JFM CU in Martin (EK 609/2010) and National Veterinary Board of Slovak Republic (Ro-1254/10-221).

Animals were anesthetized with intramuscular

ketamine (20 mg/kg; Narketan, Vétoquinol Ltd., UK) and xylazine (5 mg/kg; Xylarium, Riemsler, Germany), followed by i.v. infusion of ketamine (20 mg/kg/h). A tracheostomy was performed and an endotracheal cannula was inserted. Catheters were inserted into the femoral artery for blood sampling and monitoring blood pressure and into the femoral vein for continuous ketamine infusion. After abortion of spontaneous breathing by pipecuronium bromide (0.3 mg/kg/30 min i.v.; Arduan, Gedeon Richter, Hungary) animals were ventilated by ventilator Beat-2 (Chirana, Slovakia) with following settings: frequency (f) of 30/min, fraction of inspired oxygen (FiO<sub>2</sub>) 0.21, inspiration time (Ti) 50 %, peak inspiratory pressure (PIP) adjusted to keep a tidal volume (V<sub>T</sub>) of 7-9 ml/kg, and no positive end-expiratory pressure (PEEP). After 15 min of stabilization, meconium (M groups) or saline (C group) at a dose of 4 ml/kg were instilled i.t. in semi-upright right and left lateral positions of the animal. Subsequently, FiO<sub>2</sub> was increased to 1.0, PEEP to 3 cm H<sub>2</sub>O to avoid atelectasis of alveoli and PIP to supply V<sub>T</sub> of 7-9 ml/kg. Within 30 min, meconium instillation caused respiratory insufficiency defined as >30 % decrease in dynamic lung-thorax compliance (C<sub>dyn</sub>) and PaO<sub>2</sub> <10 kPa at FiO<sub>2</sub> 1.0. When the criteria for MAS model set according to the literature (Lam *et al.* 2000, Calkovska *et al.* 2008, Wang *et al.* 2010) were fulfilled, animals were treated (see below). After the treatment delivery, animals were ventilated for additional 5 h (FiO<sub>2</sub> 1.0, f=30/min, V<sub>T</sub> 7-9 ml/kg, PEEP 3 cm H<sub>2</sub>O), and then overdosed by anesthetics. The left lung was lavaged with saline (3x10 ml/kg) and diagnostic BAL fluid was centrifuged (1500 rpm for 15 min) to obtain cell-rich sediment. The right lung was assessed for lung edema formation, or stored at -70 °C natively or in RNA stabilization solution (RNAlater, QIAGEN Group) until biochemical analyses were performed.

#### *Administration of the treatment*

Surfactant therapy (M+S group) consisted of the lung lavage with diluted surfactant (Curosurf, 10 ml/kg, 5 mg PL/ml) given twice in the semi-upright right and left lateral animal positions, each followed by a tracheal suction using suction device. This maneuver served for partial washout of instilled meconium. Subsequently, a dose of undiluted Curosurf (100 mg PL/kg, 1.25 ml/kg) was administered *via* jet of ventilator using asymmetric high-frequency jet ventilation (or inpulsion regime of HFJV) (f. 300/min, Ti 20 %, PIP/PEEP 15/3 cm H<sub>2</sub>O) to supplement the loss of endogenous surfactant caused by

lavage and/or inactivation by meconium.

Budesonide (0.25 mg/kg, 0.5 ml/kg) was administered intratracheally by asymmetric HFJV as a monotherapy (M+B group) or added into the dose of undiluted Curosurf as a combined therapy (M+S+B group) by a similar manner as described above.

#### *Cell counts in the blood and BAL fluid*

Differential white blood cell counts in the arterial blood and cell counts in the sediment of diagnostic BAL fluid were estimated microscopically after staining by May-Grünwald/Giemsa-Romanowski. The counts of cells were expressed in percents (%).

#### *Biochemical analysis of oxidative modification and cytokine concentration in the lung*

Concentrations of cytokines and oxidative modification products were determined in 10 % (weight/volume) lung homogenate prepared using 0.1 M phosphate buffer (PBS, pH 7.4) by Polytron homogenizer PT 1200 E (Kinematica AG, Switzerland). Oxidative damage to proteins was determined using OxiSelect™ Nitrotyrosine ELISA Kit (Cell Biolabs, Inc., USA) and expressed as concentration of 3-nitrotyrosine in nanomoles (nM 3NT). Oxidative damage to lipids expressed as concentration of thiobarbituric acid reacting substances (TBARS) was determined by OxiSelect™ TBARS Assay Kit (Cell Biolabs Inc., USA), and was shown as concentration of malondialdehyde in micromoles (μM MDA). To evaluate the changes in oxidative status in plasma, concentrations of 3NT and MDA were detected in initial (P1) and final (PF) plasma and expressed as a ratio PF/P1. Concentrations of IL-2, IL-6, IL-13 and TNF-α were quantified in duplicate using rabbit-specific ELISA kits (USCN Life Science Inc., China) according to the manufacturer's instructions and expressed in pg/ml.

#### *Cytokines expression profile using real-time quantitative PCR*

Stabilized lung tissue was homogenized and total RNA was isolated using RNeasy® Mini Kit (QIAGEN Group) followed by cDNA producing using QuantiTect® Reverse Transcription Kit (QIAGEN Group). Rabbit-specific primer sequences for hypoxanthine phosphoribosyl transferase (HPRT) as a reference gene for normalization, forward (F): 5'-TGATAGATCCATTCCTATGACTGTAGA-3' and reverse (R): 5'-GGGTCCTTTTCACCAGCAG-3';

for IL-2, F: 5'-TGAAACATCTTCAGTGTCTAGAAG-3' and R: 5'-CATTGTAGAATTTCTGAACAGAT-3'; for IL-6, F: 5'-TAGTCCTTCTACCCAATTTCC-3' and R: 5'-TTGGTCCTTAGCCACTCCTTC-3'; for IL-13, F: 5'-GCAAATAATGAGCTTTCGAAGTTTCAGTGG-3' R: 5'-CTTCCGTGAGGACTGAATGAGACGGTC-3'; for TNF- $\alpha$ , F: 5'-GTCTTCTCTCTCACGCACC-3' and R: 5'-TGGGCTAGAGGCTTGTCAC-3'. Quantitative real-time PCR (RT-qPCR) was performed in triplicates using the iCycler iQ5<sup>®</sup> (Bio-Rad Laboratories Inc.) and QuantiTect SYBR<sup>®</sup> Green PCR Kit (QIAGEN Group). The amplification cycling conditions were as follows: an initial denaturation step at 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 72 °C for 30 s. The crossing point or the cycle number at which the fluorescence of the sample exceeded that of the background, was determined by the Bio-Rad iQ5 – Standard Edition Optical System Software 2.0 using the second derivative method.

#### *Lung edema formation*

Strips of the right lung were weighed before and after drying in an oven at 60 °C for 48 h. Wet to dry (W/D) lung weight ratio expressed an extent of lung edema formation.

#### *Statistical analysis*

Data are presented as means  $\pm$  SEM. Statistical analysis was performed using statistic software Graph Pad Prism 5 (USA). The differences among the groups were analyzed by Kruskal-Wallis non-parametric test with a Dunn's multiple all pairs comparison test. A  $p$  value  $<0.05$  was considered to be statistically significant.

## **Results**

#### *Cell counts in the arterial blood and BAL fluid*

In the blood, percentage of neutrophils decreased after meconium instillation compared to controls (M vs. C  $p<0.01$ ). The circulating neutrophils elevated after all therapies, but significant difference was observed only in both budesonide-treated groups compared to untreated group (M+B vs. M  $p<0.05$ , M+S+B vs. M  $p<0.01$ ). Percentage of monocytes/macrophages decreased slightly in all meconium-instilled groups compared to controls (Fig. 1).

In the BAL fluid, percentage of neutrophils

increased after meconium instillation compared to controls (M vs. C  $p<0.001$ ). The therapies decreased neutrophil leak into the lung whereas this reduction was significant only for combined therapy compared to untreated group (M+S+B vs. M  $p<0.05$ ) and to budesonide-treated group (M+S+B vs. M+B  $p<0.05$ ). Contrary, monocytes/macrophages decreased after meconium instillation compared to controls (M vs. C  $p<0.001$ ). Only combined therapy elevated their percentage significantly compared to untreated group and budesonide-treated group (M+S+B vs. M and M+S+B vs. M+B, for both  $p<0.05$ ) (Fig. 1).

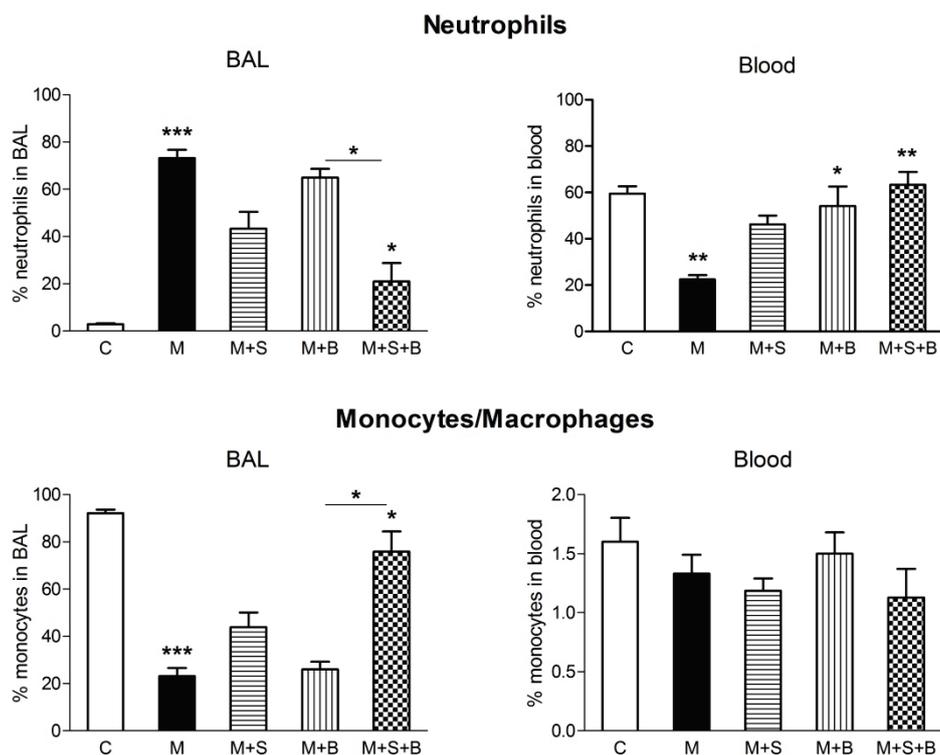
#### *Oxidative modifications in the lung tissue and plasma*

Oxidative damage to proteins in the lung and plasma expressed as 3-nitrotyrosine (3NT) increased in the meconium-instilled untreated group compared to controls (in lung M vs. C  $p<0.05$ , in plasma  $p<0.01$ ). Given therapies lowered the protein oxidation compared to the untreated animals, whereas this effect was significant only for surfactant+budesonide combination (in lung M+S+B vs. M  $p<0.001$ , in plasma  $p<0.05$ ; Fig. 2).

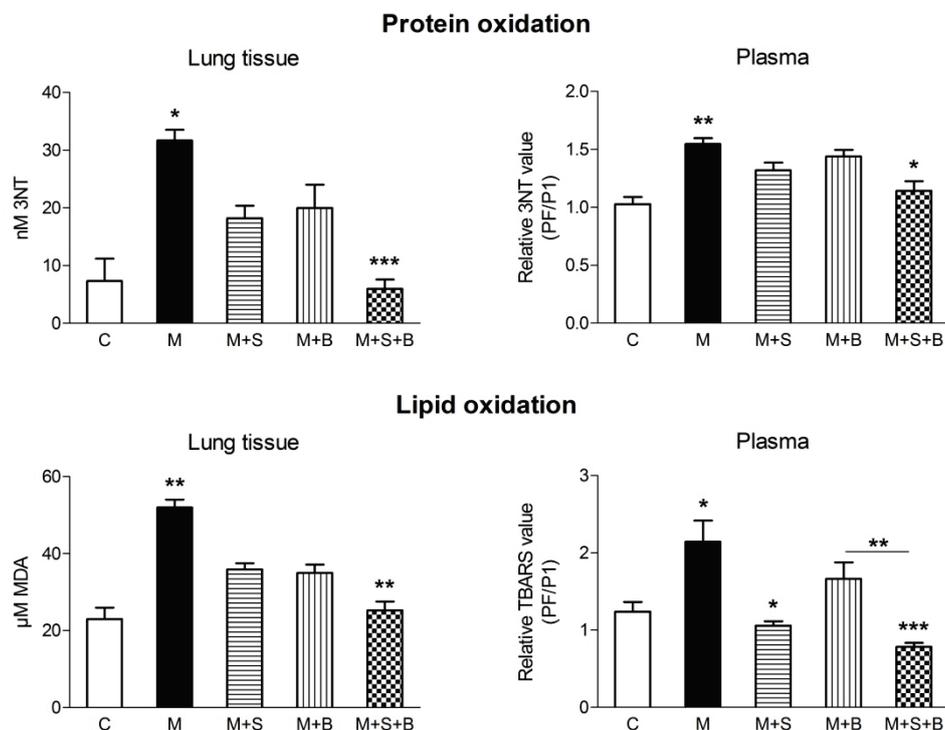
Oxidative damage to lipids expressed by formation of malondialdehyde was higher in meconium-instilled untreated group compared to controls (M vs. C  $p<0.01$  in lung,  $p<0.05$  in plasma). Lung concentration of MDA in all treated groups decreased compared to untreated group, but significantly only for combined therapy (M+S+B vs. M  $p<0.01$ ). In plasma of both surfactant-treated groups, reduced lipid damage compared to untreated group was found, whereas combined therapy was superior to surfactant alone (M+S+B vs. M  $p<0.001$ , M+S vs. M  $p<0.05$ ) and budesonide alone (M+S+B vs. M+B  $p<0.01$ ; Fig. 2).

#### *Cytokine production in the lung*

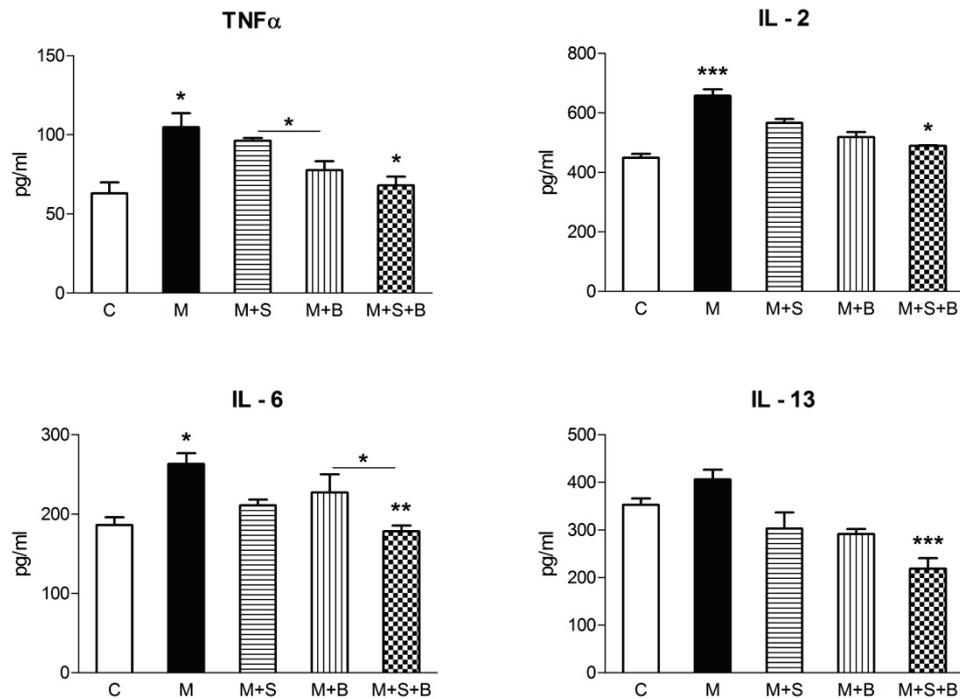
Concentrations of IL-2 ( $p<0.001$ ), IL-6 and TNF- $\alpha$  (both  $p<0.05$ ) in the lung homogenate of meconium-instilled untreated group elevated compared to controls, except IL-13 where no difference was observed (M vs. C  $p>0.05$ ). However, all therapies reduced the cytokine production, only the combined therapy showed significant effect compared to untreated group (M+S+B vs. M, for IL-6  $p<0.01$ , for IL-13  $p<0.001$ , for both TNF- $\alpha$  and IL-2  $p<0.05$ ), to surfactant monotherapy (TNF- $\alpha$ : M+S+B vs. M+S  $p<0.05$ ) and to budesonide monotherapy (IL-6: M+S+B vs. M+B  $p<0.05$ ; Fig. 3).



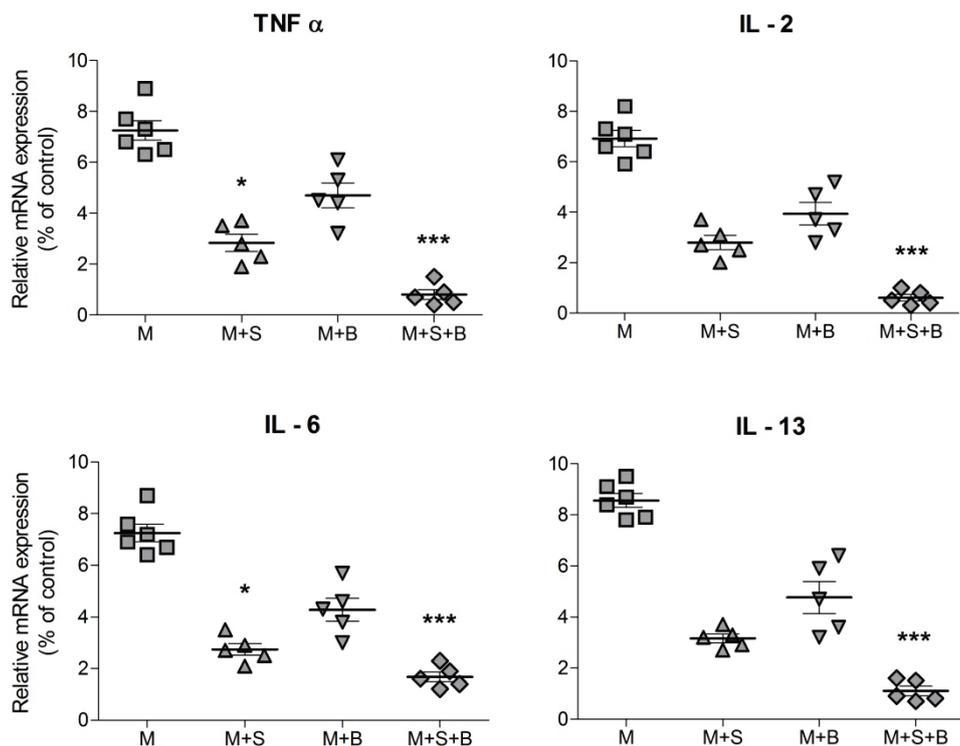
**Fig. 1.** Neutrophils and monocytes/macrophages in the blood and BAL fluid (expressed in %) at the end of experiment in saline-instilled controls (C), meconium-instilled untreated group (M), meconium-instilled and surfactant-treated (M+S) group, meconium-instilled and budesonide-treated (M+B) group, and meconium-instilled and surfactant+budesonide treated (M+S+B) group. Statistical comparisons: M vs. C; M+S and M+B and M+S+B vs. M: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



**Fig. 2.** Oxidative modifications in lung tissue and plasma of saline-instilled controls (C), meconium-instilled untreated group (M), meconium-instilled and surfactant-treated (M+S) group, meconium-instilled and budesonide-treated (M+B) group, and meconium-instilled and surfactant+budesonide treated (M+S+B) group. Marker of protein oxidation 3-nitrotyrosine [expressed in nanomole concentration (nM 3NT)] and marker of lipid oxidation thiobarbituric acid-reactive substances [TBARS expressed in micromole concentration of malondialdehyde ( $\mu\text{M}$  MDA)]. Statistical comparisons: M vs. C; M+S and M+B and M+S+B vs. M: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



**Fig. 3.** Concentrations of IL-2, IL-6, IL-13 and TNF- $\alpha$  in the lung tissue measured using ELISA method (expressed in pg/ml) in the saline-instilled controls (C), meconium-instilled untreated group (M), meconium-instilled and surfactant-treated (M+S) group, meconium-instilled and budesonide-treated (M+B) group, and meconium-instilled and surfactant+budesonide treated (M+S+B) group. Statistical comparisons: M vs. C; M+S and M+B and M+S+B vs. M: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



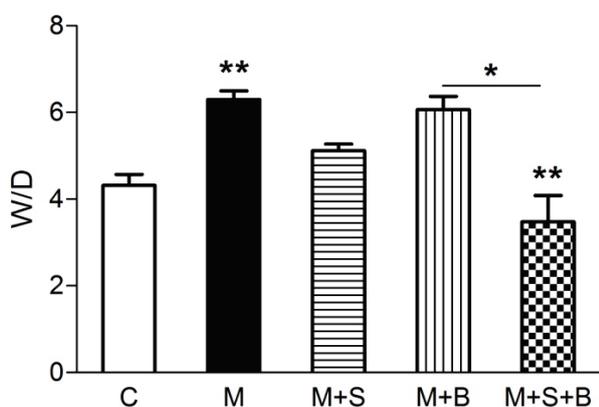
**Fig. 4.** Expression profile of cytokines IL-2, IL-6, IL-13 and TNF- $\alpha$  in the lung tissue using RT-PCR method in the meconium-instilled untreated group (M), meconium-instilled and surfactant-treated (M+S) group, meconium-instilled and budesonide-treated (M+B) group, meconium-instilled and surfactant+budesonide treated (M+S+B) group. The each geometric symbol represents the relative expression value of control (C=1.0) (% C) and is normalized to HPRT level for each animal separately. The horizontal line represents the average value of each group. Statistical comparisons: M+S and M+B and M+S+B vs. M: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

### mRNA expression profile of cytokines in the lung tissue

To evaluate the relative changes of mRNA expression of cytokines in the lung tissue, saline-instilled animals (controls, C) were used as a calibrator group with an absolute genetic expression value of 1, with values over or below representing higher or lower gene expression. Thus, the cytokine expression was expressed in percents derived from controls and data are shown in Figure 4. Expression of all cytokines was obviously elevated in meconium-instilled untreated group compared to controls. Compared to untreated group, gene expression of all cytokines decreased significantly after combined therapy (M+S+B vs. M  $p < 0.001$ ) and partially also after surfactant monotherapy (IL-6 and TNF- $\alpha$ : M+S vs. M  $p < 0.05$ ). No differences between the therapies were observed (Fig. 4).

### Lung edema formation

Formation of lung edema expressed as W/D ratio was higher in meconium-instilled untreated group compared to controls (M vs. C  $p < 0.01$ ). The W/D ratio significantly decreased in combined therapy compared to untreated group (M+S+B vs. M  $p < 0.01$ ) and to budesonide-treated group (M+S+B vs. M+B  $p < 0.05$ ; Fig. 5) what indicates antiedematous potential of the combined treatment.



**Fig. 5.** Lung edema formation expressed as wet-dry (W/D) lung weight ratio in the saline-instilled controls (C), meconium-instilled untreated group (M), meconium-instilled and surfactant-treated (M+S) group, meconium-instilled and budesonide-treated (M+B) group, and meconium-instilled and surfactant+budesonide treated (M+S+B) group. Statistical comparisons: M vs. C; M+S and M+B and M+S+B vs. M: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

## Discussion

Mechanisms of pathophysiology of MAS interact to a large extent. Properly chosen therapy can

suppress the individual factors and thereby to improve the status of neonates. This experimental study showed that exogenous surfactant enriched with budesonide reduces the meconium-induced neutrophil migration into the lung, production of pro-inflammatory cytokines and oxidation modifications of proteins and lipids, and formation of lung edema.

Substitution of inactivated pulmonary surfactant by the exogenous one has become a part of the therapeutic protocol in severe MAS (Dargaville *et al.* 2003). In this study, lung lavage with diluted surfactant was performed twice to remove a part of instilled meconium. The lavage was followed by administration of undiluted surfactant to substitute the components of surfactant lost by inactivation and/or lavage procedure. Nevertheless, due to oxidative modifications and on-going inflammation the surfactant therapy can be insufficient and repetitive doses of surfactant are required (Nkadi *et al.* 2009). To avoid this situation, surfactant can be delivered together with suitable anti-inflammatory drug. We have recently shown that anti-inflammatory drugs (budesonide, N-acetylcysteine) added to surfactant improve respiratory functions and mitigate inflammation and oxidation in animals with MAS (Mikolka *et al.* 2013, Mikolka *et al.* 2016). In this article, effects of surfactant+budesonide combination on oxidative modifications and inflammatory profile on protein and expression levels in the lung tissue are reported.

Aspiration of meconium is an initial factor for MAS. Particles of meconium obstruct the airways and contribute to hypoxemia, hypercapnia, and respiratory acidosis leading to pulmonary vasoconstriction and increase in right-to-left pulmonary shunts (Kattan *et al.* 2013). Furthermore, meconium is a source of deleterious substances such as bile acids, bile salts, bilirubin, cholesterol, tri-, di- and monoglycerides, free fatty acids, heme, enzymes including pancreatic phospholipase A<sub>2</sub>, and cytokines (de Beaufort *et al.* 2003) which inactivate pulmonary surfactant, trigger oxidation and inflammation, and damage the endothelial and epithelial cells. In experimental animals with meconium instillation, similar symptoms expressing a significant deterioration of the lung function can be observed. Meconium in a concentration of 25 mg/ml and a dose of 4 ml/kg b.w. decreases lung compliance of about 40-50 % compared to the initial value and worsens gas exchange within 30 min after meconium instillation (Mikolka *et al.* 2013).

As mentioned above, MAS is associated with a local inflammation involving several types of immune

cells. As the earliest cells recruited to the site of injury or inflammation neutrophils have been identified. Homing of leukocytes is mediated through adhesion molecules, e.g.  $\beta_2$ -integrins CD11b/CD18 which also indicate polymorphonuclear activation. Enhanced expression of CD11b in neutrophils was detected in *in vitro* exposure to meconium and increased expression of CD11b and CD18 was identified in granulocytes sequestered in the lung (Castellheim *et al.* 2005). Other potent chemoattractant for neutrophils is IL-8 which is present in meconium (de Beaufort *et al.* 2003). In accordance with the mentioned facts, we have found an increased percentage of neutrophils in the diagnostic BAL fluid of meconium-instilled untreated animals compared to controls which was associated with their decreased percentage in the blood.

Surfactant lung lavage removes a portion of instilled meconium (Dargaville *et al.* 2003), and thereby reduces the pro-inflammatory and chemotactic stimuli in the lung and prevent a leak of cells into the lung linked with their increase in the peripheral blood as we could observe it in the surfactant-treated groups compared to untreated animals. Neutrophil sequestration into the lung is further inhibited by budesonide *via* inhibition of expression of vascular cell adhesion molecule-1 (VCAM-1) (Wilson *et al.* 2001) and inhibition of neutrophil chemotaxis and production of pro-inflammatory proteins (e.g. IL-6, IL-8 and CXCL1) (Strandberg *et al.* 2010). In this study, actions of both budesonide and surfactant were additive as we could observe it on significant decrease of neutrophils in the BAL fluid only in combined therapy in contrast to monotherapies using multiple all pairs comparison test. Similar results were found in combination of surfactant and N-acetylcysteine (Mikolka *et al.* 2016).

Although neutrophil activation is vital for a host defence, overzealous activation leads to a tissue damage. Neutrophils release various agents such as proteinases, cationic polypeptides, cytokines, and RONS through their oxidant-generating systems comprising the phagocyte NADPH oxidase and nitric oxide synthase (NOS). Neutrophil-induced lung damage and increased RONS in the alveolar spaces were demonstrated in patients with ALI/ARDS (Lang *et al.* 2002). In our experiments, meconium aspiration provoked oxidative changes in the lung and plasma, whereas protein nitrosylation was verified as increased 3-nitrotyrosine and lipid peroxidation as elevated TBARS concentrations. In agreement to our results, stimulated neutrophil oxidative

burst as a meconium-mediated effect was previously published in *in vitro* studies (Soukka *et al.* 2002, Craig *et al.* 2005).

Considering the mentioned facts, administration of GCs reducing neutrophil activation and sequestration seems to be reasonable. In this study, exogenous surfactant combined with budesonide prevented oxidative damage in the lung and plasma when significantly reduced the levels of 3NT and TBARS compared to untreated group. Pro-inflammatory cytokines and oxidative stress could interact through redox sensitive transcription factor NF- $\kappa$ B. Production of 3NT results from induction of NOS mediated through NF- $\kappa$ B, which is inhibited by GCs (Wilson *et al.* 2001, Fernandes *et al.* 2005).

The inflammatory response associated with MAS could be detected early after meconium aspiration. Meconium activates TLR4 receptor complex CD14/TLR4/MD-2, which is also associated with lipopolysaccharide (LPS)-induced response (Salvesen *et al.* 2010). In addition, meconium itself is a source of IL-1, IL-6, IL-8, and TNF- $\alpha$  which may induce inflammation directly or through the stimulation of neutrophil oxidative burst. Similarly, NF- $\kappa$ B could be induced by IL-1 and TNF- $\alpha$  contained in meconium (Soukka *et al.* 2002, de Beaufort *et al.* 2003). In our study, cytokine mRNA expression and concentrations of IL-2, IL-6, IL-13, and TNF- $\alpha$  in the lung significantly increased in the meconium-instilled group compared to controls, what corresponds with our recent work and findings of other authors (Bhatia and Moochhala 2004, Mikolka *et al.* 2016). The given treatments down-regulated the cytokine expression what correlated with lower concentrations of cytokines in the lung. However, superior effect was observed after combined treatment what is likely related to partial removal of meconium and local anti-inflammatory action of budesonide, finally leading to reduced activation of NF- $\kappa$ B (Wilson *et al.* 2001, Fernandes *et al.* 2005). Significant differences were observed between M and M+S group using non-parametric Mann-Whitney U test, but significance did not survive the multiple comparison.

The complex action of meconium and pro-inflammatory substances damage the endothelial and epithelial cells and thereby increase the permeability across the alveolar-capillary membrane, resulting in a pulmonary edema (Dargaville *et al.* 2003). For instance, RONS induced in inflammation and phospholipase A<sub>2</sub> present in meconium can disrupt intercellular connections

in endothelium and/or induce apoptosis and necrosis of alveolar cells (Kääpä and Soukka 2008). In our study, extent of lung edema formation was expressed from the wet-dry (W/D) lung weight ratio. Significantly higher W/D value in the meconium-instilled group indicated increased accumulation of liquid in the tissue. Despite there was a trend to decrease W/D ratio also in surfactant-treated group (M+S), only surfactant combined with budesonide reduced significantly the edema formation compared to untreated group.

Nevertheless, there are several limitations to direct applicability of our results for clinical practice. First, use of young animals avoids postnatal changes of the lung and hemodynamics which could be observed in neonatal animals. However, due to technical problems with instrumentation of the neonatal animals and due to ethical problems, a part of researchers including our research group (Ohama and Ogawa 1999, Lam *et al.* 2000, Calkovska *et al.* 2008) prefer the use of several days to weeks-old animals, especially in the studies where artificial ventilation should be used (as it is in our study). Second limitation of the study is the difference in the dynamics of artificially delivered meconium in animal model vs. spontaneous aspiration of meconium by neonate. And finally, we have to accept some inter-species differences in the immune system between humans and rabbits which may result in some differences

in response to induction of the model or to delivered therapy. Considering all the limitations and potential benefits of the study we feel that the results of our study bring new information for research on MAS, for better understanding of the pathophysiology of the syndrome and for evaluation of potentially beneficial treatment approaches. Concluding, budesonide added to exogenous surfactant enhanced the efficacy of surfactant therapy on lung inflammation and oxidation. Administration of the therapy was not accompanied with any obvious adverse effects and we can presume that this combination could be beneficial also in the newborns with MAS.

### Conflict of Interest

There is no conflict of interest.

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