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N-ACETYLCYSTEINE EFFECTIVELY DIMINISHED MECONIUM-INDUCED OXIDATIVE STRESS IN ADULT RABBITS

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Since inflammation and oxidative stress are fundamental in the pathophysiology of neonatal meconium aspiration syndrome (MAS), various anti-inflammatory drugs have been used in experimental and clinical studies on MAS. This pilot study evaluated therapeutic potential of N-acetylcysteine in modulation of meconium-induced inflammation and oxidative lung injury. Oxygen-ventilated adult rabbits were intratracheally given 4 ml/kg of meconium (25 mg/ml) or saline (Sal, n = 6). Thirty minutes later, meconium-instilled animals were treated with intravenous N-acetylcysteine (10 mg/kg, Mec + NAC, n=6) or were non-treated (Mec, n = 6). All animals were oxygen-ventilated for additional 5 hours. Total and differential blood leukocyte counts were determined at baseline, and at 1, 3 and 5 h of the treatment. After sacrificing animals, left lung was saline-lavaged and total and differential cell counts in the bronchoalveolar lavage fluid were determined. Right lung was used for biochemical analyses and for estimation of wet-dry weight ratio. In lung tissue homogenate, thiobarbituric acid-reactive substances (TBARS), dityrosine, lysine-lipid peroxidation (LPO) products, and total antioxidant status (TAS) were detected. In isolated lung mitochondria, TBARS, dityrosine, lysine-LPO products, thiol group content, conjugated dienes, and activity of cytochrome c oxidase were estimated. To evaluate systemic effects of meconium instillation and NAC treatment, TBARS and TAS were determined also in plasma. To evaluate participation of eosinophils in the meconium-induced inflammation, eosinophil cationic protein (ECP) was detected in plasma and lung homogenate. Meconium instillation increased oxidation markers and ECP in the lung and decreased TAS (all $P < 0.05$). NAC treatment reduced ECP and oxidation markers (all $P < 0.05$, except of dityrosine in homogenate and conjugated dienes in mitochondria) and prevented a decrease in TAS ($P < 0.01$) in lung homogenate compared to Mec group. In plasma, NAC decreased TBARS ($P < 0.001$) and ECP, and increased TAS (both $P < 0.05$) compared to Mec group. Concluding, N-acetylcysteine diminished meconium-induced inflammation and oxidative lung injury.

Key words: *N-acetylcysteine, meconium aspiration syndrome, oxidative stress, reactive oxygen species, inflammation, lung injury, polymorphonuclears*

INTRODUCTION

Meconium aspiration syndrome (MAS) is a severe respiratory disorder that occurs in the term and post-term neonates. When meconium, the first faeces of the newborn, is aspirated, it obstructs the peripheral airways, deteriorates the surfactant function, and induces inflammation. Finally, it results in oxidative lung injury, lung edema formation, pulmonary vasoconstriction, and airway hyperresponsiveness (1).

Inflammation and damage of the lung tissue can be caused by both fractions of meconium: hydrophilic (consisting of gastrointestinal enzymes including pancreatic phospholipase A₂ or bile acids) and hydrophobic (consisting of cholesterol, free fatty acids etc.). In addition, meconium contains variable amounts of cytokines (IL-1 β , IL-6, IL-8, TNF α), and heme (2). Consequently, components of meconium enhance chemotactic activity of polymorphonuclears (PMNs), particularly of neutrophils, and stimulate their leak through the alveolocapillary

membrane (3). However, meconium may induce inflammation also indirectly, as it stimulates expression of cytokines (e.g. TNF α , IL-1, IL-6, IL-8), pro-inflammatory enzymes (phospholipase A₂, proteases), and other bioactive substances such as derivatives of arachidonic acid, endothelin-1, platelet activating factor, etc. from the activated cells. Furthermore, the cells generate large amounts of reactive oxygen species (ROS) (4, 5). All the mentioned substances dispose to subsequent surfactant dysfunction, parenchymal damage, edema, pulmonary vasoconstriction, and bronchial smooth muscle contraction (1). Furthermore, oxidative injury is partly iatrogenic and results from high concentrations of oxygen used for resuscitation of the patients with MAS.

Considering the role of inflammation in MAS, various anti-inflammatory drugs including antioxidants (6-14) have been used in research studies on MAS. For example, intratracheal administration of recombinant human superoxide dismutase (rhSOD) decreased myeloperoxidase activity, nitric oxide and 8-

isoprostane levels and lung injury score in the meconium-instilled rats (9). In the newborn lambs with persistent pulmonary hypertension, rhSOD increased oxygenation, and reduced vasoconstriction and oxidative injury (10).

We have presumed that other representative of antioxidants, N-acetylcysteine (NAC), can be also beneficial in MAS, as it possesses many valuable properties. NAC is N-acetyl derivative of amino acid L-cysteine. Thanks to content of -SH group, NAC acts as a direct scavenger of hydrogen peroxide, hydroxyl radicals, and hypochlorous acid. In addition, NAC is fastly deacetylated to cysteine, a precursor of glutathione synthesis in cells, hence regulating redox status in the cells (15). Furthermore, NAC may reduce generation of pro-inflammatory substances (e.g. TNF α and IL-1) in the cells (16). Alongside potent antioxidant and anti-inflammatory effects, NAC decreases viscosity and elasticity of mucus because of its ability to reduce disulphide bonds (17), what might be useful for meconium clearance from the airways (18). In addition, NAC may partially prevent surfactant dysfunction as it stimulates expression of surfactant protein (SP)-A mRNA (19). Thanks to low bioavailability, NAC is virtually non-toxic. Adverse effects, such as anaphylaxis, tachycardia and hypotension, or reduced chemotaxis and increased cytotoxicity to PMNs are rare and limited to very high concentrations (20). The mentioned favorable properties have designated NAC as potentially beneficial in the treatment of MAS. Its mucolytic effects may improve the removal of highly viscous meconium from the airways and complex antioxidative and anti-inflammatory properties may reduce the meconium-induced lung injury.

As there is no relevant information up to now on the use of NAC in the meconium-induced lung injury, purpose of this pilot study was to determine efficacy of NAC to reduce formation of ROS and to prevent oxidative damage in relation to leukocyte migration into the lung of experimental animals with MAS. The antioxidative action of NAC was detected in the lung tissue homogenate and subsequently in the lung mitochondria fraction, as the oxidation processes are most prominent in the mitochondria. To assess meconium-induced oxidation and antioxidative effect of the treatment on the systemic level, concentrations of TBARS as a marker of lipid peroxidation and total antioxidant status (TAS) were estimated also in the blood plasma.

MATERIAL AND METHODS

General design of experiments

Fresh meconium was collected from 20 healthy neonates, lyophilized and stored at -20°C . Before use, meconium was suspended in 0.9% NaCl at a concentration of 25 mg/ml.

Design of the experiments was approved by Local Ethics Committee of Jessenius School of Medicine. Adult rabbits (chinchilla) of 2.5 ± 0.3 kg were anesthetized with intramuscular ketamine (20 mg/kg; Narkamon, Spofa, Czech Republic) and xylazine (5 mg/kg; Rometar, Spofa, Czech Republic) followed by a continuous infusion of ketamine (20 mg/kg/h). A tracheotomy was performed and catheters were inserted into the femoral artery for sampling arterial blood, and into the femoral vein to administer anesthetics. The animals were then paralyzed with pipecuronium bromide (0.3 mg/kg/30 min; Arduan, Gedeon Richter, Hungary) and subjected to a pressure-controlled ventilator (Beat-2, Chirana, Slovakia). Animals were ventilated with a frequency of 30/min, fraction of inspired oxygen (FiO $_2$) of 0.21, inspiration time 50%, peak inspiratory pressure (PIP) of 0.6 kPa to keep a tidal volume (V $_T$) between 7–9 ml/kg, and no positive end-expiratory pressure (PEEP) in this stage of the

experiment. After stabilization, ventilatory parameters were recorded and sample of arterial blood was taken for blood gas analysis (RapidlabTM348, Bayer Diagnostics, Germany). Then, 4 ml/kg of saline (Sal group, n=6) or meconium suspension (25 mg/ml) were instilled into the tracheal tube homogeneously into the right and left lung lobes during positioning of the animal. From this moment on, settings of ventilation were changed: FiO $_2$ was increased to 1.0, PIP/PEEP to 1.5–1.7/0.3 kPa in meconium-instilled animals and to 1.0/0.2 kPa in saline-instilled animals to keep V $_T$ between 7–9 ml/kg and arterial pCO $_2$ in the range of 5.3–7.3 kPa. Within 30 minutes after meconium instillation, respiratory failure developed, defined as >30% decrease in dynamic lung-thorax compliance (C $_{dyn}$) and arterial partial pressure of oxygen (PaO $_2$) <10 kPa at FiO $_2$ 1.0. Sample of arterial blood was taken for blood gas analysis and parameters were recorded again. Meconium-instilled animals then intravenously received N-acetylcysteine (ACC Injekt, Salutas Pharma GmbH, Germany; Mec + NAC group, n=6) at a dose of 10 mg/kg 30 min after meconium instillation, or were left without treatment (Mec group, n=6). Animals were oxygen-ventilated for additional 5 hours.

Tracheal airflow and V $_T$ were measured by a Fleisch head connected to a pneumotachograph. Airway pressure was registered *via* a pneumatic catheter placed below the end of tracheal tube and connected to an electromanometer. C $_{dyn}$ was calculated as a ratio between V $_T$ adjusted per kg b.w. and airway pressure gradient (PIP-PEEP) (21). Samples of arterial blood were taken at the end of experiment and centrifuged at 3000 rpm for 15 min. Blood plasma was stored at -70°C till the biochemical analyses were performed. Then, the animals were euthanized by an overdose of anesthetics. The trachea and lung were excised. The right lung was cut to small pieces. Pieces from both more and less affected regions were equally used for further investigations, i.e. for estimation of wet/dry weight ratio (see below), or were stored at -70°C for biochemical analyses. Left lung was lavaged with saline (3×10 ml/kg, 0.9% NaCl), and bronchoalveolar lavage (BAL) fluid was centrifuged at 1500 rpm for 10 min.

Cells in the bronchoalveolar lavage fluid and blood

Samples of arterial blood were taken before meconium instillation, and at 1, 3, and 5 h of the treatment. Total blood leukocyte count was determined microscopically in a counting chamber after staining by Turck. Differential leukocyte count was estimated microscopically after staining by May-Grunwald/Giemsa-Romanowski.

Total number of cells in the BAL fluid was determined microscopically in a counting chamber. Differential cell count in the sediment was evaluated microscopically after staining by May-Grunwald/Giemsa-Romanowski.

Lung edema formation (wet/dry lung weight ratio)

Strips of the right lung tissue were weighed and dried at 60°C for 24 hours. The ratio between wet and dry weight expressed severity of lung edema.

Biochemical analyses

Lung tissue was washed, minced and homogenized in 50 mM of phosphate buffer (pH=7.4) and 1 mM of butylated hydroxytoluene (BHT) in a ratio 1:5 using homogenizer (Potter, B. Braun Melsungen A.G., Germany) at a temperature $0-4^{\circ}\text{C}$. A protein assay was performed by method of Lowry *et al.* (22). For dilution of the homogenate, 1% sodium dodecyl sulfate (SDS) was used. The protein concentration was calculated using bovine serum albumine (BSA) as a standard.

Mitochondria fraction was prepared from the tissue homogenate by differential centrifugation. Lung homogenate was centrifuged at 400 g for 5 min and supernatant was collected. The supernatant was then centrifuged at 12,000 g for 10 min. The resulting pellet was resuspended in a homogenisation buffer (25 mM 4-morpholinepropanesulfonic acid, 250 mM sucrose, 4 mM MgCl₂, 0.05 mM EGTA, pH 7.4) and centrifuged at 12,000 g for 10 min. The final pellet was resuspended in the homogenisation buffer (see above) and stored on ice. The protein concentration was determined by methods of Lowry *et al.* (22).

Fluorescence measurements were performed in a solution containing 50 µg of membrane protein per ml, 10 mM HEPES, 100 mM KCl, pH 7.0 at 25°C using a spectrofluorimeter (RF-540, Shimadzu, Japan). Fluorescence emission spectra (380–440 nm, slit width 5 nm) of di-tyrosine, a product of tyrosine oxidation, were measured at an excitation wavelength 325 nm (slit width 5 nm). Emission spectra (from 425 to 480 nm, slit width 5 nm) of lysine conjugates with lipid peroxidation (LPO) products were recovered at an excitation of 365 nm (5 nm slit width). Excitation spectra (from 325 to 380 nm, 5 nm slit width) were measured at 440 nm (5 nm slit width). Fluorescence intensity was expressed in arbitrary units (A.U.) (23).

A determination of thiobarbituric acid-reactive substances (TBARS) formation was performed according to Das (24). TBARS concentrations in the lung homogenate and in the mitochondria were determined from the absorbance at 532 nm and expressed in nM/mg protein, TBARS in the plasma were expressed in nM/ml.

The formation of conjugated dienes in the mitochondria was estimated from the absorbance ratio $A_{233\text{nm}}/A_{215\text{nm}}$ of mitochondria (0.02 mg protein per ml) dispensed in 10 mM phosphate buffer containing 1% Lubrol (25). The total thiol group content in the mitochondria was determined spectrophotometrically at 412 nm ($\epsilon=13.6 \text{ mM}^{-1}\text{cm}^{-1}$) as described by Hu (26), and expressed in µM/mg protein.

The activity of cytochrome c oxidase (COX) in the mitochondria was measured by monitoring of ascorbate reduced cytochrome c. Membrane proteins were resuspended in a reaction buffer (50 mM Tris/HCl (pH 8.0, 0.01% n-dodecyl-β-D-maltoside). The reaction was initialized by addition of reduced cytochrome c to the final concentration of 0.05 mM. Oxidation of cytochrome c was monitored spectrophotometrically at 550 nm by Ultrospec III (Pharmacia-Amersham) and a rate of oxidation was calculated from the slope of absorbance dependence on the incubation time using the value of $19.6 \text{ M}^{-1}\text{cm}^{-1}$ as a molar extinction coefficient of oxidized cytochrome c at 550 nm. Cytochrome c was reduced with ascorbic acid and separated on a Sephadex G-25 column. The concentration of reduced cytochrome c was controlled by the measurement of the absorbance ratio $A_{550\text{nm}}/A_{565\text{nm}}$. COX activity was expressed in nM of oxidized cytochrome c per mg of protein per min (27).

Quantification of total antioxidant status (TAS) in the lung homogenate and in the blood plasma was carried out by ELISA kit using ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) radical formation kinetics (Randox TAS kit, Randox Laboratories Ltd., UK) and expressed in mM/l. Eosinophil cationic protein (ECP) in the plasma and lung homogenate was detected by ELISA kits (Diagnostics Development, Sweden) and were expressed in µg/l.

Statistical analysis

Between-group differences were evaluated by one-way analysis of variance (ANOVA) with the post-hoc Fisher's LSD test, within-group differences were evaluated by Wilcoxon's test. Strength of association between the markers was expressed by

Pearson's correlation coefficient (r) and Bonferroni probability (P). $P<0.05$ was considered statistically significant. Data were expressed as means \pm S.E.M.

RESULTS

Cell counts in the arterial blood

The total leukocyte count in the blood gradually decreased after meconium instillation and was lower in Mec vs. Sal group at 1 and 3 h ($P<0.01$) and at 5 h of the treatment ($P<0.001$). NAC increased total circulating leukocytes compared to Mec group at the end of experiment ($P<0.05$; *Fig. 1*).

At 5 h of the treatment, relative number of neutrophils was lower and numbers of lymphocytes and eosinophils were higher in the Mec vs. Sal group (all $P<0.001$). NAC elevated circulating neutrophils and decreased lymphocytes (both $P<0.001$). In addition, NAC increased monocytes ($P<0.05$ or 0.01) during the experiment and showed a tendency to reduce eosinophils ($P<0.05$) compared to the Mec group (*Table 1*).

Cell counts in the bronchoalveolar lavage fluid

Total count of cells in the BAL fluid was higher in the Mec vs. Sal group ($1842 \pm 358 \times 10^3/\mu\text{l}$ in Mec group vs. $155 \pm 45 \times 10^3/\mu\text{l}$ in Sal group; $P<0.001$). NAC decreased the number of cells migrating into the alveolar space compared with Mec group ($913 \pm 217 \times 10^3/\mu\text{l}$ in Mec+NAC group; $P<0.05$).

The presence of meconium in the lungs elevated the relative numbers of neutrophils ($P<0.001$) and eosinophils ($P<0.01$) and decreased the monocyte-macrophages ($P<0.001$) in the BAL fluid compared to the Sal group. NAC decreased neutrophils ($P<0.001$) and eosinophils ($P<0.01$) and elevated the relative number of monocyte-macrophages ($P<0.001$) compared to the Mec group (*Fig. 2*).

Oxidation markers in the lung homogenate

Meconium aspiration caused significant oxidative stress in the lung, as indicated by increased TBARS, a representative of lipid oxidation, and by increased concentrations of di-tyrosines and lysine-LPO products, markers of protein oxidation (all $P<0.001$ in Mec vs. Sal group). Higher production of ROS was additionally proven by decreased content of -SH groups ($P<0.01$) and lower total antioxidant status (TAS) ($P<0.001$). Activation of PMNs was expressed also by higher ECP in the lung homogenate ($P<0.05$; *Table 2*). NAC decreased formation of TBARS ($P<0.001$) and lysine-LPO products ($P<0.05$) and showed a trend to decrease di-tyrosines ($P>0.05$). NAC elevated the total content of thiol groups ($P<0.001$ vs. Mec group) and prevented a decrease in TAS ($P<0.01$; *Table 2*). In addition, NAC decreased ECP in the lung tissue ($P<0.05$; *Table 2*).

Oxidation markers in the lung mitochondria

To elucidate the oxidative processes in the meconium-injured lungs more in detail, oxidative stress was determined also in the isolated lung mitochondria. In the Mec group, higher concentrations of conjugated dienes and TBARS, indicators of lipid peroxidation, as well as higher concentrations of -SH groups, di-tyrosines and lysine-LPO products were found in comparison to Sal group (all $P<0.001$). In addition, activity of cytochrome c oxidase in the mitochondria decreased after meconium instillation compared to the saline-instilled animals ($P<0.001$; *Table 3*). NAC prevented a decrease in cytochrome c oxidase activity

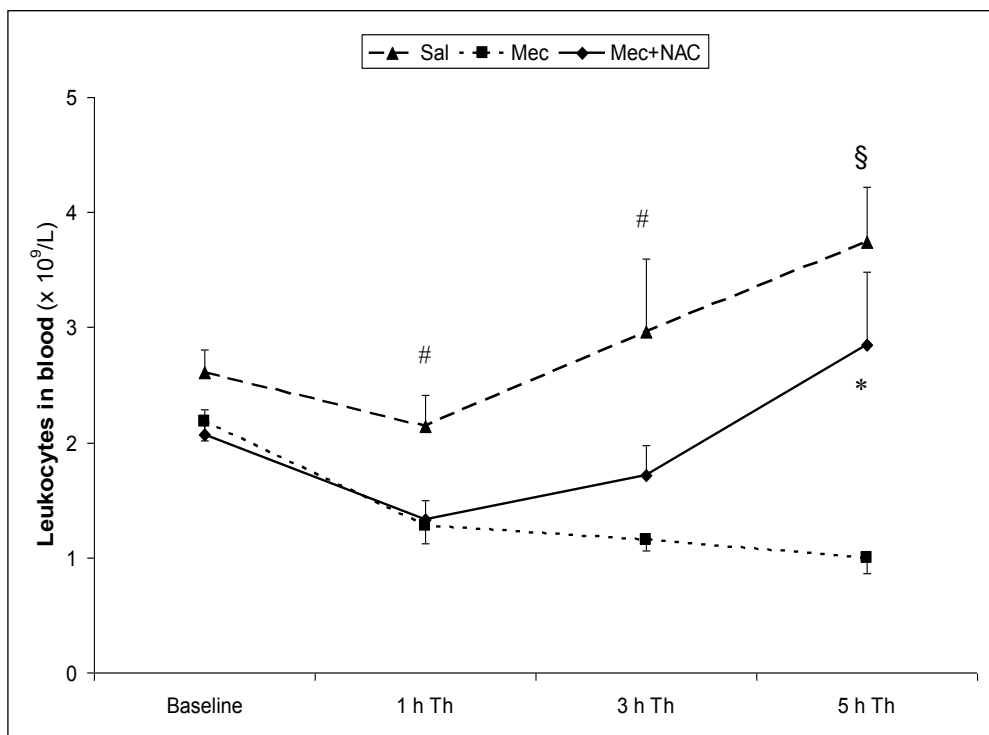


Fig. 1. Total leukocyte count in the arterial blood ($\times 10^9/l$) at the end of experiments in the saline-instilled group (Sal), in the meconium-instilled non-treated (Mec) group, and in the meconium-instilled and N-acetylcysteine-treated group (Mec + NAC). For comparisons vs. Mec: * $P < 0.05$, # $P < 0.01$, § $P < 0.001$.

Table 1. Relative counts (in %) of leukocyte subpopulations in the arterial blood during experiment.

	Baseline	1 h Th	3 h Th	5 h Th
Sal group				
Neutrophils	6.07±1.65	14.1±4.44	50.7±6.72 [§]	69.6±2.38 [§]
Lymphocytes	91.1±1.82	84.5±4.45	47.7±6.85 [§]	28.8±2.41 [§]
Monocytes	2.73±0.30	1.99±0.36	1.49±0.23	1.44±0.27
Eosinophils	0.11±0.05	0.10±0.06	0.13±0.06 [#]	0.11±0.05 [§]
Mec group				
Neutrophils	4.73±1.11	6.53±1.21	8.33±0.89	13.4±2.39
Lymphocytes	91.0±1.35	91.9±1.16	90.0±1.11	84.1±2.55
Monocytes	2.42±0.40	1.30±0.33	0.92±0.26	1.37±0.29
Eosinophils	0.18±0.09	0.45±0.17	0.80±0.19	1.22±0.25
Mec+NAC group				
Neutrophils	5.88±1.45	10.6±1.48	35.1±9.69 [*]	44.2±10.1 [#]
Lymphocytes	92.1±1.47	86.6±1.55	62.6±9.72 [*]	53.3±9.80 [#]
Monocytes	1.88±0.43	2.62±0.27 [*]	1.88±0.18 [#]	1.83±0.33
Eosinophils	0.13±0.09	0.18±0.12	0.42±0.16	0.70±0.23

Statistical differences vs. Mec group: * $P < 0.05$, # $P < 0.01$, § $P < 0.001$.

compared to the non-treated Mec group ($P < 0.001$) and significantly reduced the concentrations of TBARS and fluorescence intensity of dityrosines and lysine-LPO products (all $P < 0.001$). The conjugated dienes slightly decreased ($P > 0.05$) and total content of -SH groups was lower than in the Mec group ($P < 0.01$; Table 3).

Oxidation markers in the plasma

To estimate impact of inflammation and oxidative changes in the lungs on the systemic level, some markers were determined also in the blood plasma taken at the end of experiments. Concentrations of TBARS ($P < 0.001$; Fig. 3) and ECP ($P < 0.05$; Fig. 4) increased and TAS decreased ($P < 0.05$; Fig. 5) in the Mec vs. Sal group. NAC administration decreased TBARS ($P < 0.001$; Fig. 3) and ECP ($P < 0.05$; Fig. 4)

and prevented elevation in TAS ($P < 0.05$; Fig. 5) in comparison to Mec group.

Lung edema formation

Meconium instillation increased edema formation expressed as a wet/dry lung weight ratio (W/D ratio) compared to saline instillation in the Sal group ($P < 0.001$). NAC diminished the wet-dry ratio in comparison to the non-treated Mec group ($P < 0.001$; Fig. 6).

Correlations between the measured parameters

Pearson's evaluation showed significant correlations between counts of total cells, neutrophils and eosinophils in the BAL fluid vs. biochemical markers in the lung homogenate,

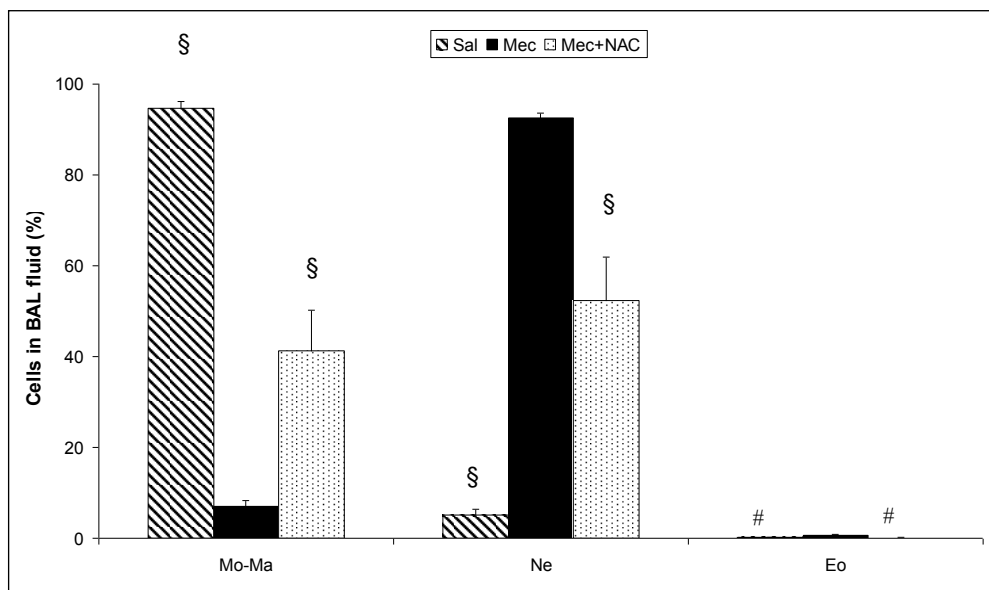


Fig. 2. Differential leukocyte count (Mo-Ma; monocyte-macrophages, Ne; neutrophils, Eo; eosinophils) in the BAL fluid (%) at the end of experiments in the saline-instilled group (Sal), in the meconium-instilled non-treated (Mec) group, and in the meconium-instilled and N-acetylcysteine-treated group (Mec + NAC). For comparisons vs. Mec: #P<0.01, §P<0.001.

Table 2. Concentrations of markers of oxidative stress in the lung homogenate.

	Sal group	Mec group	Mec+NAC group
TBARS (nM/mg protein)	1.03±0.08 [§]	1.83±0.08	1.20±0.09 [§]
Dityrosine (A.U.)	21.7±0.67 [§]	31.2±1.02	28.9±1.38
Lysine-LPO products (A.U.)	6.58±0.19 [§]	9.63±0.53	8.03±0.36*
-SH groups (μM/mg protein)	0.096±0.004 [#]	0.068±0.006	0.165±0.010 [§]
TAS (mM/l)	1.86±0.04 [§]	0.41±0.07	1.12±0.28 [#]
ECP (μg/l)	8.23±1.69*	14.9±1.65	7.88±1.99*

Statistical differences vs. Mec group: *P<0.05, #P<0.01, §P<0.001.

Table 3. Concentrations of markers of oxidative stress in the lung mitochondria.

	Sal group	Mec group	Mec + NAC group
TBARS (nM/mg protein)	1.70±0.05 [§]	2.19±0.07	1.59±0.10 [§]
Dityrosine (A.U.)	29.9±1.03 [§]	48.7±1.39	27.9±1.43 [§]
Lysine-LPO products (A.U.)	17.4±0.47 [§]	25.6±1.36	16.7±1.16 [§]
-SH groups (μM/mg protein)	0.072±0.003 [§]	0.052±0.002	0.039±0.003 [#]
Conjugated dienes (μM/mg protein)	0.35±0.02 [§]	0.76±0.02	0.72±0.01
Cytochromoxidase activity (nM/mg protein/min)	87.9±3.34 [§]	50.2±2.15	76.4±1.75 [§]

Statistical differences vs. Mec group: #P<0.01, §P<0.001.

mitochondria, or in the plasma. Clear correlations were also found between wet/dry lung ratio and cells in the BAL fluid and in the blood at the end of experiment; and between wet/dry ratio vs. almost all biochemical markers in the lung homogenate, in the mitochondria, and in the plasma (all P<0.05).

DISCUSSION

Inflammation and oxidative stress play a key role in the development of severe MAS. In the meconium-instilled rabbits, NAC decreased number of cells migrating into the alveolar space, particularly of neutrophils. In addition, NAC reduced formation of oxidation products in the lung homogenate and isolated mitochondria, prevented a meconium-induced decrease in total antioxidant status and diminished lung edema formation.

Meconium acts as a potent chemoattractant for PMNs. Higher counts of neutrophils may be observed in the lung within

several hours after meconium instillation (11). In this study, relative numbers of neutrophils and eosinophils in the BAL fluid increased after meconium instillation compared to saline-instilled controls, presumably due to their demargination and migration through the capillary wall into the interstitium, and further into the alveolar spaces. Subsequently, decreased neutrophils in the blood were detected, while eosinophils remained increased. This discrepancy may be explained by different mechanisms regulating the influx of these two types of leukocytes into the alveolar space (28, 29).

Decrease in circulating PMNs and extent of their accumulation in the lung may be proportional to severity of the lung injury (30). In this study, clear correlations were found between the lung edema formation and cells in the BAL fluid and cells circulating in the blood, respectively. NAC reduced both neutrophils and eosinophils in the BAL fluid in the treated animals in comparison to the non-treated meconium-instilled rabbits. Similarly, decreased

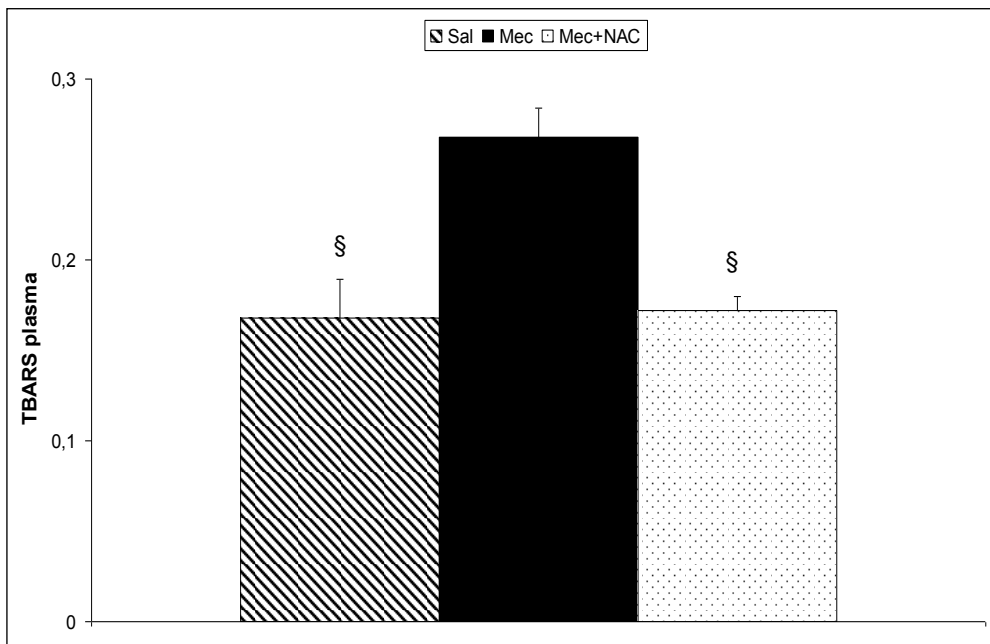


Fig. 3. Thiobarbituric acid-reactive substances (TBARS, in nM/ml) in the plasma at the end of experiments in the saline-instilled group (Sal), in the meconium-instilled non-treated (Mec) group, and in the meconium-instilled and N-acetylcysteine-treated group (Mec + NAC). For comparisons vs. Mec: §P<0.001.

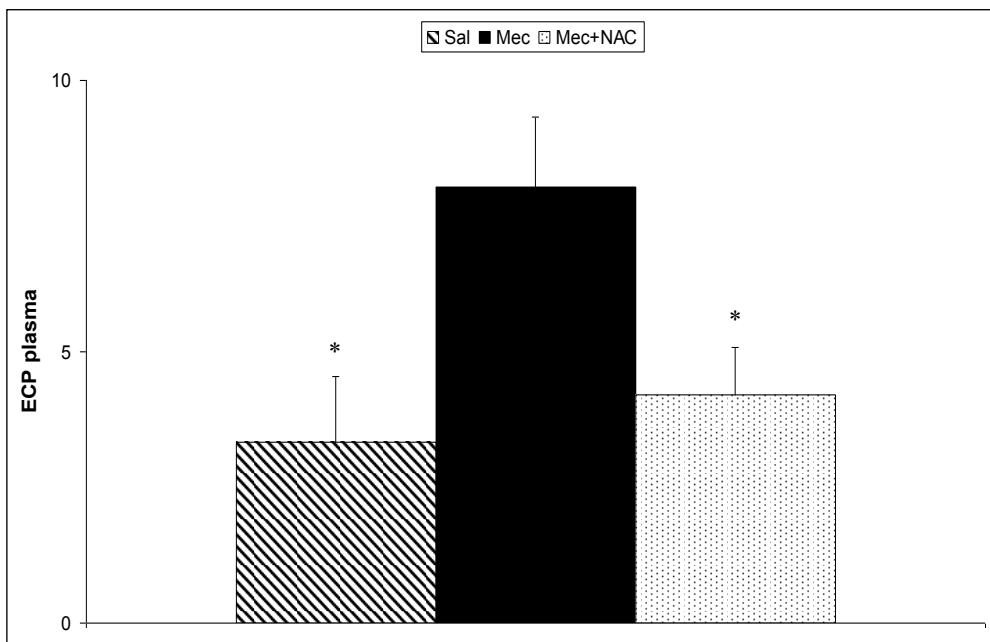


Fig. 4. Eosinophil cationic protein (ECP, in µg/l) in the plasma at the end of experiments in the saline-instilled group (Sal), in the meconium-instilled non-treated (Mec) group, and in the meconium-instilled and N-acetylcysteine-treated group (Mec + NAC). For comparisons vs. Mec: *P<0.05.

neutrophils in the alveolar space after NAC were found in other models of acute lung injury (31-33). On the other hand, NAC showed no effect on neutrophil population in the BAL fluid in rats with lung contusion (34), or LPS-induced lung injury (35).

The influx of PMNs into the meconium-exposed lung is associated with production of a wide range of bioactive substances. To estimate participation of eosinophils in the meconium-induced lung injury, ECP as a marker of eosinophil activation was detected (36). In both the lung homogenate and plasma, concentrations of ECP increased after meconium instillation and decreased after NAC. ECP in the lung homogenate significantly correlated with the number of eosinophils in the BAL fluid, while the relationship to the number of BAL neutrophils was non-significant (P = 0.078, r = 0.426). On the other hand, plasma ECP correlated significantly with neutrophils in the BAL fluid (P = 0.019, r = 0.563) and non-significantly with their decrease in the blood (P = 0.084, r = -0.431). These findings might be partially

explained by the fact that ECP may be produced in lower amounts also by neutrophils (37).

Activated leukocytes generate also ROS, which may directly injure the lung tissue. In these experiments, meconium instillation increased markers of protein and lipid oxidation in the lung homogenate and isolated lung mitochondria compared to saline instillation.

Lipoperoxidation (LPO) of membrane lipids was expressed by the production of conjugated dienes (CD) and malonyldialdehyde (MDA). CD are products of interaction of ROS with unsaturated bonds of lipids (24). Free MDA molecules react with free thiol (-SH) groups of cysteine or amino (-NH₂) groups of lysine and generate complexes reacting with thiobarbituric acid (TBA), which create thiobarbituric acid-reactive substances (TBARS) (24). Thus, an obvious increase in TBARS and CD in this study clearly showed overproduction of ROS after meconium instillation.

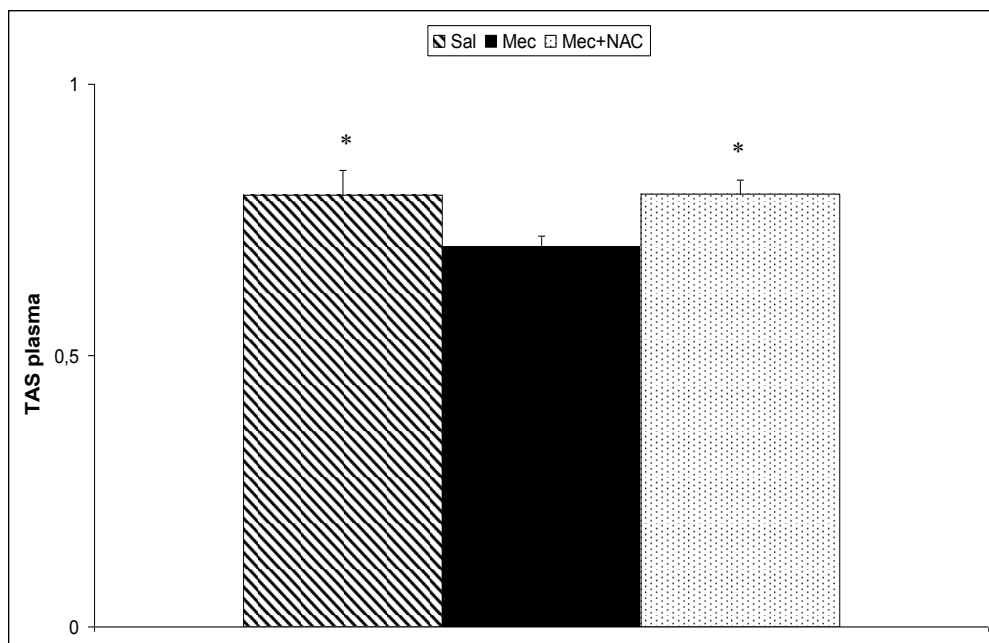


Fig. 5. Total antioxidant status (TAS, in mM/l) in the plasma at the end of experiments in the saline-instilled group (Sal), in the meconium-instilled non-treated (Mec) group, and in the meconium-instilled and N-acetylcysteine-treated group (Mec + NAC). For comparisons vs. Mec: * $P < 0.05$.

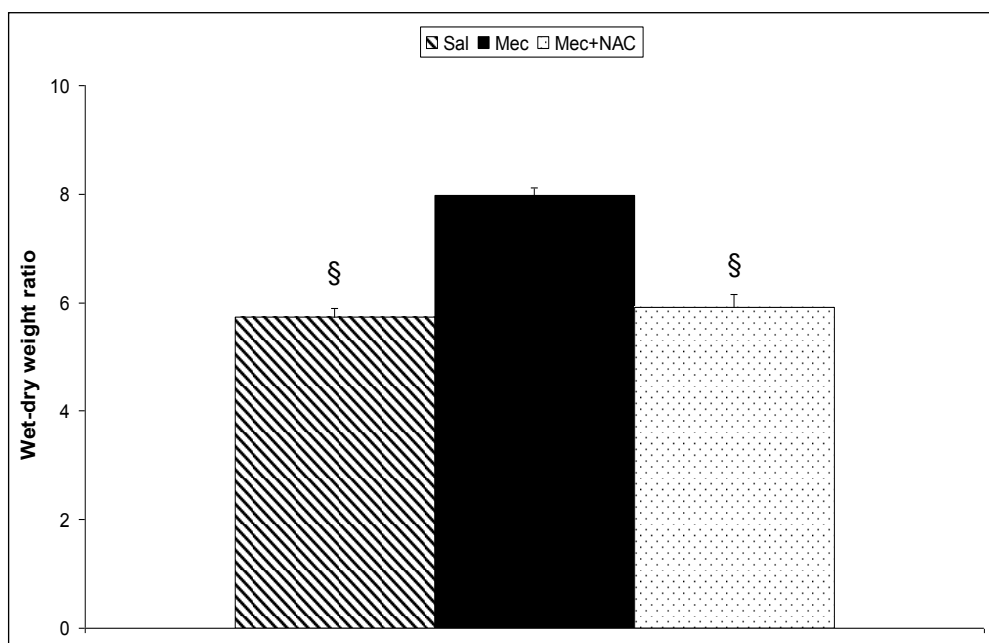


Fig. 6. Formation of lung edema expressed by wet/dry lung weight ratio in the saline-instilled group (Sal), in the meconium-instilled non-treated (Mec) group, and in the meconium-instilled and N-acetylcysteine-treated group (Mec + NAC). For comparisons vs. Mec: § $P < 0.001$.

Intravenous NAC significantly reduced TBARS in both the lung homogenate and mitochondria, while CD in the mitochondria decreased just slightly. Similarly, in various models of acute lung injury, NAC reduced the generation of MDA in the lung tissue and serum of rats (32, 34, 38) and lowered LPO-markers in the lungs of rats with LPS-induced lung injury (35) and in mice with hyperoxic lung injury (33). However, up to this moment, there are no studies evaluating antioxidative effects of NAC in MAS.

As overproduction of ROS caused by meconium aspiration may subsequently oxidize proteins, several indicators of protein oxidation were determined, too. Content of thiol groups, components of cysteine and glutathione, i.e. amino acids important in the antioxidant protection system, is a sensitive marker of oxidative injury. In the presence of ROS, free -SH groups are easily oxidized and make disulfide bonds, what decreases their concentration (26). In our study, thiol groups significantly

decreased after meconium instillation in both the lung homogenate and mitochondria. Administration of NAC increased the content of -SH groups in the homogenate, presumably because NAC itself contains -SH group in the molecule. However, we may just speculate on the mechanisms responsible for a slight decrease in content of -SH groups in the mitochondria compared to the Mec group. Probably, the concentration of free radicals in the mitochondria, site of the pronounced oxidation, was so high due to inflammation and oxygen ventilation that free -SH groups were occupied, and that process was irreversible during the time of observation. Furthermore, -SH groups originated from NAC may be utilized sooner than they reach mitochondria.

Protein oxidation by ROS may cause modification of amino acid side chains and formation of new groups, cleavage of peptide bonds, or formation of covalent protein-protein cross bonds. Products of oxidative modification of aromatic amino acids (e.g. dityrosines or lysine-LPO products) may be easily

detected (23, 39). In this study, the fluorescence intensity of dityrosine, a result of tyrosine oxidation, and lysine conjugates with LPO products increased after meconium instillation and diminished after NAC treatment.

Metabolic functions of mitochondria in the presence of ROS may be determined by estimation of activity of Krebs cycle enzymes participating in the production of ROS or individual enzymatic complexes of the Krebs cycle. Cytochrome c oxidase (COX) is the last enzyme in the respiratory electron transport chain of mitochondria located in the membrane. As COX is highly sensitive to oxidative stress (27), decreased activity of COX after meconium instillation indirectly indicates inhibition of its enzymatic activity due to accumulation of ROS, which may cause the post-translational modification of proteins. NAC treatment partially prevented a decrease in COX activity. To estimate the antioxidant capacity of the lung tissue and blood plasma, total antioxidant status (TAS) was measured. TAS includes various antioxidants, such as glutathione, vitamins C, A, and E, and enzymes (e.g. catalase, superoxide dismutase and peroxidases). Low concentrations of antioxidants or inhibition of the antioxidant enzymes cause oxidative stress and cell damage or death (40). In this study, meconium instillation decreased TAS in both lung homogenate and plasma, while NAC increased the antioxidant capacity, probably due to promoting cellular glutathione production (20, 41), and/or due to reduced production of oxidants. In addition to TAS, concentration of TBARS in the plasma was determined to evaluate the systemic effects of meconium instillation. High concentrations of TBARS detected not only in the lungs, but also in the plasma after the meconium instillation support the previous observations that the distant organs and tissues (e.g. hippocampus) may be damaged by oxidation, as well (42). Similarly to our results, NAC increased TAS and decreased serum MDA in rat models of oxidative lung injury (38, 43).

In addition, ROS can activate several signaling pathways including mitogen-activated protein kinase (MAPK) signaling, which may ultimately promote inflammation (44). Furthermore, end-products of lipid peroxidation activate extracellular signal-regulated kinase p44/42 (Erk1/2), c-Jun N terminal kinase, and p38MAPK, whereas this activation may be blocked by NAC (45, 46). Activation of the mentioned kinases is accompanied by increased activity of various transcription factors including activating protein (AP)-1 and nuclear factor (NF)- κ B (47, 48). Supporting this hypothesis, NAC decreased concentration of NF- κ B in the lung tissue in a recent study (34). In addition, NAC is known to inhibit activity of proteolytic enzymes (e.g. matrix metalloproteinases or urokinase-type plasminogen activator) (49, 50). As endogenous and meconium-derived proteases have been suggested as playing part in the pathogenesis of MAS, some of the protective effects offered by NAC in these experiments might be partially explained also by inhibition of these enzymes.

Reduced generation of ROS and other pro-inflammatory substances following NAC treatment finally resulted in decreased formation of lung edema. However, diminished lung edema is also attributed to other effects of NAC, particularly to the ability to reduce pulmonary vasoconstriction and right-to-left pulmonary shunts (34, 51). In the models of acute lung injury, NAC decreased pulmonary vascular resistance and lung edema in dogs with oxygen-induced lung injury (51), reduced alveolar disruption and edema in rats with lung contusion (34), and decreased edema formation in LPS-induced lung injury in rats (35).

NAC exerted complex antioxidative action in the meconium-induced acute lung injury on all measured levels: mitochondrial, organ, and systemic. As preventing mitochondrial oxidative stress (52) has become fundamental for effective therapeutic strategy in disorders with mitochondrial dysfunction, NAC acting as a direct radical scavenger and a stimulator of mitochondrial glutathione and other thiol-based

antioxidants can be useful in a wide spectrum of diseases including COPD, interstitial lung diseases, hyperoxic lung injury, fever, cardiovascular, oncological or neurodegenerative diseases (50, 53-59). In this moment, we can just hypothesize which of the mentioned mechanisms of NAC protects the lung from oxidative stress and in what extent, or whether NAC protects from ventilation-induced oxidative stress rather than from meconium-induced oxidative stress etc. Nevertheless, antioxidative and anti-inflammatory effects of NAC appeared to be significant in animal model of MAS, therefore we can suggest that it might be useful also in the newborns suffering from MAS.

Concluding, instillation of meconium caused significant lung inflammation, oxidative stress, and lung edema. Intravenous NAC reduced the influx of PMNs into the lung and decreased concentrations of markers of oxidative stress in the lung homogenate, in isolated mitochondria, as well as in the plasma. Thus, our results suggest that NAC may be perspective used also in the treatment of MAS.

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