

# Budesonide Added to Modified Porcine Surfactant Curosurf May Additionally Improve the Lung Functions in Meconium Aspiration Syndrome

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## Summary

Severe meconium aspiration syndrome (MAS) in newborns is often treated by exogenous surfactant. Because its efficacy is reduced by meconium-induced inflammation, glucocorticoid budesonide was added into surfactant preparation Curosurf to enhance efficacy of the surfactant therapy in experimental model of MAS. Oxygen-ventilated rabbits were intratracheally given meconium (25 mg/ml, 4 ml/kg) to induce respiratory failure. Thirty minutes later, animals were treated by intratracheal budesonide (0.25 mg/kg); or surfactant lung lavage (10 ml/kg, 5 mg phospholipids/ml) repeated twice, followed by undiluted Curosurf (100 mg phospholipids/kg); or by the above mentioned surfactant treatment with the last surfactant dose fortified with budesonide (0.25 mg/kg); or were untreated. Animals were ventilated for additional 5 hours and respiratory parameters were measured regularly. After sacrificing animals, wet-dry lung weight ratio was evaluated and plasma levels of interleukins (IL)-1beta, -6, -8, and TNF-alpha were measured by ELISA method. Efficacy of the given therapies to enhance lung functions and to diminish lung edema formation and inflammation increased from budesonide-only and surfactant-only therapy to surfactant+budesonide therapy. Combined therapy improved gas exchange from 30 min of administration, and showed a longer-lasting effect than surfactant-only therapy. In conclusions, budesonide additionally improved the effects of exogenous surfactant in experimental MAS.

## Key words

Budesonide • Curosurf • Exogenous surfactant • Inflammation • Meconium aspiration

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## Introduction

Meconium aspiration syndrome (MAS) is a serious disorder in the term and post-term newborns, resulting from intrauterine or postnatal aspiration of the first feces of the newborn – meconium. Meconium contains a number of substances, such as bile acids and bile salts, bilirubin, cholesterol, tri-, di- and monoglycerides, free fatty acids, heme, enzymes including pancreatic phospholipase A<sub>2</sub>, and pro-inflammatory interleukins IL-1, -6, -8, and tumor necrosis factor (TNF)- $\alpha$  (de Beaufort *et al.* 2003). The mentioned substances have a great potential to induce dysfunction of pulmonary surfactant and to trigger inflammation, which finally contribute to lung edema formation. In addition, mucopolysaccharides as a major component of meconium increase viscosity and reduce transportability of aspirated meconium, and thereby participate in airway obstruction. Hand-in-hand with worsened gas exchange and impairment of the lung parenchyma, pulmonary vasoconstriction and increased right-to-left pulmonary shunts occur. As a result, hypoxemia, hypercapnia, and respiratory acidosis may be observed shortly after meconium aspiration.

Therapeutic interventions in severe MAS may include airway suctioning, oxygen delivery, or ventilatory support. In severe cases, exogenous surfactant should be

delivered as undiluted bolus or as bronchoalveolar (or lung) lavage by diluted exogenous surfactant. Exogenous surfactant substitutes functional loss of pulmonary surfactant due to inactivation by meconium and other surfactant inhibitors, such as plasma proteins leaking through injured alveolocapillary membrane, pro-inflammatory cytokines, and reactive oxygen species. If surfactant lung lavage is used, it removes a significant portion of aspirated meconium and proteinaceous debris from the lungs, resulting in lower airway obstruction, diminished lung injury, and reduced ventilation-perfusion mismatch. To enhance the favorable effects of the treatment, lung lavage by diluted surfactant may be followed by slow administration of undiluted surfactant (Kaneko *et al.* 2001, Szymankiewicz *et al.* 2004).

Because inflammation plays an important role in the pathogenesis of MAS, anti-inflammatory agents, such as glucocorticoids, may be useful in the treatment of MAS. For example, intratracheal administration of budesonide has improved lung functions in the experimental animals (Mokra *et al.* 2007a) and in the newborns (Basu *et al.* 2007, Tripathi and Saili 2007) with MAS. Budesonide using exogenous surfactant as a carrier has been used in respiratory distress syndrome or chronic lung disease (Yeh *et al.* 2008, Yang *et al.* 2013). However, up to date no study has combined budesonide with exogenous surfactant in the treatment of MAS.

Based on the references and our previous experience with surfactant lung lavage (Sevecova-Mokra *et al.* 2004, Calkovska *et al.* 2008) and administration of glucocorticoids (Mokra *et al.* 2007a,b) in a rabbit model of MAS, we have supposed that combined administration of exogenous surfactant and budesonide may result in accentuated improvement in the lung functions compared with these treatments given separately. This pilot study has evaluated changes in the lung function parameters and some inflammatory markers after the surfactant lung lavage followed by a substitution dose of surfactant enriched with budesonide in comparison with surfactant-only and budesonide-only treatments, as well as differences between the treated groups and non-treated meconium-instilled animals.

## Methods

### *Meconium*

First-pass meconium was collected from 30 healthy term neonates. The samples were pooled, lyophilized, and stored at  $-20^{\circ}\text{C}$ . Before use, meconium

was suspended in 0.9 % NaCl at a concentration of 25 mg/ml.

### *Surfactant/budesonide*

For lung lavage, modified porcine surfactant (Curosurf<sup>®</sup>, Chiesi Farmaceutici, Italy; 80 mg phospholipids (PL)/ml) was diluted in saline to a PL concentration of 5 mg/ml. After the lavage, undiluted Curosurf was given at a dose of 100 mg/kg body weight (b.w.) as a supplementation dose.

Budesonide (Pulmicort suspension for inhalation, AstraZeneca, 0.5 mg/ml) at a dose of 0.25 mg/kg b.w. was used as a monotherapy (Mec+Bud group) or added to Curosurf (Mec+Surf+Bud).

### *General design of experiments*

Protocol of experiments was approved by the local Ethics Committee of Jessenius Faculty of Medicine. Experiments were carried out on about 8-week-old New Zealand white rabbits of both genders and mean body weight of  $2.5\pm 0.2$  kg (SAV Dobra Voda, Slovakia). Animals were anesthetized with intramuscular ketamine (20 mg/kg; Narketan, Vétouinol Ltd., UK) and xylazine (5 mg/kg; Xylarium, Riemsler, Germany), followed by ketamine infusion (20 mg/kg/h). A tracheotomy was performed and an endotracheal tube was inserted into the trachea. Catheters were inserted into the femoral artery for monitoring blood pressure and sampling arterial blood, into the femoral vein for administration of drugs and anesthetics, and through the jugular vein into the right atrium for sampling mixed venous blood. Animals were paralyzed with pipecuronium bromide (Arduan, Gedeon Richter, Hungary) at a dose of 0.3 mg/kg/30 min i.v. to avoid spontaneous breathing and were subjected to the pressure-controlled ventilator (Beat-2, Chirana, Slovakia). Animals were ventilated with conventional or IPPV (intermittent positive pressure ventilation) mode of ventilation with a frequency (f) of 30/min, fraction of inspired oxygen ( $\text{FiO}_2$ ) of 0.21, time of inspiration ( $T_i$ ) 50 %, and peak inspiratory pressure (PIP) adjusted to keep a tidal volume ( $V_T$ ) of 7-9 ml/kg. No positive end-expiratory pressure (PEEP) was used in this stage of experiments. After 15 min stabilization, lung function parameters ( $V_T$ , PIP, PEEP) were recorded and baseline values of arterial and venous blood gases, hemoglobin, and parameters of acid-base balance were measured by a blood analyzer (RapidLab<sup>TM348</sup>, Bayer Diagnostics, Germany). A suspension of meconium at a dose of 4 ml/kg b.w. was divided into two equal portions and

instilled into the lungs in the semi-upright (elevation up to 45°), right and left lateral positions of the animal.  $\text{FiO}_2$  was increased to 1.0, PEEP to 0.3 kPa and PIP to supply a tidal volume of 7-9 ml/kg b.w. Respiratory failure developed within 30 min after meconium administration, defined as >30 % decrease in dynamic lung-thorax compliance ( $C_{\text{dyn}}$ ) and  $\text{PaO}_2 < 10$  kPa at  $\text{FiO}_2$  of 1.0. Blood gases and lung function parameters were measured at this time point as baseline values before treatment administration.

#### *Administration of the treatment*

The animals were divided according to the treatment into four groups: 1. meconium without treatment (Mec,  $n=6$ ), 2. meconium with surfactant treatment (Mec+Surf,  $n=5$ ), 3. meconium with budesonide treatment (Mec+Bud,  $n=6$ ), 4. meconium with combined surfactant and budesonide treatment (Mec+Surf+Bud,  $n=5$ ). In all surfactant-treated animals, treatment was given in two steps. Initially, bronchoalveolar lavage with 10 ml/kg of diluted exogenous surfactant (Curosurf, concentration of 5 mg PL/ml) was performed twice. First dose of diluted Curosurf was administered using a syringe in semi-upright (45°), right and left lateral positions of the animal to ensure a proportional distribution of the fluid. Then, animal was connected to the ventilator and ventilated with following settings: f. 30/min, PIP/PEEP 1.5/0.3 kPa,  $\text{FiO}_2$  1.0. The lavage fluid was suctioned (Suction Professional, Elletromedicali, Italy) with a pressure of -40 kPa within 30 s after the administration and 1 min after the first suctioning. After stabilization of cardiorespiratory parameters, second dose of the lavage fluid was administered by a same way as the first dose. Volume of the removed lavage fluid was measured and recovery of the lavage fluid was calculated and expressed in %. After stabilization, the third dose of Curosurf (concentration of 100 mg PL/kg, 1.25 ml/kg) was administered slowly into a jet of ventilator using asymmetric high-frequency jet ventilation (or impulsion regime of HFJV) (f. 300/min, Ti 20 %, PIP/PEEP 1.5/0.3 kPa) to homogeneously spread surfactant throughout the lungs. In animals with combined treatment (Mec+Surf+Bud group), budesonide (Pulmicort susp. inh., AstraZeneca, 0.25 mg/kg b.w., 0.5 ml/kg b.w.) was added into the dose of undiluted Curosurf. The mixture was administered intratracheally by means of asymmetric HFJV, as described above. In budesonide-only-treated animals (Mec+Bud group), budesonide (Pulmicort susp.

inh., AstraZeneca, 0.25 mg/kg b.w., 0.5 ml/kg b.w.) was administered intratracheally by means of asymmetric HFJV (f. 300/min, Ti 20 %, PIP/PEEP 1.5/0.3 kPa), by a similar manner as described above. After the treatment administration, animals were ventilated ( $\text{FiO}_2$  1.0, frequency 30/min,  $V_T$  7-9 ml/kg b.w.) for additional 5 hours. Arterial and venous blood samples were analyzed and lung function parameters were recorded 30 min, 1, 2, 3, 4, and 5 hours after the treatment. At the end of experiment, animals were sacrificed by an overdose of anesthetics.

#### *Measurements and calculations of lung function parameters*

Tracheal airflow and tidal volume were measured by a heated Fleisch head connected to a pneumotachograph (UMMT SAV, Slovakia), placed temporarily between the endotracheal tube and outlet of the ventilatory circuit. Airway pressure was registered *via* a pneumatic catheter placed 0.5 cm below a distal tip of the tracheal tube and connected to an electromanometer (Tesla, Czech Republic). Blood pressure was measured through the catheter placed in the femoral artery and recorded using an electromanometer (Tesla, Czech Republic). Heart rate was recorded using subcutaneous needle electrodes. The biosignals were transferred to a multi-channel recorder PowerLab 8/30 (AD Instruments, Germany).

Mean airway pressure (MAP) was calculated as:  $\text{MAP} = (\text{PIP} + \text{PEEP})/2$ , and oxygenation index (OI) as:  $\text{OI} = \text{MAP} \times \text{FiO}_2/\text{PaO}_2$ .  $C_{\text{dyn}}$  was calculated as a ratio between the tidal volume adjusted per kg b.w. and the airway pressure gradient (PIP - PEEP). Right-to-left shunts (RLS) were calculated by a computer program using the Fick equation:  $(\text{CcO}_2 - \text{CaO}_2)/(\text{CcO}_2 - \text{CvO}_2) \times 100$ , where  $\text{CcO}_2$ ,  $\text{CaO}_2$  and  $\text{CvO}_2$  are the concentrations of oxygen in the pulmonary capillaries, arterial and mixed venous blood.  $\text{CcO}_2$  was calculated by using  $\text{P}_{\text{A}}\text{O}_2$  (alveolar partial pressure of oxygen) from the equation:  $\text{P}_{\text{A}}\text{O}_2 = (\text{PB} - \text{PH}_2\text{O}) \times \text{FiO}_2 - \text{PaCO}_2 \times [\text{FiO}_2 + (1 - \text{FiO}_2)/\text{R}]$ , where PB is a barometric pressure and  $\text{PH}_2\text{O}$  the pressure of water vapor. Respiratory exchange ratio (R) was assumed to be 0.8 and the value of hemoglobin necessary for calculating the oxygen concentration in the blood was measured by analyzer RapidLab<sup>TM348</sup> (Bayer Diagnostics, Germany).

#### *Wet-dry lung weight ratio*

After sacrificing the animals, strips from the

**Table 1.** Mean airway pressure (MAP), dynamic lung-thorax compliance (C<sub>dyn</sub>), and right-to-left pulmonary shunts (RLS) before and after meconium (Mec) instillation and within 5 hours after administration of the therapy (Th) in the meconium-instilled non-treated group (Mec), budesonide-only treated group (Mec+Bud), surfactant-only treated group (Mec+Surf), and surfactant+budesonide treated group (Mec+Surf+Bud).

	Before Mec	After Mec	30 min Th	1 h Th	2 h Th	3 h Th	4 h Th	5 h Th
<i>MAP (kPa)</i>								
<i>Mec</i>	0.32±0.02	0.96±0.02	0.99±0.02	0.99±0.02	0.98±0.02	0.98±0.02	0.98±0.02	0.98±0.02
<i>Mec+Bud</i>	0.28±0.02	0.92±0.02	0.86±0.03 <sup>c</sup>	0.88±0.03 <sup>b</sup>	0.88±0.02 <sup>b</sup>	0.88±0.03 <sup>c</sup>	0.89±0.04	0.89±0.03 <sup>c</sup>
<i>Mec+Surf</i>	0.29±0.01	0.95±0.05	0.80±0.03 <sup>b</sup>	0.78±0.02 <sup>af</sup>	0.80±0.03 <sup>af</sup>	0.80±0.03 <sup>a</sup>	0.81±0.02 <sup>b</sup>	0.84±0.03 <sup>b</sup>
<i>Mec+Surf+Bud</i>	0.29±0.02	0.95±0.05	0.76±0.06 <sup>a</sup>	0.72±0.03 <sup>ad</sup>	0.75±0.03 <sup>ae</sup>	0.81±0.04 <sup>a</sup>	0.83±0.05 <sup>b</sup>	0.86±0.03 <sup>b</sup>
<i>C<sub>dyn</sub> (ml/kPa/kg)</i>								
<i>Mec</i>	13.1±0.7	6.0±0.1	5.7±0.2	5.7±0.2	5.7±0.2	5.8±0.2	5.8±0.2	5.8±0.2
<i>Mec+Bud</i>	14.3±1.0	6.2±0.3	6.9±0.3 <sup>c</sup>	6.9±0.3 <sup>c</sup>	6.9±0.3 <sup>c</sup>	6.9±0.3 <sup>c</sup>	6.8±0.4	6.7±0.3 <sup>c</sup>
<i>Mec+Surf</i>	13.8±0.5	6.0±0.3	7.1±0.2 <sup>b</sup>	7.3±0.3 <sup>b</sup>	7.2±0.3 <sup>b</sup>	7.1±0.3 <sup>c</sup>	7.1±0.3 <sup>c</sup>	6.9±0.3 <sup>c</sup>
<i>Mec+Surf+Bud</i>	14.2±1.1	6.0±0.3	7.7±0.5 <sup>a</sup>	7.7±0.5 <sup>a</sup>	7.6±0.5 <sup>a</sup>	7.4±0.5 <sup>b</sup>	7.3±0.6 <sup>b</sup>	7.0±0.3 <sup>b</sup>
<i>RLS (%)</i>								
<i>Mec</i>	15.6±3.7	47.4±3.5	47.4±3.3	50.0±2.3	49.5±2.2	49.1±1.8	47.6±2.3	48.8±1.9
<i>Mec+Bud</i>	11.9±2.5	43.1±2.3	43.8±4.5	42.6±5.2	45.1±4.8	41.2±3.5	40.3±3.8	33.6±3.8 <sup>b</sup>
<i>Mec+Surf</i>	13.8±2.0	46.7±4.0	42.6±5.7	36.9±2.0 <sup>c</sup>	34.1±3.7 <sup>c</sup>	36.3±2.7 <sup>b</sup>	37.8±3.6 <sup>c</sup>	35.0±4.3 <sup>b</sup>
<i>Mec+Surf+Bud</i>	14.2±3.1	42.7±5.0	33.4±4.1 <sup>c</sup>	35.7±3.1 <sup>b</sup>	33.3±5.4 <sup>bf</sup>	33.1±4.1 <sup>b</sup>	33.7±3.6 <sup>b</sup>	30.0±4.6 <sup>a</sup>

Statistical comparisons: for Mec+Bud, Mec+Surf and Mec+Surf+Bud vs. Mec: <sup>a</sup>  $P < 0.001$ , <sup>b</sup>  $P < 0.01$ , <sup>c</sup>  $P < 0.05$ ; for Mec+Surf and Mec+Surf+Bud vs. Mec+Bud: <sup>d</sup>  $P < 0.001$ , <sup>e</sup>  $P < 0.01$ , <sup>f</sup>  $P < 0.05$ .

right lung lobes were cut, weighed, and dried at 60 °C for 24 hours. Then, weight of dry tissue was measured and wet-dry (W/D) lung weight ratio was calculated, expressing an extent of lung edema formation.

#### Biochemical analyses

Concentrations of pro-inflammatory markers in the blood plasma, IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  were determined by ELISA kits for rabbit (USCN Life Science Inc., China) and expressed in pg/ml.

#### Data analysis

Statistical analyses were performed by SYSTAT For Windows (SPSS Inc., USA). One-way analysis of variance (ANOVA) with post-hoc Fisher's LSD test was used for intergroup analysis. Furthermore, two-way ANOVA for repeated measures (with factors „group“ and „time“) was used. Association between wet-dry lung weight ratio and inflammatory markers vs. lung function parameters was evaluated by Pearson's correlations and expressed as Pearson's correlation coefficient ( $r$ ) and Bonferroni probability ( $P$ ). A value of  $P < 0.05$  was considered statistically significant. Data are expressed as means±SEM.

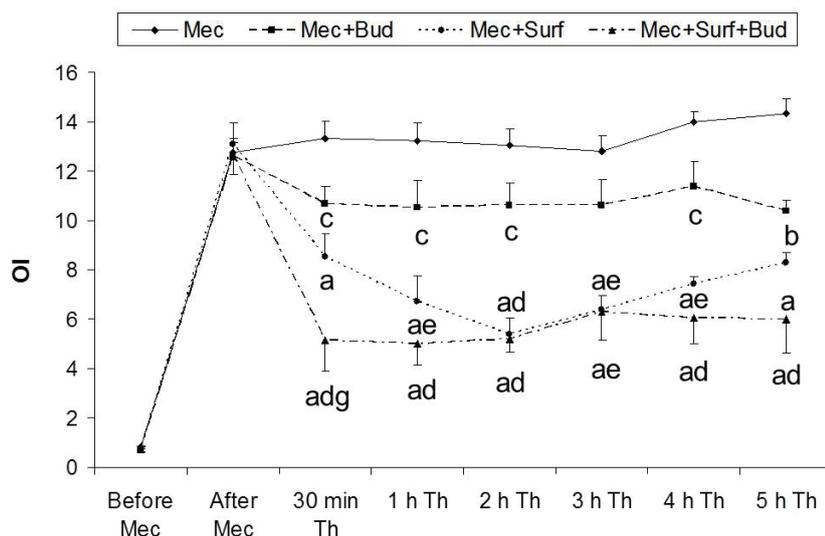
## Results

### *Lung function parameters before and after meconium instillation*

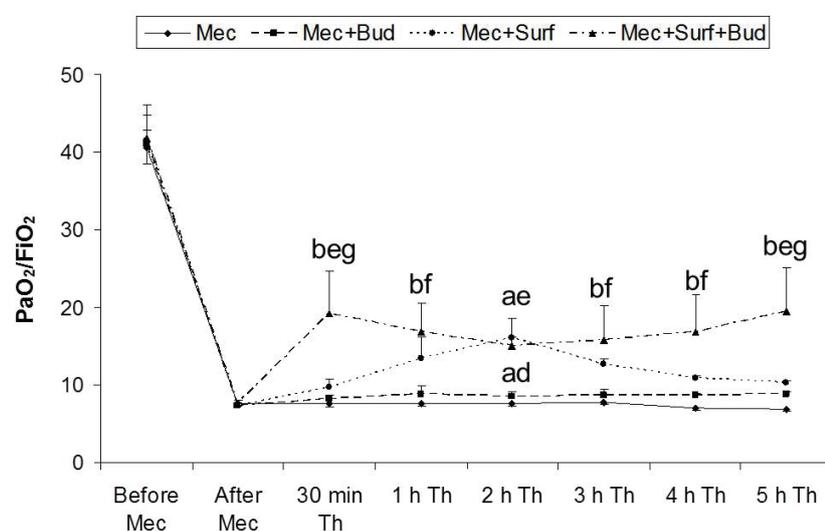
In total, twenty-two rabbits of both genders were used for final data analysis. There were no differences in entry parameters: body weight, gender, and lung function parameters before and after meconium instillation were comparable between the groups (all  $P > 0.05$ ). The ANOVA for repeated measures revealed significant effect of factor „time“ on ventilatory pressures PIP ( $F=880.59$ ,  $P=0.000$ ) and MAP ( $F=1121.47$ ,  $P=0.000$ ), right-to-left pulmonary shunts ( $F=175.33$ ,  $P=0.000$ ), PaO<sub>2</sub> ( $F=7.813$ ,  $P=0.012$ ), OI ( $F=1198.16$ ,  $P=0.000$ ), O<sub>2</sub> saturation ( $F=31.91$ ,  $P=0.000$ ), and lung-thorax compliance ( $F=274.83$ ,  $P=0.000$ ) after instillation of meconium in comparison to initial values.

### *Recovery of the therapeutic bronchoalveolar lavage (BAL) fluid*

In both surfactant-treated groups, therapeutic BAL using 10 ml/kg of diluted exogenous surfactant (Curosurf) was performed twice. There was no significant difference between the groups in the recovery of BAL fluid (67.4±3.4 % in Mec+Surf group vs. 69.2±2.6 % in Mec+Surf+Bud group,  $P > 0.05$ ).



**Fig. 1.** Oxygenation index (OI) before and after meconium (Mec) instillation and during 5 hours after administration of the therapy (Th) in the meconium-instilled non-treated group (Mec), budesonide-only treated group (Mec+Bud), surfactant-only treated group (Mec+Surf), and surfactant+budesonide treated group (Mec+Surf+Bud). Statistical comparisons: for Mec+Bud, Mec+Surf and Mec+Surf+Bud vs. Mec: <sup>a</sup>  $P < 0.001$ , <sup>b</sup>  $P < 0.01$ , <sup>c</sup>  $P < 0.05$ ; for Mec+Surf and Mec+Surf+Bud vs. Mec+Bud: <sup>d</sup>  $P < 0.001$ , <sup>e</sup>  $P < 0.01$ , <sup>f</sup>  $P < 0.05$ ; for Mec+Surf+Bud vs. Mec+Surf: <sup>g</sup>  $P < 0.05$ .



**Fig. 2.** PaO<sub>2</sub>/FiO<sub>2</sub> before and after meconium (Mec) instillation and during 5 hours after administration of the therapy (Th) in the meconium-instilled non-treated group (Mec), budesonide-only treated group (Mec+Bud), surfactant-only treated group (Mec+Surf), and surfactant+budesonide treated group (Mec+Surf+Bud). PaO<sub>2</sub>: arterial partial pressure of oxygen, FiO<sub>2</sub>: fraction of inspired oxygen. Statistical comparisons: for Mec+Bud, Mec+Surf and Mec+Surf+Bud vs. Mec: <sup>a</sup>  $P < 0.001$ , <sup>b</sup>  $P < 0.01$ , <sup>c</sup>  $P < 0.05$ ; for Mec+Surf and Mec+Surf+Bud vs. Mec+Bud: <sup>d</sup>  $P < 0.001$ , <sup>e</sup>  $P < 0.01$ , <sup>f</sup>  $P < 0.05$ ; for Mec+Surf+Bud vs. Mec+Surf: <sup>g</sup>  $P < 0.05$ .

#### Effect of therapy on the lung function parameters

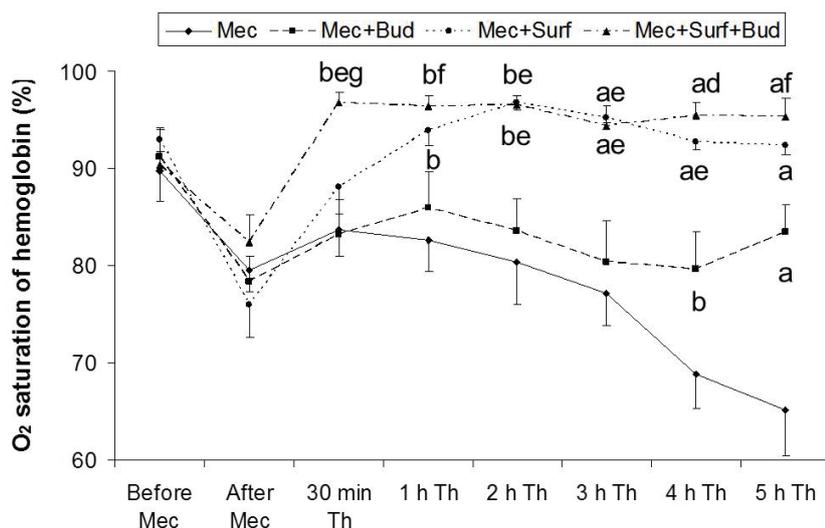
After budesonide administration (Mec+Bud group), lower MAP ( $P < 0.05$ , Table 1) and OI ( $P < 0.05$ , Fig. 1), and non-significantly higher PaO<sub>2</sub>/FiO<sub>2</sub> ( $P > 0.05$ , Fig. 2) were observed from 30 min of the therapy, whereas improvements in O<sub>2</sub> saturation of hemoglobin (Fig. 3) and pulmonary shunts (Table 1) compared with Mec group were more obvious at the end of experiment (all  $P < 0.05$ , 0.01, or 0.001).

Surfactant-only treatment (Mec+Surf group) enhanced the lung functions more effectively than budesonide (Table 1, Figs 1, 2, and 3), with the most potent effect observed at 1-3 hours of the treatment. In some parameters, the differences between these two treatments were significant, e.g. in MAP ( $P < 0.05$ , Table 1), OI ( $P < 0.001$  or 0.01, Fig. 1), PaO<sub>2</sub>/FiO<sub>2</sub> ( $P < 0.05$ , Fig. 2), or O<sub>2</sub> saturation of hemoglobin ( $P < 0.01$ ) (Fig. 3).

Combined treatment with surfactant and budesonide led to an immediate improvement in OI ( $P < 0.001$ ), PaO<sub>2</sub>/FiO<sub>2</sub>, and O<sub>2</sub> saturation of hemoglobin ( $P < 0.001$  or 0.01) in comparison with both Mec and Mec+Bud groups within 30 min of the treatment administration. OI and PaO<sub>2</sub>/FiO<sub>2</sub> at 30 min ( $P < 0.01$ ) and PaO<sub>2</sub>/FiO<sub>2</sub> at 5 hours ( $P < 0.05$ ) of the treatment were in Mec+Surf+Bud group even better than in Mec+Surf group (Table 1, Figs 1 and 2). Superior effect of combined treatment was also demonstrated in pulmonary shunts and lung compliance ( $P < 0.001$  or 0.01), with significant differences compared with Mec group observed at 30 min of the treatment ( $P < 0.05$  or 0.001) (Table 1).

#### W/D ratio

Different efficacy on formation of lung edema according to the treatment was found. Whereas



**Fig. 3.** Oxygen saturation of hemoglobin (sat. O<sub>2</sub>) before and after meconium (Mec) instillation and during 5 hours after administration of the therapy (Th) in the meconium-instilled non-treated group (Mec), budesonide-only treated group (Mec+Bud), surfactant-only treated group (Mec+Surf), and surfactant+budesonide treated group (Mec+Surf+Bud). Statistical comparisons: for Mec+Bud, Mec+Surf and Mec+Surf+Bud vs. Mec: <sup>a</sup>  $P < 0.001$ , <sup>b</sup>  $P < 0.01$ , <sup>c</sup>  $P < 0.05$ ; for Mec+Surf and Mec+Surf+Bud vs. Mec+Bud: <sup>d</sup>  $P < 0.001$ , <sup>e</sup>  $P < 0.01$ , <sup>f</sup>  $P < 0.05$ ; for Mec+Surf+Bud vs. Mec+Surf: <sup>g</sup>  $P < 0.05$ .

budesonide decreased W/D ratio just non-significantly ( $P > 0.05$ ), surfactant therapy ( $P < 0.05$ ) and combined surfactant+budesonide therapy ( $P < 0.01$ ) significantly reduced lung edema compared with Mec group (Table 2).

Formation of lung edema was in relation to the lung function parameters. For example, at 5 hours of the treatment W/D ratio positively correlated with MAP ( $P = 0.033$ ,  $r = 0.457$ ), OI ( $P = 0.013$ ,  $r = 0.519$ ), RLS ( $P = 0.032$ ,  $r = 0.459$ ), and negatively correlated with compliance ( $P = 0.006$ ,  $r = -0.568$ ) and O<sub>2</sub> saturation of hemoglobin ( $P = 0.019$ ,  $r = -0.495$ ). In addition, W/D ratio correlated well with pro-inflammatory cytokines: with IL-1 $\beta$  ( $P = 0.002$ ,  $r = 0.674$ ), IL-6 ( $P = 0.001$ ,  $r = 0.672$ ), IL-8 ( $P = 0.000$ ,  $r = 0.760$ ), and with TNF- $\alpha$  ( $P = 0.001$ ,  $r = 0.695$ ).

#### Markers of inflammation

All the treatments reduced plasma levels of IL-1 $\beta$  and IL-8, with superior effect observed in combined surfactant+budesonide treatment. However, no effect of the treatment was observed on the concentrations of IL-6 and TNF- $\alpha$  (Table 2).

Plasma levels of both IL-1 $\beta$  and IL-8 well correlated with oxygenation index ( $P = 0.017$ ,  $r = 0.540$  for IL-1 $\beta$ ;  $P = 0.015$ ,  $r = 0.548$  for IL-8), O<sub>2</sub> saturation of hemoglobin ( $P = 0.002$ ,  $r = -0.657$  for IL-1 $\beta$ ;  $P = 0.002$ ,  $r = -0.665$  for IL-8), and lung compliance ( $P = 0.018$ ,  $r = -0.535$  for IL-1 $\beta$ ;  $P = 0.014$ ,  $r = -0.555$  for IL-8), but had no significant relation to MAP and RLS.

## Discussion

Surfactant dysfunction plays a significant role in MAS. Therefore, administration of exogenous surfactant has become a part of the therapeutic protocol in severe

MAS. However, MAS is associated with inflammation, lipid and protein oxidation, and pulmonary vasoconstriction, which may reduce efficacy of surfactant therapy. As glucocorticoids may diminish the mentioned changes, we have supposed that addition of budesonide to exogenous surfactant may potentiate an improvement in the lung functions compared with these two treatments given separately. In agreement with our hypothesis, combined administration of surfactant and budesonide enhanced lung functions and alleviated inflammation more effectively than budesonide-only and surfactant-only treatments.

Surfactant dysfunction may be partially overcome by sufficiently high concentrations of exogenous surfactant (Sun *et al.* 1993). Surfactant may be delivered as a bolus or as a lung lavage by diluted surfactant. In the lung lavage, higher volume of the lavage fluid partially removes aspirated meconium, and thereby reduces airway obstruction, and provides better distribution of the following dose of the treatment, or it may even reduce needs for additional doses of surfactant (Meister *et al.* 2004). On the other hand, the lung lavage procedure may be accompanied by a transient decrease in oxygenation and systemic blood pressure. Therefore, the possible risks versus benefits should be carefully considered (Dargaville and Mills 2005). Administration of a bolus, particularly when given slowly in small portions, may be associated with less acute side effects and no loss of given surfactant as it is in the lung lavage. However, meconium decreasing efficacy of the therapy is not removed from the lungs. To take advantages of both approaches, lung lavage with diluted surfactant removing meconium from the lungs may be followed by a slow instillation of undiluted surfactant, replacing the

**Table 2.** Wet-dry (W/D) lung weight ratio and plasma concentrations of pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  (in pg/ml) in the meconium-instilled non-treated group (Mec), budesonide-only treated group (Mec+Bud), surfactant-only treated group (Mec+Surf) and surfactant+budesonide treated group (Mec+Surf+Bud).

	W/D ratio	IL-1 $\beta$	IL-6	IL-8	TNF- $\alpha$
<i>Mec</i>	6.0 $\pm$ 0.2	222.2 $\pm$ 111.4	26.9 $\pm$ 3.1	221.6 $\pm$ 95.7	254.5 $\pm$ 23.2
<i>Mec+Bud</i>	5.9 $\pm$ 0.2	52.2 $\pm$ 29.1 <sup>b</sup>	24.4 $\pm$ 0.8	75.2 $\pm$ 39.0 <sup>b</sup>	212.5 $\pm$ 19.4
<i>Mec+Surf</i>	5.4 $\pm$ 0.2 <sup>b</sup>	32.4 $\pm$ 7.3 <sup>b</sup>	20.5 $\pm$ 1.2 <sup>b</sup>	50.8 $\pm$ 14.3 <sup>b</sup>	234.7 $\pm$ 11.2
<i>Mec+Surf+Bud</i>	5.2 $\pm$ 0.1 <sup>ac</sup>	19.5 $\pm$ 2.6 <sup>a</sup>	23.8 $\pm$ 2.6	29.4 $\pm$ 5.7 <sup>a</sup>	220.5 $\pm$ 8.2

Statistical comparisons: for Mec+Bud, Mec+Surf and Mec+Surf+Bud vs. Mec: <sup>a</sup>  $P < 0.01$ , <sup>b</sup>  $P < 0.05$ ; for Mec+Surf+Bud vs. Mec+Bud: <sup>c</sup>  $P < 0.05$ .

inactivated surfactant (Kaneko *et al.* 2001, Szymankiewicz *et al.* 2004).

In these experiments, lung lavage with diluted surfactant was performed twice to recover majority of accessible meconium. The third lavage, with the smallest recovery of meconium (Cochrane *et al.* 1998), was replaced by homogenous delivery of undiluted surfactant using asymmetric high-frequency jet ventilation (Sevecova-Mokra *et al.* 2004, Calkovska *et al.* 2005). Exogenous surfactant substitutes the loss of pulmonary surfactant due to inactivation and represents a suitable transport medium for budesonide. Within 30 min of the surfactant therapy we could observe a significant improvement in oxygenation, probably due to stabilization or re-opening the collapsed alveoli and small airways, providing enhanced ventilation. Redistribution of the blood flow into better-aerated lung areas and higher oxygenation resulted in diminishing right-to-left pulmonary shunts and further increase in oxygenation. In agreement with our findings, rapid improvement in oxygenation was demonstrated also after surfactant lung lavage (Cochrane *et al.* 1998, Ohama *et al.* 1999, Lam *et al.* 2000, Sevecova-Mokra *et al.* 2004, Rey-Santano *et al.* 2011) and after surfactant lung lavage followed by instillation of undiluted surfactant (Kaneko *et al.* 2001, Szymankiewicz *et al.* 2004, Gadzinowski *et al.* 2008). However, similarly to other authors (Cochrane *et al.* 1998, Lam *et al.* 2000, Gadzinowski *et al.* 2008) a worsening in the lung functions from 1-2 hours after the treatment was found, probably due to on-going inflammation and inactivation of surfactant from the persistence of meconium in the lungs. To minimize surfactant inactivation, modified natural surfactant containing specific proteins was used in this study, and BAL fluid contained 5 mg of PL/ml to be resistant to inhibition (Dargaville and Mills 2005). Despite use of the

natural surfactant in a sufficient concentration and dose, surfactant inactivation and inflammation were not overcome in the surfactant-only treated group.

Addition of glucocorticoid budesonide to surfactant accentuated improvement in oxygenation, lung compliance, and right-to-left pulmonary shunts, which persisted longer than the effect observed after surfactant-only treatment. It is likely attributed to complex anti-inflammatory, antioxidative, vasodilation and antiedematous action of glucocorticoids, which is even more pronounced in local instillation (Newton *et al.* 2010). Thanks to its pharmacological properties, high lipophilicity, good solubility in surfactant, and high affinity for the lungs budesonide possesses an exceptional potential to reduce inflammation, oxidation stress, and lung edema (Wiedmann *et al.* 2000, Braga *et al.* 2005, Mokra *et al.* 2007a). In addition, budesonide increases expression of surfactant proteins SP-A and SP-B (Yu and Zhang, 2008) and has negligible effect on the surface activity of Curosurf (Zhang *et al.* 2012).

Positives of glucocorticoid therapy have been previously shown in neonates with MAS (Wu *et al.* 1999, da Costa *et al.* 2001, Basu *et al.* 2007, Tripathi and Saili 2007), and in various animal models of MAS (Khan *et al.* 1999, Holopainen *et al.* 2001, Mokra *et al.* 2007a,b). In the present study, combined surfactant+budesonide treatment led to a bi-phasic improvement in the lung functions. An early improvement after the surfactant therapy may be partially explained by the lavage procedure, removal of meconium, or short-term use of high-frequency jet ventilation enhancing ventilation. However, the obvious difference between the surfactant+budesonide vs. surfactant-only treatments indicates that these rapid changes are likely caused by nongenomic action of budesonide (Stellato 2004) influencing the processes in the inflammatory, endothelial

and smooth muscle cells (Long *et al.* 2005, Sun *et al.* 2006) within several minutes. The late improvement in the lung functions in favor of combined vs. surfactant-only treatment 4-5 hours after the treatment administration might be related to genomically mediated effects of budesonide, which require several hours until some changes may be observed on the systemic level (Stellato 2004).

Because of on-going inflammation, surfactant dysfunction, lung edema formation, and vasoconstriction, differences between the non-treated and treated animals become more prominent at the end of experiment. Each treatment showed smaller or bigger effect on the gas exchange, lung compliance, lung edema formation, or right-to-left pulmonary shunts, with potential of the treatment increasing from budesonide-only and surfactant-only treatments to surfactant+budesonide. Superior effect of the combined therapy was observed also in reducing plasma levels of pro-inflammatory cytokines. We found significant decrease in IL-1 $\beta$  and IL-8, but just non-significant changes in IL-6 and TNF- $\alpha$ . Similarly to our results, surfactant lung lavage reduced plasma concentrations of IL-1 $\beta$ , but had no obvious effect on TNF- $\alpha$  and IL-6 in the newborn piglets with MAS (Wang *et al.* 2010). We may only speculate whether the absence of significant differences in these markers was attributed to relatively small numbers of animals included in the groups in this pilot study, short time of observation, or different response of the cells producing cytokines to the given therapy. However, changes in cytokines showed strong association with lung functions.

Of course, we are aware of the limitations of our study. First, we used young-to-adult animals as a model of MAS, which is a neonatal disease, instead of neonatal animals. In the use of such a model, postnatal transformational changes of the lung parenchyma and hemodynamics are missing. In addition, artificial instillation of meconium suspension into the lungs has another dynamics than its spontaneous aspiration by the fetus or newborn. However, due to technical difficulties

and ethics problems associated with handling animals early after delivery, several-week-old rabbits may serve as an acceptable model of MAS (Cochrane *et al.* 1998, Ohama and Ogawa 1999, Lam *et al.* 2000), because of the body weight and diameter of the airways similar to those in the newborns. Taking the limitations into account, results of this experimental study may be useful also for clinicians, as the principle changes accompanying meconium aspiration, effects of meconium on pulmonary surfactant, triggering inflammation and lung edema, as well as changes related to the treatment administration are comparable between the experimental animals and neonates.

In conclusion, this is the first study showing that early surfactant lung lavage followed by undiluted surfactant enriched with budesonide can accentuate and prolong improvement in the lung functions compared with surfactant-only therapy. Nevertheless, further studies are needed to evaluate whether the mentioned combination may prevent a progression of the disease and improve an outcome in the newborns with severe MAS. In addition, potential side effects of the treatment including lavage procedure should be carefully investigated before this treatment may be recommended for clinical practice.

### Conflict of Interest

There is no conflict of interest.

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