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SINGLE-DOSE VERSUS TWO-DOSE DEXAMETHASONE EFFECTS ON LUNG INFLAMMATION AND AIRWAY REACTIVITY IN MECONIUM-INSTILLED RABBITS

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Two doses of the corticosteroid dexamethasone may alleviate meconium-induced acute lung injury more effectively than a single dose. Meconium-instilled rabbits intravenously received dexamethasone (0.5 mg/kg) at one dose 0.5 hours after meconium instillation or at two doses 0.5 hours and 2.5 hours after meconium instillation or were left without treatment, and were oxygen-ventilated for additional 5 hours. At the end of experiment, lungs and trachea were excised. Two doses of dexamethasone effectively diminished meconium-induced lung edema, tracheal hyperreactivity to histamine, neutrophil count in bronchoalveolar lavage fluid, and decreased oxidative modifications of proteins and lipids in lung homogenate compared with the non-treated group. Single-dose dexamethasone also reduced lung edema, lung neutrophils, and tracheal hyperreactivity to histamine, but these effects were weaker than those after two-dose dexamethasone. We conclude that two-dose dexamethasone is superior to single-dose dexamethasone in prevention lung injury in meconium-instilled rabbits.

Key words: airway hyperreactivity, dexamethasone, inflammation, meconium aspiration, oxidative damage

INTRODUCTION

Severe aspiration of meconium in the term and post-term newborns may result in an acute lung injury. Initially, mechanical obstruction of the airways by meconium may lead to alveolar atelectasis, air-trapping, and air leak. With
breathing, aspirated meconium may reach the alveoli, where triggers inflammation and inactivates surfactant. Surfactant dysfunction is potentiated by plasma proteins leaking through the injured alveolocapillary membrane, and by cytokines, proteolytic enzymes, reactive oxygen and nitrogen species, and other substances released from activated cells including neutrophils during the inflammatory response (1). Moreover, meconium alone is a source of cytokines (2) and phospholipase A₂ (3) and enhances expression of inducible cyclooxygenase-2 and inducible nitric oxide synthase (4, 5). In addition, hypoxia and higher levels of thromboxane A₂, leukotrienes, prostaglandins, and endothelin-1 (6-8) may result in pulmonary vasoconstriction, while bronchoactive substances such as leukotrienes and platelet-activating factor may be responsible for airway hyperreactivity (9). Oxidative damage to lung tissue is further aggravated by ventilation with high oxygen concentrations.

Since inflammation plays a key role in the pathogenesis of neonatal meconium aspiration syndrome (MAS), administration of drugs with anti-inflammatory effects, e.g., corticosteroids (CS), may be of benefit. CS molecules cross into the cytoplasm and, after binding to a specific receptor, interact with nuclear factor (NF)-κB and protein activator-1 in the cell nucleus. As a result, CS inhibit an expression of cytokines, proinflammatory enzymes, and other biologically active substances (10, 11) important in the pathogenesis of MAS (12). In addition, nongenomic interactions with cytosolic cGCR receptors and membrane-bound mGCR receptors are probably responsible for rapid CS action until the effects mediated by genomic mechanisms occur (13). Thus, neonates with severe MAS could benefit from CS administration. However, timing of the treatment is critical for ideal pulmonary response. Although pretreatment with CS before meconium instillation reduces pulmonary hypertension and edema formation and improves oxygenation in piglets with MAS (14, 15), early treatment with dexamethasone after meconium instillation is less effective (15). On the other side, two-dose dexamethasone treatment in piglets enhances gas exchange and reduces oxygen requirements (16). Similarly, in newborns with MAS dexamethasone given for several days in a reducing schedule improves lung function and facilitates weaning from the ventilator (17). Considering these findings, we hypothesized that two doses of dexamethasone could alleviate the meconium-induced inflammatory and oxidative lung injury and bronchoconstriction more effectively than a single dose of it.

**MATERIAL AND METHODS**

The design of experiments was approved by a local Ethics Committee of Jessenius Faculty of Medicine in Martin, Slovakia. Meconium was collected from 20 healthy term neonates, lyophilized, stored at -20°C, and before use, suspended in 0.9% NaCl at a concentration of 25 mg/ml.

Adult rabbits (chinchilla) of 2.3±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 ketamine infusion (20 mg/kg/h). Tracheal tube was placed and catheters were inserted into the femoral artery and right atrium for sampling the blood, and into the femoral vein to administer anesthetics. The animals were then paralyzed with pipercuronium bromide (0.3 mg/kg/30 min; Arduan, Gedeon Richter, Hungary) and subjected to pressure-controlled ventilator (Beat-2, Chirana, Slovakia). They were ventilated with a frequency of 30/min, fraction of inspired oxygen (F_{O_2}) of 0.21, peak inspiratory pressure (PIP) to keep a tidal volume (V_{T}) between 7-9 ml/kg and no positive end-expiratory pressure (PEEP) at this stage of experiment. After 15 min stabilization, ventilatory parameters were recorded and blood gases analysed (Rapidilab^3348, Bayer Diagnostics, Germany). Then, 4 ml/kg of saline (Sal group, n=5) or meconium suspension (25 mg/ml) was instilled into the tracheal tube. From this moment on, animals were ventilated with 100% oxygen and PEEP about 0.3 kPa. Within 30 min after meconium instillation, respiratory failure developed, defined as >30% decrease in dynamic lung-thorax compliance (C_{dyn}) and PaO_{2}<10 kPa at F_{O_2} 1.0. The rabbits with MAS received intravenously one dose of dexamethasone (0.5 mg/kg) 0.5 h after meconium instillation (Mec+Dex1, n=7), two doses of dexamethasone (each of 0.5 mg/kg) 0.5 h and 2.5 h after meconium instillation (Mec+Dex2, n=8), or were left without treatment (Mec, n=8). All animals were oxygen-ventilated for additional 5 h after the first dose of treatment. Tracheal airflow and V_{T} were measured by a Fleisch head connected to pneumotachograph. Airway pressure was registered via a pneumatic catheter placed below the tracheal tube and connected to electromanometer. C_{dyn} was calculated as a ratio between V_{T} adjusted per kg body weight and airway pressure gradient (PIP-PEEP). Samples of arterial blood were taken before meconium instillation and 1, 3, and 5 h of the treatment and the total WBC count was determined in Bürker's chamber after staining by Türk. Differential WBC count was estimated microscopically after Pappenheim's staining.

At the end of experiments, animals were killed by an overdose of anesthetics and lungs and trachea were excised. Left lungs were lavaged by saline (0.9% NaCl, 37°C) 3 x 10 ml/kg, bronchoalveolar lavage (BAL) fluid was centrifuged at 1150 rpm for 10 min and differential WBC count in sediment was evaluated microscopically after Pappenheim's staining. Right lungs were cut, strips of the tissue were weighed and dried at 60°C for 24 h to determine the wet/dry weight ratio. They also were used for the estimation of lung tissue reactivity or were homogenized and used for biochemical analyses. Airway smooth muscle reactivity was estimated in vitro (9). Tracheal and lung tissue strips were placed into organ chambers with Krebs-Henseleit's buffer. The chambers were kept at 36.5±0.5°C and aerated with a mixture of 95% O_{2} and 5% CO_{2} to maintain pH 7.5±0.1. One of the hooks was connected to a force transducer and an amplifier, and tension changes were recorded by computer software (all equipment RES Martin, Slovakia). Tissue strips were initially set to 4 g of tension for 30 min (loading phase) and then readjusted to a baseline value of 2 g for another 30 min (adaptation phase). During both periods, tissue strips were washed at 10 min intervals. Thereafter, cumulative doses of histamine (10^{-6}-10^{-4} mol/l, subst. Sigma-Aldrich, Germany) were added and continual recording of contractions was made. Data of tracheal and lung tissue reactivity were expressed in grams (g) of the smooth muscle tension. Products of lipid and protein oxidation were determined in the homogenate of the right lungs. Concentration of lipid peroxidation (LPO) products (thiobarbituric acid-reactive substances, TBARS) was determined from the absorbance at 532 nm and expressed in nmol/mg protein (18). Protein assay was performed using bovine serum albumine as standard (19). Accumulation of dityrosine and lysine-LPO products demonstrating oxidative modification of proteins was determined by fluorescence method. Fluorescence measurement was performed in solution containing 50 µg of homogenate protein per ml, 10 mmol/l HEPES, 100 mmol/l KCl at pH 7.0 at 25°C using spectrofluorometer (RF-540, Shimadzu, Japan). Fluorescence emission spectra (380-440 nm, slit width 5 nm) of dityrosine, a product of tyrosine oxidation, were measured with excitation at 325 nm (slit width 5 nm) (20). Emission spectra (425-480 nm, slit width 5 nm) of lysine conjugates with LPO product were
recovered at excitation of 365 nm (5 nm slit width). Excitation spectra (325-380 nm, 5 nm slit width) were measured at 440 nm (5 nm slit width) (21).

All data were expressed as means ±SE. Differences between groups were evaluated by ANOVA with a post-hoc LSD test, within-group differences were evaluated by Wilcoxon’s test. A P<0.05 was considered statistically significant.

RESULTS

Intratracheal instillation of meconium increased fluid accumulation in the lungs in Mec vs. Sal group (P<0.001). Single-dose dexamethasone decreased lung

![Fig. 1. Lung wet/dry (W/D) weight ratio. Sal, saline-instilled, Mec, meconium-instilled, Mec+Dex, dexamethionium-treated animals. Data are means ±SE. For between-group differences: *P<0.05, **P<0.01, ***P<0.001.](image1)

![Fig. 2. Number of white blood cells (WBC) in arterial blood before saline/meconium instillation and at 1 h, 3 h, and 5 h of dexamethasone treatment. Data are means ±SE. For Sal vs. other three groups, **P<0.01; for Sal vs. Mec+Dex1, *P<0.05; for Sal vs. Mec, #P<0.01; for Mec+Dex2 vs. Mec, †P<0.05; for Mec+Dex2 vs. Mec, ‡P<0.01. Abbreviations of experimental groups as in Fig. 1.](image2)
edema compared with the Mec group (P<0.01), but a more pronounced effect was observed after two doses of dexamethasone (P<0.001; Fig. 1). A total number of WBC in the arterial blood was lower in all meconium-instilled groups (Mec, Mec+Dex1, and Mec+Dex2) compared with the Sal group at 1 h of the treatment (P<0.01). At 3 h and 5 h of the treatment in the dexamethasone-treated groups, particularly in the Mec+Dex2 group, an increase in WBC count was observed compared with the Mec group (Mec+Dex2 vs. Mec P<0.05 or 0.01, respectively; Fig. 2). Differential WBC count in arterial blood showed a gradual increase in
neutrophils in all groups. At the end of experiments, the number of neutrophils was lower in Mec group vs. other three groups (all P<0.001; Fig. 3). In BAL fluid, percentage of neutrophils was higher in all meconium-instilled groups vs. Sal group (all P<0.001). One-dose (P<0.05), and particularly two-dose (P<0.001) dexamethasone treatment decreased neutrophils in BAL compared with the Mec group, with a significant difference found also between Mec+Dex1 and Mec+Dex2 groups (P<0.05; Fig. 3).

Cumulative doses of histamine progressively increased the contractile responses in all groups of animals. Tracheal reactivity to cumulative doses of histamine was significantly higher in Mec vs. Sal group at histamine concentrations of 10^-8 and 10^-7 mol/l (P<0.05). Administration of dexamethasone (both regimen) decreased tracheal reactivity to histamine vs. Mec group at histamine concentration of 10^-8-10^-3 mol/l (P<0.05). Furthermore, a significant difference in suppression of tracheal strips contraction to histamine at concentrations of 10^-4-10^-3 mol/l was observed also between one-dose and two-dose dexamethasone (P<0.05; Fig. 4). Dexamethasone (one-dose and two-dose) did not change significantly lung tissue reactivity to histamine compared with the Mec group, although there was a tendency to decrease the reactivity at histamine concentrations of 10^-5-10^-3 mol/l (data not shown; P>0.05). An analysis of the oxidative damage of lipids and proteins in lung homogenate showed increased levels of TBARS (expressing lipid peroxidation) and higher accumulation of dityrosine and lysine conjugates with LPO products (expressing protein oxidation) in all meconium-instilled groups vs. Sal group (all P<0.001). While two-dose dexamethasone significantly reduced TBARS, dityrosine, and lysine-LPO products (P<0.01), single-dose dexamethasone treatment had only a minor effect on the markers of lipid and protein oxidation (Fig. 5).
DISCUSSION

In this study, we hypothesized that early administration of repetitive doses of CS could improve lung function in MAS more effectively than a single-dose treatment. We found that dexamethasone, given intravenously in two doses, effectively alleviated lung inflammation, as expressed by decreases in lung edema, the number of neutrophils in BAL fluid, the meconium-induced airway hyperreactivity, and in the markers of lipid and protein oxidation in lung homogenate.

Meconium instillation resulted in a significant lung edema formation compared with saline-instilled animals. Even a single-dose of dexamethasone 0.5 h after meconium instillation reduced the fluid accumulation compared with the non-treated animals. An anti-edematous effect of dexamethasone is related to stabilizing the membranes and diminishing the microvascular permeability and to decreasing the levels of cytokines, proteolytic enzymes and reactive species, all of which may damage surfactant and alveolarcapillary membrane (10, 11, 22). The fact that the same dose of dexamethasone given 1 h after meconium instillation in piglets showed just a non-significant decrease in lung edema (15) indicates that the changes following meconium aspiration become severe very early and there is a narrow time window to overcome nascending inflammation. Two-dose dexamethasone was more effective in reducing lung edema, suggesting that CS could also act well in established MAS, but repetitive doses are needed. Similarly to our results, two doses of dexamethasone administered 2 h and 8 h after meconium instillation significantly increased lung compliance in piglets (8) and dexamethasone given for several days in a reducing schedule improved lung function in newborns with MAS (17).

Meconium instillation also increased the number of neutrophils in the lungs compared with the saline-instilled controls, whereas a higher percentage of neutrophils in BAL fluid was associated with a lower neutrophil number in the blood. Dexamethasone treatment in one dose, and particular in two doses, reversed these changes in the number of neutrophils in both compartments. Comparable findings have been observed by other authors after CS treatment in the meconium-instilled piglets (14, 15). It is supposed that CS increase the number of circulating neutrophils due to their decreased adherence to vascular endothelium and reduced leak into the alveolar spaces (10, 11). In lung diseases, loss of circulating neutrophils and the extent of their accumulation in the lung are proportional to the magnitude of lung injury (23). In addition to reduced migration of neutrophils into the lung, CS diminish the release and action of bioactive substances, which may modulate the smooth muscle tone and its responses to contractile mediators, and may thus contribute to airway hyperreactivity (24). In this study, we observed increased airway reactivity to histamine, confirming the importance of inflammation and bronchoconstriction in the pathogenesis of MAS. Dexamethasone decreased the meconium-induced airway hyperreactivity to histamine, which was more pronounced in tracheal
smooth muscle strips. A significant difference in tracheal strip reactivity to histamine noted between one-dose and two-dose dexamethasone suggests a beneficial effect of repeated administration of corticosteroids in MAS.

Since we supposed that reactive species may be of key importance in the meconium-induced acute lung injury, we also investigated oxidative damage to lipids and proteins in the lung homogenate. Peroxidation of lipids by free oxygen radicals leads to release of lipid peroxidation products, e.g., thiobarbituric acid-reactive substances (TBARS) (18). Protein oxidation by reactive species may result in modification of amino acid side chains and formation of new groups or covalent protein-protein cross bonds (25). Aromatic amino acids like tyrosine and tryptofan are modified by oxidation and can be estimated by fluorescence of dityrosine and lysine-LPO products (20, 21). Instillation of meconium in our study dramatically increased the markers of both lipid and protein oxidation compared with the saline-instilled control animals. While single-dose dexamethasone showed only minor anti-oxidative effects, treatment with two doses of dexamethasone significantly reduced the oxidative injury of the lung tissue.

In conclusion, meconium instillation increased edema formation, the number of neutrophils, airway reactivity, and lipid and protein oxidation in the lungs. Treatment with two doses of dexamethasone significantly reduced these effects; the reduction being more effective than that after single-dose dexamethasone.

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