Effects of hypothermia on lung inflammation in a rat model of meconium aspiration syndrome[1]

Ali Haydar Turhan[I], Aytuğ Atıcı[II], Serra Sürmeli[III]

[I]Associate Professor, Department of Pediatrics, Division of Neonatology, School of Medicine, University of Başkent, Istanbul, Turkey. Scientific, intellectual, conception and design of the study; acquisition of data, analysis and interpretation of data; technical procedures; statistics analysis; manuscript preparation and writing.
[II]Professor, Department of Pediatrics, Division of Neonatology, School of Medicine, University of Mersin, Turkey. Scientific and intellectual content of the study, manuscript preparation.
[III]MD, Department of Pediatrics, Division of Neonatology, School of Medicine, University of Mersin, Turkey. Acquisition, analysis and interpretation of data; technical procedures.

Abstract

Purpose: To evaluate the effects of hypothermia treatment on meconium-induced inflammation.
Methods: Fifteen rats were instilled with human meconium (MEC, 1.5 mL/kg, 65 mg/mL) intratracheally and ventilated for 3 hours. Eight rats that were ventilated and not instilled with meconium served as a sham group. In MEC-hypothermia group, the body temperature was lowered to 33±0.5°C. Analysis of the blood gases, interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α in bronchoalveolar lavage (BAL) fluid samples, and histological analyses of the lungs were performed.
Results: The BAL fluid TNF-α, IL-1β, IL-6 and IL-8 concentrations were significantly higher in the MEC-hypothermia group than in the MEC-normothermia ($p < 0.001$, $p < 0.001$, $p = 0.001$, $p < 0.001$, respectively) and sham-controlled groups ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, respectively).
Conclusion: Meconium-induced inflammatory cytokine production is affected by the body temperature control.
Key words: Hypothermia. Meconium. Lung. Respiratory Distress Syndrome, Adult. Rats.

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■ Introduction

Meconium aspiration syndrome (MAS) is a severe respiratory disorder that occurs in the term and post-term neonates, and it is still a significant cause of respiratory morbidity and mortality in newborn infants[1,2]. The etiology of the MAS involves inflammatory response characterized by a predominantly neutrophilic infiltrate and stimulation of inflammatory cytokines (e.g., interleukin [IL]-1, IL-6, IL-8 and tumor necrosis factor [TNF]-α)[3-5]. The aim of MAS treatment focuses on minimizing ongoing injury, maintaining oxygenation, and reducing inflammation. Various anti-inflammatory drugs (such as glucocorticoids, phosphodiesterase inhibitors, angiotensin-converting enzyme inhibitors, erythropoietin) have been tested as possible MAS therapies[6-9]. However, therapy of MAS has controversies to show beneficial effects.

In some series, meconium stained liquor has been still reported in 22% to 55% of infants with hypoxic-ischemic encephalopathy (HIE)[10]. Therapeutic hypothermia has widely recommended in the standard of care for neonates with HIE[11]. The mechanisms underlying hypothermia's protective effects are complex and it is thought to attenuates secondary injury of inflammation, decreases vasogenic edema and hemorrhage, prevents neutrophil infiltration, decreases apoptosis and prevents free oxygen radical production[12,13]. Most of the interest focused on brain function and relatively little information has been gained on the effect of therapeutic hypothermia on other body organs and more so on the lungs[14]. Recent experimental studies suggest that hypothermia might have a protective role in several models of lung injury, including ventilator-induced lung injury (VILI) and endotoxin-induced acute lung injury[15-18]. However, the effect of therapeutic hypothermia on inflammatory injury in the meconium-exposed lungs remains unknown.

Considering the above facts, we have analyzed the suitability of the administration of hypothermia on MAS. In this study, we induced a lung injury with local meconium instillation as described previously[19] and examined the effects of therapeutic hypothermia on pulmonary inflammation on MAS in rats.

■ Methods

This study was approved by the Institutional Committee of Animal Care in Research of Mersin University.

Twenty-seven adult male Wistar rats, weighing 160-240g were studied. Animals were anesthetized with ketamine (20 mg/kg) and xylazine (5 mg/kg) intraperitoneally (i.p.). Tracheotomy was performed; a 16-G internal diameter catheter was inserted into the trachea, and the animals were placed on a volume-controlled mechanical ventilator (Inspira ASV; Harvard Apparatus, Saint-Laurent, QC, Canada). The initial respiratory settings were as follows: fraction of inspired oxygen (FiO_2), 1.0; rate, 40 breaths/min; tidal volume, 4 mL/kg; and positive end-expiratory pressure (PEEP), 4 cmH_2O. Paralysis was induced with vecuronium (2 mg/kg) i.p. A 26 G polyethylene catheter was inserted into the right carotid artery to obtain blood gas samples and to measure the blood pressure continuously.

Wistar rats were divided into three groups, according to whether meconium had been administered and whether animals were exposed to hypothermia. The study groups were as follows: Sham group; without meconium instillation and maintained in normothermia. Meconium-normothermia group; with meconium instillation and maintained in normothermia. Meconium-hypothermia group; with meconium instillation and maintained in hypothermia.

Meconium preparation

Meconium was collected from the first stools of healthy term human neonates. The samples were initially frozen and later pooled and lyophilized. Before the experiment, meconium was diluted with sterile saline to a concentration of 65 mg/mL[20]. The meconium batch was sterile in culture.

Meconium and hypothermia induction

In a supine position, a bolus (1.5 mL/kg) of meconium was instilled slowly through the catheter in the meconium-hypotermia and meconium groups. Animals in the sham-controlled group was ventilated without meconium instillation. After the stabilization period, ventilator frequency, tidal volume, and PEEP were adjusted to keep partial pressure of arterial carbon dioxide ($PaCO_2$) between 35 and 45 mmHg throughout the study. Five percent glucose in saline was infused intra-arterial (i.a.) at 100 mL/kg/day.

Rectal temperatures were measured continuously by a temperature probe inserted 0.5 cm rectally. Body temperature was maintained between 37°C and 38°C with a radiant warmer in the meconium and sham-controlled groups. Hypothermia was established by cooling circulating water in a closed system. In the meconium-hypotermia group, the rectal temperature was reduced to 33±0.5°C and animals outside the targeted temperature range were excluded from the study. The target temperature was reached in 10 to 15 min.

Tissue samples

Animals were sacrificed at the end of the study by intravenous administration of a lethal dose of ketamine (50 mg/kg), after extracting of whole blood. The heart and lungs were removed en bloc. Right lung was saved for later biochemical analyses. Left lung tissue samples were fixed in 10% buffered formalin, dehydrated, cleared, and embedded in paraffin according to a routine process. Five-micrometer sections were stained with hematoxylin and eosin for light microscopic analysis. To determine the extent and severity of the lung tissue injury, a pathologist (AP) blinded to the grouping of the rats examined the samples and assigned a score from 0 to 4 for three different characteristics: *I*. extension of leukocyte infiltration (0 = 0%, 1 = 0% to 25%, 2 = 25% to 50%, 3 = 50% to 75%, and 4 = 75% to 100%); *II*. amount of intra-alveolar leukocytes (0 = none, 1 = occasional, 2 = several leukocytes in the alveoli, 3 = alveoli almost full of leukocytes, and 4 = alveoli distended by tightly packed leukocytes); and *III.* amount of exudative debris (0 = alveoli open, 1 = exudate scarcely seen, 2 = exudate clearly visible, 3 = alveoli almost full of exudate, and 4 = alveoli distended by exudate). The histologic lung injury score is the sum of these scores[19].

Bronchoalveolar lavage samples

Lavage of right lung was performed by instillation of 15 mL of 0.9% NaCl three times and immediate aspiration. BAL fluid was centrifuged (350 G, + 4°C for 10 min), and the total alveolar cell counts from cellular debris were performed. Differential cell counts were performed after cytocentrifugation and May–Grünwald–Giemsa staining. Supernatant was collected, frozen at –70°C, and later used for determinations of biochemical measurements of TNF-α, IL-1β, IL-6, and IL-8 concentration[20].

Cytokines

Rat TNF-α concentration in BAL fluid supernatant was measured with *in vitro* enzyme-linked immunosorbent assay (Endogen, Inc, Woburn, MA, USA) according to the manufacturer's instructions. Absorbance

was read at 450 nm on an EIA reader (Spectra II; Wallac, Turku, Finland) with suitable statistical software (version 2.4; MultiCalc Advanced, Perkin Elmer, Turku, Finland) to calculate the results[19]. IL-6, IL-1β, and IL-8 levels were measured using an electrochemiluminescence method with Immulite 2000 systems (Diagnostic Products Corp., Los Angeles, CA, USA).

Statistical analysis

Statistical analysis was carried out using PASW statistical software (v. 18; SPSS Inc, Armonk, NY, USA). Averages of the results and standard deviations were measured using descriptive statistical methods. Normal distribution for the continuous variable was assessed by Shapiro–Wilk's test, and all data were analyzed with nonparametric tests according to the test results. The median values were compared using Mann–Whitney's *U* test and Kruskal–Wallis test followed by Dunn's test. Comparison of partial pressure of O_2 (PaO_2) levels and oxygenation index (OI) before and after instillation of meconium were performed with Wilcoxon's signed-rank test. *P* values less than 0.05 were regarded as statistically significant.

■ Results

Twenty-seven male rats were used in the study. Three rats died from hemorrhage during carotid artery catheterization and one rat died before the study was completed. Twenty-three rats completed the study; there were 8, 7 and 8 rats in sham-controlled, MEC-normothermia and MEC-hypothermia groups, respectively.

Mean weight, mean arterial blood pressure, mean body temperature, mean heart and respiratory rates and were similar between groups at baseline. After 60 minutes of cooling, the mean respiratory rates were significantly higher in MEC-normothermia group [54±6 (45 – 60)] than the MEC-hypothermia group [44±5 (40 – 50)] ($p = 0.002$).

Oxygenation index

Results are shown in Figure 1. Intrapulmonary instillation of meconium resulted in increased arterial OI (FiO_2 × mean airway pressure/PaO_2) in MEC-normothermia and MEC-hypothermia groups ($p < 0.001$ and $p < 0.001$, respectively), whereas no difference was seen in the sham-controlled group. This result suggests that MAS model was successfully constructed. At the end of the study (after three hours) OI values significantly are still significantly higher in MEC-normothermia group ($p = 0.009$).

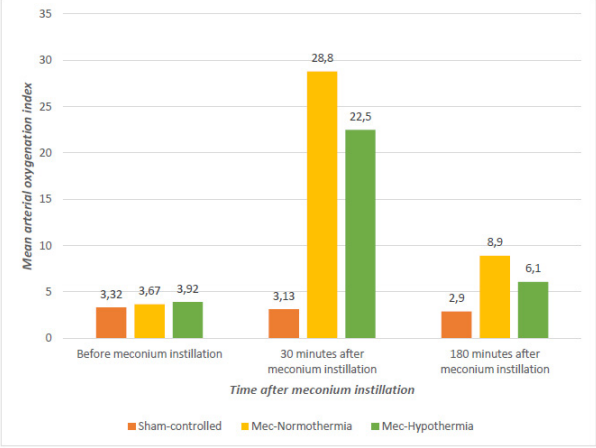

Figure 1 – The mean arterial oxygenation index levels.

Cytokines levels

As shown in Table 1, intrapulmonary instillation of meconium resulted in an increase in TNF-α ($p < 0.001$) and IL-6 concentrations ($p < 0.001$) in BAL fluid in MEC-normothermia group compared with the sham-controlled group (Table 1).

The BAL fluid TNF-α, IL-1β, IL-6 and

IL-8 concentrations were significantly higher in the MEC-hypothermia group than in the MEC-normothermia ($p < 0.001$, $p < 0.001$, $p = 0.001$, $p < 0.001$, respectively) and sham groups ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, respectively) (Table 1).

Table 1 - BAL fluid levels of IL-1β, IL-6, IL-8 and TNF-α.

	TNF-α (pg/ml), median (%25-75)	IL-1β (pg/ml), median (%25-75)	IL-6 (pg/ml), median (%25-75)	IL-8 (pg/ml), median (%25-75)
Meconium Non-Instilled lungs + Normothermia (Sham, n: 8)	59 (31-81)	49 (32-67)	27 (13-36)	16 (13-23)
Meconium Instilled lungs + Normothermia (MEC-Normothermia, n: 7)	283* (205-461)	19,6 (18-21,2)	395* (330-544)	87* (72-202)
Meconium Instilled lungs + Hypothermia (MEC-Hypothermia, n: 8)	1153*,a (972-1475)	2340*,a (1286-3627)	818*,£ (668-919)	1538*,a (1499-1584)

BAL: bronchoalveolar lavage fluid, IL: interleukin, MEC: meconium, TNF: tumor necrosis factor.
*p < 0.001 (Compared to the Sham group).
ap < 0.001, £p = 0.001 (Compared to the Meconium+ Normothermia group).

Histological markers

No histological differences were found between groups, neither overall score nor specific histologic variables examined.

■ Discussion

Although the NICU applications improved, the overall prevalence of morbidity in children born with meconium stained amniotic fluid is still high[1,10]. This present study indicated for the first time the therapeutic effect of hypothermia on meconium induced lung injury in a newborn rat model.

The pathogenesis of MAS is involves that obstruction of the peripheral airways, inactivation of the surfactant function, and induction an inflammatory response through increasing chemotactic activity of polymorphonuclear neutrophils, alveolar macrophages, and stimulating of inflammatory cytokines (e.g., interleukin [IL]-1, IL-6, IL-8 and tumor necrosis factor [TNF]-α)[3,4]. This inflammatory reaction is associated with decreases in lung compliance and the impairment of pulmonary function[21,22]. In our study, alveolar cytokine levels significantly increased in the meconium-instilled lungs compared to non-instilled controls.

In various models of acute lung injury and injurious mechanical ventilation, hypothermia-treated animals have shown different results. In some of models of acute lung injury, hypothermia-treated animals have shown improved gas exchange and lung mechanics, less pulmonary edema and inflammation, lower histological score of lung injury, and less oxidative stress damage[15,17,23,24]. On the other hand, these beneficial effects have not been shown in the other similar models[18,25-27].

Altınsoy *et al.*[15] suggested that mild hypothermia therapy may be protective in lipopolysaccharide induced lung injury in immature rats. This effect may be related to milder hypothermia (34.5±0.5°C) and younger (seven days old) rats studied without mechanical ventilation. In study of Cruces et

al.[27], animals that underwent hypothermia and two hours of deleterious mechanical ventilation (PIP 40 cmH_2O and zero PEEP) had lower serum IL-1β levels, but there had no difference in BAL fluid IL-1β levels. In the study of Morita et al.[18], hypothermia had not reduce BAL cytokine levels in spite of reducing serum cytokines, during mechanical ventilation BAL levels of TNF-α and IL-1β are higher in hypothermic rats than normothermic ones.

This present study is the first study that shows the effects of hypothermia treatment in a meconium induced lung injury model. Unlike to the of the previous studies on ALI, surprisingly, our results indicated the inflammatory role of hypothermia on pulmonary inflammation related to MAS. The lung injury models mentioned above are too different from meconium-induced lung injury model. This may be related to the effect of hypothermia on meconium and the effect of mechanical ventilation. The association of meconium and hypothermia might be causing more obstruction, atelectasis and increasing inflammation in the lung.

A previous report suggested that hyperventilation and reduction of $PaCO_2$ could directly enhance pulmonary impairment[28]. In the present study, the respiratory rate in the MEC-hypothermia group was reduced to keep $PaCO_2$ uniform among subjects, within the normal range of 35–45 torr. As for the effects of different respiratory rates in the initial setting, there has been a theory called ‘‘lung rest” that states that the lower the breathing rate, the less the occurrence of lung damage, if the same $PaCO_2$ levels are maintained[18]. In this experiment, the MEC-normothermia group had higher respiratory rates and the MEC-hypothermia group had lower respiratory rates to maintain the same $PaCO_2$ level. In terms of respiratory rates, the MEC-hypothermia group was more protective from lung damage than the MEC-normothermia group. Therefore, the differences in the respiratory rates might strengthen the significance of the results in the present study.

On histological examination of the lungs, no differences were found between the groups. This may indicate that in lungs, cytokine production occurs before histological alterations are clearly noted.

The major limitation of this study is the dissimilarity of this rat model to a human newborn infant, because of the complex pathophysiology of MAS development. Second, the internal fluid requirement normally varies depending on the body temperature and degree of stress. In the present study, however, the intravenous fluid administration was the same in all groups. Third, FiO_2 was set at 1.0 during ventilation in all subjects to avoid effects of reduction of PaO_2 arising from pulmonary dysfunction in this study. Some investigators have reported that fever enhanced the toxicity of oxygen[29].

■ Conclusions

Meconium-induced inflammatory cytokine production is affected by the body temperature control. When hypothermia therapy associate with meconium aspiration may induced the inflammatory effects.

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Correspondence:
Ali Haydar Turhan
Istanbul Hospital of Başkent University, Neonatal Intensive Care Unit
34662, Istanbul Turkey
Phone: 0090 530 527 1997
alihaydarturhan@baskent.edu.tr

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