INTRODUCTION

Aspiration of meconium-stained amniotic fluid (MSAF) into the lung of the newborn may cause a serious life-threatening condition called meconium aspiration syndrome (MAS). Incidence of MSAF is about 10 – 15%, whereas MAS develops in 0.10% in the 37th week of gestation and elevates up to 0.31% in the 41st week of gestation (1). Pathophysiology of MAS is complex. Mechanical airway obstruction by particulate meconium is followed by surfactant dysfunction, pneumonitis, pulmonary hypertension, and ventilation/perfusion mismatch what results in progressing acidosis and hypoxemia (2, 3). Initial injury to endothelium and epithelial alveolar cells increases permeability of alveolar-capillary membrane and migration of inflammatory cells into the interstitium and bronchoalveolar space (4). Migration of cells is influenced by various factors, including cell adhesion molecules, proteinases, oxidants, cytokines, and chemokines (5, 6). Additionally, it may be potentiated by interleukin-8 (IL-8), a potent chemoattractant present in aspirated meconium (7). Activated cells (neutrophils, alveolar macrophages, endothelial and epithelial cells etc.) produce a wide spectrum of biologically-active substances (5) which may further worsen the lung injury (4). Inflammatory response is triggered also by meconium components, as some processes are mediated through TLR4/MD-2 CD14 signalling complex (8).

Meconium-induced lung injury and inflammation lead to oxidative stress resulting from activation of phagocytes and endothelial cells, with overproduction of reactive oxygen (ROS) and nitrogen (RNS) species mediated through several oxidant-generating systems, such as NADPH oxidase, nitric oxide (NO) synthase, xanthine oxidase, myeloperoxidase etc. (9). Oxidative
inflamatory cytokines (TNF-α, IL-1, IL-6, IL-8, etc.), chemokines (IL-8, chemotactant protein-1, etc.), and growth factors: transforming growth factor β (TGF-β), monocyte connective tissue growth factor, etc., through activating protein kinases, which amplify inflammatory changes and associated oxidative damage (9, 14). By this way, pro-inflammatory responses and oxidative stress can provoke each other and participate in the development of MAS.

Beside other changes, overproduction of ROS and pro-inflammatory substances deteriorate the alveolar epithelium and surfactant function (15, 16). While injury to type I cells promotes fluid accumulation in the alveolar space and interstitium, injury to type II cells impairs synthesis and metabolism of pulmonary surfactant, finally leading to increased surface tension and alveolar collapse. Additional inactivation of surfactant is caused by plasma proteins from edematous fluid (17) and meconium itself (18), changing the ultrastructure of surfactant and deteriorating its surface properties.

To reduce surfactant dysfunction and to improve ventilation in severe MAS, replacement therapy with exogenous surfactant delivered either as a bolus or as a bronchoalveolar lavage (BAL) with diluted surfactant can be used (19). In infants with MAS, surfactant therapy reduces the severity of respiratory distress and decreases the number of infants requiring support with ECMO (20). However, effectiveness of the treatment may be limited by inactivation of exogenous surfactant, which proceeds by a similar manner than inactivation of pulmonary surfactant mentioned above (21, 22).

In view of these findings, suppression of inflammation with suitable anti-inflammatory agent seems to be promising. For instance, intratracheal or intravenous glucocorticoids have improved lung functions in the experimental animals (23-26) and the newborns (27-30) with MAS. Additional improvement may be expected for glucocorticoids combined with exogenous surfactant, as it was observed in animal models (31, 32) and preterm infants (33, 34) with respiratory distress syndrome (RDS). However, there is a lack of information on their use in MAS. In a small clinical study, one BAL with diluted surfactant following a dose of intravenous dexamethasone improved oxygenation and decreased days of oxygenation and ventilation compared to standard therapy (35). In our previous studies, effects of surfactant BAL (36) and glucocorticoids (25, 26) on a rabbit model of MAS were tested separately. In the actually realized project, BAL with diluted surfactant is performed twice to remove meconium from the airways. BAL is followed by a dose of undiluted surfactant, which replaces surfactant removed by the lavage or inactivated by meconium and/or plasma proteins. The undiluted surfactant is enriched with budesonide to enhance anti-inflammatory and antioxidative potential of the treatment. Pilot results have recently demonstrated that this kind of combined therapy can additionally improve the lung functions in comparison with surfactant-alone (37). In this article, further data on effects of the mentioned surfactant-plus-budesonide therapy on meconium-induced migration of neutrophils and oxidative changes are provided. To show a complex action of the therapy on meconium-induced oxidative injury, oxidative status was detected on three levels - in the lung homogenate, isolated lung mitochondria, and blood plasma - by measuring concentrations of dityrosine, lysine-LPO products, TBARS, conjugated dienes, and total antioxidant capacity (TAC).

### Materials and Methods

#### Animals

Twenty four healthy, 8-week-old New Zealand white rabbits of both genders, with a mean body weight (bw) of 1.9 ± 0.2 kg were supplied by Laboratory Animal Breeding Station, Department of Toxicology, Slovak Academy of Sciences, Dobra Voda, Slovakia. Animals were divided into 4 groups (each of n = 6) according to the used treatment: meconium-instilled group without therapy (M), meconium-instilled group treated with surfactant (M + S), meconium-instilled group treated with surfactant plus budesonide (M + S + B), and control group with saline instillation instead of meconium (C).

The experimental protocol was performed in accordance with the ethical guidelines and authorized by the local Ethics Committee of Jessenius School of Medicine in Martin and National Veterinary Board.

#### Meconium

First-pass meconium was collected from 20 healthy term neonates born at Clinics of Neonatology, University Hospital in Martin. Samples were pooled, lyophilized and stored at −20°C. Before use, meconium was suspended in saline (0.9% NaCl, 37°C) at a concentration of 25 mg/ml.

#### Surfactant/budesonide

Modified porcine surfactant (Curosurf®, Chiesi Farmaceutici, Italy; 80 mg phospholipids (PL)/ml) was diluted in saline (0.9% NaCl, 37°C) to a PL concentration of 5 mg/ml for bronchoalveolar lavage (BAL). Undiluted Curosurf was administered at a dose of 100 mg/kg bw as a supplementation dose after BAL. Synthetic glucocorticoid budesonide (Pulmicort suspension for inhalation, AstraZeneca, 0.5 mg/ml) at a dose of 0.25 mg/kg bw. was added into a dose of undiluted Curosurf after finished BAL with diluted surfactant (M + S + B group).

#### General protocol of the experiments

Animals were anesthetized with intramuscular ketamine (20 mg/kg b.w.; Narketan, Vétoquinol Ltd., UK) and xylazine (5 mg/kg b.w.; Xylariem, Riemser, Germany), followed by a continuous infusion of ketamine (20 mg/kg b.w/h). A tracheostomy was performed and an endotracheal cannula was inserted into the trachea. Thin polypropylene catheters were placed into the femoral artery for monitoring blood pressure and sampling the blood and into the femoral vein for administration of drugs and anesthetics by intravenous pump. Subsequently, spontaneous breathing of animals was paralyzed with pipercuronium bromide (0.3 mg/kg b.w/30 min iv; Arduan, Gedeon Richter, Hungary) and animals were ventilated using a pressure-controlled ventilator (Beat-2, Chirana, Slovakia) with following settings: a frequency (f) of 30/min, fraction of inspired oxygen (FiO₂) of 0.21, time of inspiration (Ti) 50 %, and peak inspiratory pressure (PIP) adjusted to keep a tidal volume (V₁) of 7 - 9 ml/kg bw without positive end-expiratory pressure (PEEP) in this stage of experiment. After 15 min of stabilization, lung function parameters and blood gases were measured. Then, suspension of meconium (or saline in controls) at a dose of 4 ml/kg bw. was instilled into the endotracheal cannula in the semi-upright right and left lateral positions of the animal to ensure homogenous distribution of meconium/saline throughout the lung. From this moment on, FiO₂ was increased to 1.0. PEEP to 0.3 kPa and PIP to the value supplying a tidal volume of 7 – 9 ml/kg bw.
Within 30 min after meconium administration, respiratory failure defined as 30% decrease in dynamic lung-thorax compliance (Cdyn) and PaO₂ at FiO₂ of 1.0 developed (38).

Administration of the treatment

Surfactant therapy was given in two steps in all surfactant-treated animals (M + S and M + S + B groups). Bronchoalveolar lavage (BAL) with diluted exogenous surfactant (Curosurf, 5 mg PL/ml) was performed twice, each with a volume of 10 ml/kg b.w. Lavage fluid was instilled with a high flow syringe in the semi-upright right and left lateral positions of the animal to ensure a proportional distribution in the lung. Then, the lavage fluid was evacuated using aspirator (Suction Professional, Ellettomedicali, Italy) with a pressure of ~40 kPa at 30 s and 90 s after the instillation. After stabilization of the cardiovascular parameters, a dose of undilated Curosurf (100 mg PL/kg b.w., 1.25 ml/kg b.w.) representing the second step of surfactant therapy was injected into a jet of ventilator and using a regime of asymmetric high-frequency jet ventilation (or inpulsion regime of HFJV) (t. 300/min, Ti 20%, PIP/PEEP 1.5/0.3 KPa) was homogeneously spread throughout the lung. In the group with combined treatment (M + S + B group), budesonide (0.25 mg/kg b.w., 0.5 ml/kg b.w.) was added into the dose of undiluted surfactant therapy and was given by the same way as previously described.

Animals were oxygen-ventilated for an additional 5 h after administration of the treatment. At the end of experiment, samples of blood were taken and centrifuged at 3000 rpm for 15 min and plasma was stored at ~70°C. Then, animals were euthanized by an overdose of anesthetics. The trachea and lung were excised. Left lung was lavaged with saline (3 × 10 ml/kg b.w., 0.9% NaCl), and diagnostic BAL fluid was centrifuged at 1500 rpm for 10 min. The right lung was cut to small pieces and stored at ~70°C until following biochemical analyses.

Cells in the diagnostic bronchoalveolar lavage fluid and blood at the end of experiment

Differential white blood cell (WBC) count in the blood and differential count of cells in the sediment of diagnostic BAL were estimated microscopically after staining by May-Grunwald/ Giemsa-Romanowski and results were expressed in %.

Preparation of the lung tissue homogenate and isolation of mitochondria

Lung tissue was washed, chopped, and homogenized as described elsewhere (39). Dissected tissue was homogenized (5-times for 25 s, 1200 rpm) in an ice-cold solution containing 0.32 M sucrose, 5 mM HEPES (pH 7.4), 1 mM EDTA, and 0.3 mM phenylmethylsulphonyl fluoride as a proteases inhibitor in a ratio 1:5 using the Tefon-glass Potter homogenizer (B. Braun Melsungen A.G, Germany) chilled with ice. Butylated hydroxytoluene (1 mM) as an inhibitor of oxidative damage was added into the homogenate after completion of the process. Homogenates were stored at ~70°C.

Mitochondria were isolated from the lung homogenate by differential centrifugation as described previously (40). The homogenate was centrifuged at 3200 rpm for 10 min at 4°C and supernatant was fractionated by one-step Percoll gradient (8 ml 16% Percoll, 0.25 M sucrose) centrifugation at 25,000 rpm for 30 min at 4°C. The sediment containing mitochondria was resuspended in 0.25% BSA and stored at ~70°C.

Protein concentrations in the lung homogenates and mitochondria samples were determined according to the method of Lowry (41), using bovine serum albumin as a standard.

Fluorescence measurements of protein oxidation

Fluorescence measurements were performed in the homogenates and mitochondria samples containing 0.05 mg of protein per ml in solution of 10 mM HEPES, 100 mM KCl, pH 7.0 at a room temperature using a spectrofluorometer (PerkinElmer LS 55RF-540). Fluorescence emission spectra of dityrosine (in the range of 380 – 440 nm ± 5 nm), a product of tyrosine oxidation, were measured at the excitation wavelength of 325 nm ± 5 nm. Fluorescence intensity of dityrosine was expressed in relative fluorescence units (RFU) (42). Emission spectra (in the range of 425 – 480 nm ± 5 nm) of protein conjugates with lipid peroxidation products (LPO) were measured at the excitation of 365 nm ± 5 nm. Fluorescence intensity of lysine-LPO products was expressed in relative fluorescence units (RFU) (43).

Measurement of lipid peroxidation

Determination of lipid peroxidation was performed according to the formation of conjugated dienes and thiobarbituric acid-reactive substances (TBARS) in the homogenates and mitochondria fraction as described previously (39). Conjugated dienes were determined from the absorbance ratio A₃₃₅nm/A₃₇₅nm of 0.02 mg proteins dissolved in 10 mM phosphate buffer (pH 7.1) containing 1% Lubrol. TBARS in the lung homogenate and mitochondrial fraction were analysed using OxiSelect™ TBARS Assay Kit (Cell Biolabs Inc., USA) by measuring levels of MDA (malondialdehyde), a product of lipid peroxidation generated mainly by free hydroxyl radicals, following the manufacturer instructions. TBARS concentration was determined spectrophotometrically from the absorbance at 532 nm and expressed in micromoles of MDA formed per milligram of protein (μM MDA).

Total antioxidant capacity (TAC)

TAC in the lung homogenate and blood plasma was determined spectrophotometrically using the OxiSelect TAC Assay Kit (Cell Biolabs Inc., USA) according to the manufacturer instructions. TAC assay is based on measuring the reduction of copper (II) to copper (I) by antioxidants in the sample. Reduction was determined from the absorbance at 490 nm and compared with known uric acid standard curve. Results are expressed as μM Copper Reducing Equivalents (CRE), as CRE sample values are proportional to TAC.

Statistical analysis

Data are presented as means ± S.E.M. Differences between the groups were analyzed using one-way ANOVA followed by a Bonferroni multiple comparison test. The P-values < 0.05, after Bonferroni correction, were considered statistically significant. Statistical calculations were carried out using Graph Pad Prism 5 (San Diego, California, USA).

RESULTS

Neutrophils (%) in the diagnostic bronchoalveolar lavage fluid and arterial blood at the end of experiment

Differential numbers of neutrophils were determined in the diagnostic BAL fluid and arterial blood at the end of experiment and were expressed in percents (Fig. 1). In the blood, percentage of neutrophils decreased after meconium instillation compared to controls (for M versus C, P < 0.001). The used therapies elevated
circulating neutrophils compared to untreated meconium group (for M + S versus M P < 0.01; for M + S + B versus M P < 0.001). In addition, difference between the therapies was significant (for M + S + B versus M + S P < 0.05).

Opposite to blood, the percentage of neutrophils in BAL fluid raised after meconium instillation compared to controls (for M versus C P < 0.001). Both therapies decreased the numbers of neutrophils leaking into the lung compared to untreated

Fig. 1. Percentage of neutrophils in the arterial blood and the BAL fluid. Neutrophils (expressed in %) in the diagnostic BAL fluid (1A) and arterial blood (1B) at the end of experiment in saline-instilled controls (C), meconium-instilled untreated group (M), meconium-instilled and surfactant-treated (M + S) group, and meconium-instilled and combined surfactant + budesonide treated (M + S + B) group. For between-group comparisons: M versus C; M + S & M + S + B versus M; M + S + B versus M + S: •P < 0.05, •P < 0.01, •P < 0.001.

Fig. 2. Oxidative modifications in the lung homogenate. Markers of protein oxidation (dityrosine (2A) and lysine-LPO products (2B) expressed in RFU) and lipid oxidation [TBARS (2C) in µmol MDA/mg protein; conjugated dienes (2D) in RFU] in the lung homogenate of saline-instilled controls (C), meconium-instilled untreated group (M), meconium-instilled and surfactant-treated (M + S) group, and meconium-instilled and combined surfactant + budesonide treated (M + S + B) group. For between-group comparisons: M versus C; M + S & M + S + B versus M; M + S + B versus M + S: •P < 0.05, •P < 0.01, •P < 0.001.
meconium group (for M + S versus M, P < 0.01; for M + S + B versus M, P < 0.001). Significant difference was observed also between the therapies (for M + S + B versus M + S, P < 0.05).

Oxidative modifications in the lung homogenate

Oxidative modifications of proteins and lipids in the lung homogenate are shown in Fig. 2. Oxidative damage of proteins was assessed by measurement of dityrosine formation and modification of lysine and formation of lysine LPO-end products. In untreated meconium group, dityrosine and lysine-LPO fluorescence increased markedly in comparison to control group (for dityrosine +133.7% and for lysine LPO-products +157.2%, for M versus C both P < 0.001). Both treatments decreased formation of dityrosine versus M group, but this reduction was significant only for combined therapy (for M + S versus M: −14.9%, P > 0.05; for M + S + B versus M: −32.0%, P < 0.05). In lysine-LPO fluorescence, significant decrease was shown in both treatment groups versus M group (for M + S versus M: −114.1%, P < 0.001; for M + S + B versus M: −157.0%, P < 0.001). Further significant reduction was observed in combined therapy vs surfactant-only therapy (for M + S + B versus M + S: −34.7%, P < 0.05).

Modifications in the lipid structure due to peroxidation were assessed by detection of TBARS and conjugated dienes (CD). Concentrations of TBARS and conjugated dienes elevated significantly in M group compared to controls (for M versus C: TBARS +61.9%, P < 0.01; CD +54.3%, P < 0.001). Both therapies decreased formation of TBARS in comparison to M group (for M + S versus M: −60.4%, P < 0.01; for M + S + B versus M: −67.0%, P < 0.001). Significant decrease was observed also between the treatment groups (for M + S + B versus M + S: −33.3%, P < 0.05). Formation of conjugated dienes declined after both therapies compared to untreated M group (for M + S + B versus M: −23.6%, for M + S versus M: −23.0%, both P < 0.05), but there was no statistic difference between the treated groups.

Oxidative modifications in isolated lung mitochondria

Oxidative modifications of proteins and lipids in the lung mitochondria are shown in Fig. 3. Oxidative changes of proteins in the mitochondria were determined by the same way as in the homogenates, i.e. by estimation of dityrosine and lysine-LPO products. In untreated M group, damage of proteins was significantly higher than in controls (for dityrosine: +32.9%, P < 0.01; for lysine-LPO: +114.4%, P < 0.001). Both therapies reduced tyrosine and lysine oxidation versus untreated M group, but only for dityrosine the changes were significant (for dityrosine formation: M + S versus M: −18.8%, P < 0.05; M + S + B versus M: −23.0%, P < 0.01; for lysine-LPO products: M + S versus M –12.2%, P > 0.05; M + S + B versus M −14.9%, P > 0.05). No significant differences were observed between the treated groups (for M + S + B versus M + S: dityrosine −5.3%, P > 0.05; lysine-LPO −3.1%, P > 0.05).

![Fig. 3. Oxidative modifications in the lung mitochondria. Markers of protein oxidation (dityrosine (3A) and lysine-LPO products (3B) in RFU) and lipid oxidation (TBARS (3C) in µmol MDA/mg protein; conjugated dienes (3D) in RFU) in the lung mitochondria of saline-instilled controls (C), meconium-instilled untreated group (M), meconium-instilled and surfactant-treated (M + S) group, and meconium-instilled and combined surfactant + budesonide treated (M + S + B) group. For between-group comparisons: M versus C; M + S & M + S + B versus M; M + S + B versus M + S: aP < 0.05, bP < 0.01, cP < 0.001.](image-url)
Damage of lipids was detected using measurements of TBARS and conjugated dienes. TBARS and conjugated dienes increased significantly after meconium instillation compared to controls (for M versus C: TBARS +31.8%, P < 0.01; CD +15.7%, P < 0.05). Both therapies decreased TBARS versus M group, but only combined treatment showed significant effect (M + S versus M: −7.2%, P > 0.05; M + S + B versus M: −14.9%, P < 0.05). Formation of conjugated dienes decreased only in M + S + B group compared to M group (−18.2%; P < 0.01), although slight reduction was present also in M + S versus M (−6.2%; P > 0.05). Significant difference was found also between the therapies (for M + S + B versus M + S: −12.8%; P < 0.05).

Total antioxidant score

Total antioxidant score was estimated from total antioxidant capacity (TAC) in the lung homogenate and plasma, as shown in Fig. 4. In the lung homogenate, TAC was lower in untreated meconium group compared to controls (for M versus C: −24.6%; P < 0.05). TAC slightly increased in surfactant therapy group (for M + S versus M: +26.8%; P > 0.05), but significantly elevated in combined therapy (for M + S + B versus M: +37.9%; P < 0.05) compared to untreated M group. However, no significant differences were observed between the therapies (for M + S + B versus M + S: 8.8%; P > 0.05).

Opposite situation was observed in the plasma. In untreated meconium-instilled group, TAC in plasma increased compared to controls (for M versus C: +109.4%, P < 0.01). Both therapies decreased TAC compared to M group, but only for combined treatment the difference was significant (for M + S versus M: −39.9%, P > 0.05; for M + S + B versus M: −48.9%, P < 0.05). M + S + B showed tendency to decrease TAC also in comparison to M + S, but the difference was not significant (for M + S + B versus M + S: −15.3%; P > 0.05).

DISCUSSION

Meconium aspiration syndrome (MAS) is a typical example of the disease, where the delicate balance between prooxidants-antioxidants is disrupted and the newborns suffer from oxidative stress. In the present study, intratracheal instillation of meconium provoked massive neutrophil recruitment from the blood stream into the alveolar compartment, their subsequent activation and overproduction of ROS. Administration of exogenous surfactant, but particularly the combined use of exogenous surfactant and budesonide decreased the oxidative stress in the lung tissue.

Infiltration of polymorphonuclears into the alveolar compartment is detectable within several hours after experimental instillation of meconium (44) or meconium aspiration by neonates (45). In this study, microscopic evaluation of BAL fluid showed significantly higher count of neutrophils in the BAL fluid compared to saline controls. Increased leak of cells into the lung was associated with their decrease in the peripheral blood at the end of experiments. Neutrophils are attracted into the lung by various chemotactic factors (5) including IL-8 present in meconium (7). In the tissue, neutrophils express pro-inflammatory enzymes, cytokines, and other biologically active substances deteriorating the alveolar epithelial and endothelial cells (4, 5, 46). Other potentially damaging substances come from alveolar macrophages, which produce chemokines including IL-8, enhancing neutrophil influx into the airspaces (4, 5). Further damage to lining cells may be caused by aggressive substances present in meconium, such as pancreatic PL2A, free fatty acids or bile acids (2). As a result of multifactorial injury to the endothelium and/or alveolar cells, permeability of the alveolar-capillary barrier dramatically increases and enables additional influx of neutrophils into the lung (4, 5).

Phagocytes and other cells produce vast quantities of ROS and RNS species through their oxidant-generating systems. Free radicals are released into the cytosol, where they alter the redox state of the cell and modify other cell components by oxidation (9). For instance, membrane-bound enzyme complex NADPH oxidase, which is dormant in resting cells, can be activated rapidly by chemotactant peptides or chemokines and generates high quantities of ROS. Furthermore, myeloperoxidase, which is present in the neutrophil granules, catalyzes the production of additional ROS. Other ROS may be generated through the metabolism of arachidonic acid. ROS in abundant concentrations react with other molecules (proteins, lipids, or nucleic acids), disrupt intercellular tight junctions of the endothelium, and induce cell apoptosis and necrosis of alveolar type II cells (4, 5).

In our study, meconium instillation significantly increased production of ROS. In the lung homogenate and its mitochondria, higher concentrations of markers of protein oxidation (dityrosine and lysine-LPO products) and markers of lipid oxidation (TBARS and CD) were detected in the meconium-instilled untreated group vs saline-instilled controls. Our results

![Fig. 4. Total antioxidant status of plasma (4A) and lung (4B) assessed by TAC viewed in µM CRE. TAC in the lung homogenate and plasma of saline-instilled controls (C), meconium-instilled untreated group (M), meconium-instilled and surfactant-treated (M + S) group, and meconium-instilled and combined surfactant + budesonide treated (M + S + B) group. For between-group comparisons: M versus C; M + S + B versus M: *P < 0.05, †P < 0.01.](image-url)
are in accordance with findings of other authors who found that meconium stimulates neutrophil burst (47-49) and contributes to oxidative injury of distant organs (i.e., brain structures) (50) in in vitro and in vivo conditions.

Nevertheless, the pathomechanisms of MAS, particularly within first hours after meconium aspiration, have not been fully elucidated and processes determining the role of oxidative stress in MAS are sometimes controversial. As previously shown, meconium at low concentration may inhibit neutrophil oxidative burst and phagocytosis (51), possibly due to bilirubin and ubiquinol-10 found at high concentrations in meconium (52). On the other hand, meconium at higher concentrations strongly induces oxidative stress (48), and these changes are largely dependent on activation of complement (53). However, excessive complement activation with release of C5a may be responsible for suppression of granulocyte oxidative burst, which was observed in plasma of meconium-instilled newborn piglets 1–4 hours after meconium instillation (53). Similarly, we found higher TAC in the plasma of meconium-instilled rabbits 5 hours after meconium instillation despite TAC in the lung homogenate decreased compared to controls. Considering some antioxidative effect of meconium and suppression of neutrophil oxidative burst by overactivation of complement in the first hours of meconium exposure we cannot exclude other factors which may influence oxidative burst on local or systemic level, as well. For example, meconium-induced lung injury may elicit a production of substances which may protect the lung from additional lung injury, e.g., α1-antitrypsin, an inhibitor of neutrophil elastase (54).

In severe MAS, further oxidative damage originates from fetal/neonatal hypoxia and from ventilation with high concentrations of oxygen. Clinical studies showed increased oxidative stress in hypoxic fetuses and neonates with elevated products of lipid peroxidation in expired air and in serum and decreased levels of antioxidants in red blood cells (55, 56). Subsequent resuscitation with high inspiratory fraction of oxygen causes a dose-dependent oxidative stress in the neonates (12) and in the animal models (13). In our study, animals of all groups including controls were ventilated with 100% oxygen to have constant conditions of ventilation and ventilation-induced lung injury. By this way, the influence of oxygen ventilation on between-group differences was minimized, as the parameters from meconium-instilled groups were compared to oxygen-ventilated saline-instilled controls.

Oxidative stress and inflammatory processes strongly interact and contribute to the progression of MAS. Oxidative stress potentiates cytokine production by different mechanisms. Increased pro-oxidants levels mediate inflammatory signaling by activating protein kinases, e.g., JNK, PI3 K, PKC, and PLC, which stimulate redox sensitive transcription factors, such as STAT, CREB, NF-kB, or AP-1 via a series of signaling events transduced by other kinases like MAPK, ERK, and JAK. Activation of transcription factors leads to the transcriptional activation of inflammatory cytokines, chemokines, chemoattractant protein-1, growth factors etc., acting via autocrine and paracrine pathways (9, 14).

Considering this knowledge, suppressing of inflammation by addition of anti-inflammatory drug into the dose of exogenous surfactant may decrease oxidative damage to the lung tissue and thereby improve the respiratory status. For this purpose, synthetic glucocorticoid budesonide was chosen, as it possesses potent anti-inflammatory, antioxidative, vasodilatory, and antiedematous actions in local instillation (57-59). In addition, budesonide up to 10% concentration has only limited effect on surface activity of Curosurf (60). This finding is critical for clinical applicability of this treatment combination, as glucocorticoids delivered via the respiratory system must not interfere with surface activity of the surfactant lining layer nor impair the biophysical properties of exogenous surfactant used as a carrier. Moreover, budesonide is thought to increase expression of surfactant proteins SP-A and SP-B, and may thereby enhance surfactant function in innate immune reactions (61, 62). In this study, combined treatment with exogenous surfactant enriched with budesonide appeared to be superior to surfactant-only therapy. Combined therapy significantly decreased leak of neutrophils into the alveolar compartment and their activation, as indicated by decreased oxidative damage of proteins and lipids in the lung homogenate and mitochondria fraction in comparison to untreated animals. For some markers, combined therapy showed such improvement in oxidative parameters that reached the significant difference even in comparison with surfactant monotherapy.

Of course, we are aware of several limitations for direct applicability of our results into the clinical practice. As first, from technical reasons young animals were used instead of rabbit puppies. Therefore, in this model we had to omit transitional changes of lung tissue and hemodynamics early after the labor, which underly on the background of MAS. On the other hand, handling of older rabbits is advantageous for intratracheal instillation of meconium and treatment, as the size and diameter of the airways are similar to those in the term neonate. As second limitation, the lung injury in animals is induced by a single event (instillation of meconium suspension by a syringe) in a healthy animal, while the human MAS is often associated with complex interactions between primary risk factors, comorbidities, underlying lung and vascular changes, as well as genetic determinants. As third limitation, there are several inter-species differences in the innate immune response (i.e., in TLR receptors, mononuclear-phagocyte system, NO production, and chemokines and their receptors) that may reduce the translation of results from animal studies to clinics. Nevertheless, data from this study may be useful at least for better understanding of the pathophysiology of MAS, particularly of dynamics of oxidative changes related to meconium aspiration. Moreover, the study provides an experimental basis for testing of the novel combined therapeutic approach in the clinics.

Concluding, intratracheal instillation of meconium suspension into rabbits provoked massive recruitment of neutrophils from the blood stream into the alveolar compartment, their subsequent activation and overproduction of ROS. Treatment with exogenous surfactant, but particularly the use of exogenous surfactant enriched with budesonide decreased the oxidative stress in the lung tissue. Considering the results of this experimental study, combined administration of exogenous surfactant and synthetic glucocorticoid may be an useful approach for treatment of newborns with severe MAS. However, further research is needed until this combination may be recommended for clinical practice.

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