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ORIGINAL ARTICLE

Cytokine changes in newborns with therapeutic hypothermia after hypoxic ischemic encephalopathy

CJ Moon, YA Youn, SK Yum and IK Sung

OBJECTIVE: This study aimed to examine changes in cytokines according to therapeutic hypothermia (TH) for newborn hypoxic ischemic encephalopathy (HIE).
STUDY DESIGN: We studied 20 neonates who were admitted with a diagnosis of HIE in the neonatal intensive care unit. Cytokine concentration assay was carried out for neonates ($n = 12$) who received TH and neonates ($n = 8$) who were not treated with hypothermia by collecting blood sample at 12, 48 and 120 h after birth.
RESULTS: At 48 h after birth, interleukin (IL)-6 in the normothermia group was higher than that in the hypothermia group ($P = 0.010$). At 48 h after birth, IL-10 was higher in the hypothermia group than in the normothermia group ($P = 0.038$).
CONCLUSION: This study confirmed that TH performs a role in the prevention of inflammatory process by way of maintaining proinflammatory cytokine IL-6 at low levels and anti-inflammatory cytokines IL-10 at high levels.

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INTRODUCTION

Hypoxic ischemic encephalopathy (HIE) is a disease that can cause neonatal developmental delay.[1–3] Many studies have been conducted on preventing developmental delay in HIE neonates, among which therapeutic hypothermia (TH) is one treatment modality.[4–7] An inflammatory response is triggered in the human body after asphyxia.[8–14] Particularly, proinflammation cytokines and chemokines are generated in the microglia and astrocytes of the brain.[15] Moreover, systemic inflammatory cells can trigger an inflammatory response by entering the cerebrum through the blood–brain barrier that is weakened by ischemic insult,[16] and the inflammatory response further exacerbates injuries to the brain cells. Some of the most well-known asphyxia-related cytokines include interleukin (IL)-1, IL-2, IL-6, IL-8 and tumor necrosis factor-α.[10–14,17] TH protects neuronal cells in various way, including by inhibiting inflammation.[8,18] In this study, we focused on the fact that TH and the inflammatory response, which are brain injury mechanisms in HIE neonates, are responsible for mitigating the inflammatory response that occurs after hypoxic injury. We investigated the effects of TH on the inflammatory response in HIE neonates. Although numerous studies involving animals and adult humans have investigated inflammation and changes in cytokines after hypoxic insult, few studies have been conducted in neonates. There are rare studies that have compared cytokines between groups receiving TH and groups not receiving TH. This is the study to examine changes in cytokines according to TH applied to HIE neonates.

MATERIALS AND METHODS

This study included 20 neonates who were admitted to the neonatal intensive care unit (NICU) at Seoul St Mary's Hospital between September 2013 and December 2014 for diagnosis of HIE. Among the 20 neonates, 12 received TH, whereas 8 did not receive TH. The inclusion and exclusion criteria for TH are shown in Table 1. TH was performed for 72 h. TH was performed immediately after the newborns were diagnosed with indications for treatment. None achieved body temperature lowering by passive cooling. The esophageal temperature was used as the core temperature and the target body temperature was 33.5 ± 0.5 °C. Upon completion of TH, body temperature was elevated at a rate of 0.5 °C h^{-1}, and the target body temperature during rewarming was set to 36.5 °C. Arterial blood samples were collected 3 times from all neonates at 12, 48 and 120 h after birth. Approximately 1 to 2 ml blood samples were collected and used to analyze serum cytokines. Immediately after collection, blood samples were centrifuged at 2500 r.p.m. for 10 min to separate the supernatant. Next, the supernatant was carefully extracted by pipetting and was stored at − 80 °C. All supernatant samples were used for cytokine analysis. An enzyme-linked immunosorbent assay kit (Merck Millipore, Billerica, MA, USA) was used to analyze serum cytokines. Video electroencephalography (EEG) was performed on all neonates within 5 days of birth, whereas brain magnetic resonance imaging and magnetic resonance diffusion was performed within 10 days of birth. This was a prospective study conducted after receiving approval from the institutional review board of the Catholic Medical Center.

Statistical analysis

Continuous variables were analyzed using the Mann–Whitney *U*-test, whereas categorical variables were analyzed using *t*-test and regression analysis. To compare cytokines between the experimental and control groups, nonparametric Wilcoxon test was used, whereas comparisons based on changes in cytokines were analyzed using repeated measure analysis of variance. A *P*-value of < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS for windows 15.0 (SPSS, Chicago, IL, USA).

RESULTS

This study included 20 neonates who were admitted to the NICU at Seoul St Mary's Hospital between September 2013 and December 2014 for the diagnosis of HIE. Serum cytokines of 20 neonates were analyzed using blood samples collected 3 times from each patient. Serial testing of blood samples was performed at 12, 48 and 120 h after birth. The total number of neonates

Department of Pediatrics, The Catholic University of Korea, Seoul St Mary's Hospital, Seoul, Republic of Korea. Correspondence: Dr IK Sung, Department of Pediatrics, The Catholic University of Korea, Seoul St Mary's Hospital, 505 Banpo-Dong, Seocho-Gu, Seoul 137-701, Republic of Korea.
E-mail: sinky@catholic.ac.kr
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admitted to the NICU at Seoul St Mary's Hospital between September 2013 and December 2014 for diagnosis of HIE were 101. Among these, 35 neonates had indication for TH and received TH, whereas 66 neonates had contraindication for TH, and thus did not receive TH. The tests were conducted after receiving the necessary consent from the parent for sample collection. Serial analysis of serum cytokines was performed on the hypothermia group that received TH (experimental group, $n=12$) and the normothermia group that did not receive TH (control group, $n=8$; Figure 1).

Table 1. Therapeutic hypothermia indication and contraindication

Indication	⩾35 weeks of gestation, birth weight ⩾2,000 g ⩽6 hours after birth PLUS ⩾1 of (1) PH ⩽7.0 or a base deficit >16 mmol/L (2) Apgar score <5 at 10 minutes or continued respiratory support at 10 minutes after birth PLUS ⩾1 of (1) Neurologic examination : Sarnat 2,3 (2) Seizure
Contraindication	<35 weeks of gestation, birth weight <2,000 g >6 hours after birth Congenital anomaly Metabolic disease Overt bleeding Sign of infection Need for FiO_2 >0.8 Severe persistent pulmonary hypertension

Abbreviation: FiO_2, fraction of inspired oxygen.

There were no statistically significant differences between the two groups with respect to gestational age, birth weight, gender, vaginal delivery, meconium aspiration syndrome, duration of mechanical ventilator use and clinical seizure (Table 2). All neonates were classified according to their HIE severity using the Sarnat Grading Scale. The analysis of blood cytokines was performed only on newborn with moderate HIE to minimize statistical errors resulting from difference among group. TH was being performed at 72 h from birth, the presence of hypotension was monitored and the frequency of hypotension was found to be higher in the hypothermia group than in the normothermia group ($P=0.036$). However, most of the neonates easily maintained their blood pressure with one of inotropics and no neonate suffered hypotension-related complications. HIE neonates were placed under amplitude-integrated electroencephalopathy (aEEG) for 72 h once they were admitted to the NICU. The aEEG results were interpreted as normal, moderate abnormal or severe abnormal. The number of neonates who showed abnormal results in aEEG were 10 in the hypothermia group and 6 in the normothermia group, indicating no significant difference between the two groups ($P=0.669$). Moreover, findings of video EEG acquired within 5 days of birth and brain magnetic resonance imaging taken within 10 days of birth showed no differences

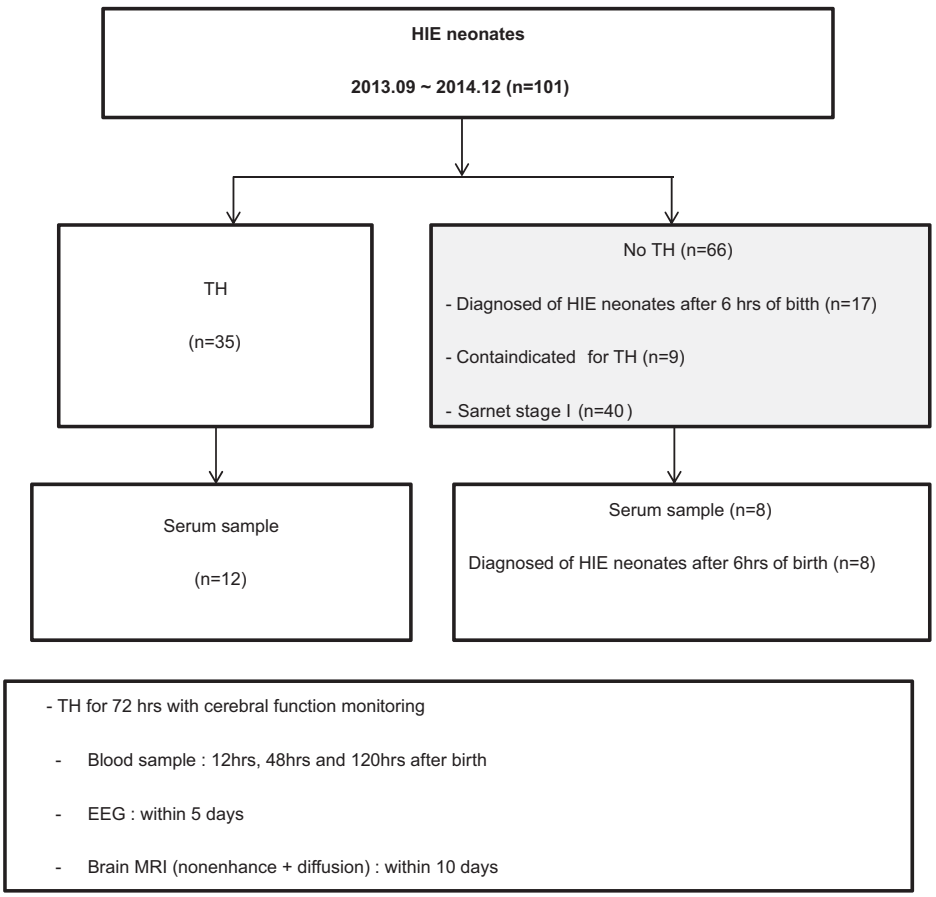


Figure 1. Diagram of the study. HIE, hypoxic ischemic encephalopathy; EEG, electroencephalography; MRI, magnetic resonance imaging; TH, therapeutic hypothermia.

Table 2. Patient characteristics and clinical manifestations

	Hypothermia (n = *12*)	*Normothermia* (n = *8*)	P-*value*
Gestational age (weeks)	39.1 ± 1.2	38.2 ± 1.4	0.185
Birth weight (g)	2996 ± 471.6	3186 ± 297.4	0.326
Sex (male) (%)	6 (50.0)	3 (37.5)	0.390
Vaginal delivery (%)	7 (58.3)	4 (50.0)	0.731
Outborn (%)	3 (25.0)	8 (100.0)	0.010*
Apgar score at 1 min	5.08 ± 2.7	7.75 ± 0.8	0.015*
Apgar score at 5 min	6.92 ± 2.3	9.13 ± 0.7	0.018*
Meconium aspiration syndrome (%)	3 (25.0)	4 (50.0)	0.296
Mechanical ventilator (day)	5.25 ± 1.6	5.00 ± 1.7	0.742
Clinical seizure (%)	9 (75.0)	8 (100.0)	0.074
Abnormal aEEG during 72 h (%)	10 (83.3)	6 (75.0)	0.669
Hypotension during 72 h (%)	10 (83.3)	3 (37.5)	0.036*
DIC in first 72 h of birth (%)	10 (83.3)	6 (75.0)	0.438
EEG abnormality (%)	10 (83.3)	8 (100.0)	0.246
HI lesions in brain MRI (%)	10 (83.3)	6 (75.0)	0.669

Abbreviations: aEEG, amplitude integrated electroencephalography; DIC, disseminated intravascular coagulopathy; EEG, electroencephalography; HI, hypoxic ischemic; MRI, magnetic resonance imaging. Values are expressed as mean ± s.d. or number (%) of neonates. **P*-value < 0.05 comparing hypothermia group with normothermia group.

between the two groups (Table 2, $P = 0.246$ and $P = 0.669$). The normothermia group that did not receive TH had a higher rate of outborn ($P = 0.010$) and showed higher 1 and 5 min Apgar scores (Table 2, $P = 0.015$ and $P = 0.018$).

The concentrations of IL-1, IL-4, IL-6, IL-8 and IL-10 were identified through blood samples collected 3 times from each subject. In three serial tests of cytokine concentrations, IL-1, IL-4 and IL-8 showed no differences between the two groups (Table 3). IL-6, a proinflammatory cytokine, showed no difference between the two groups for the blood samples collected at 12 and 120 h after birth; however, at 48 h after birth, IL-6 in the normothermia group was higher than that in the hypothermia group (5.17 ± 1.1 vs 2.58 ± 0.7 pg ml^{-1}, respectively; $P = 0.010$).

IL-10, an anti-inflammatory cytokine, showed no difference between the two groups at 12 and 120 h after birth. However, at 48 h after birth, IL-10 was higher in the hypothermia group than in the normothermia group (43.45 ± 14.8 vs 2.42 ± 0.12 pg ml^{-1}, respectively; $P = 0.038$).

Changes in IL-6 and IL-10 over time are shown in Figure 2. The solid line in the graph was determined by connecting the mean values of the cytokines. IL-6 and IL-10 gradually decreased over time. IL-6 and IL-10 at 12 and 120 h after birth did not show a statistically significant difference between the two groups. However, at 48 h after birth, IL-6 was higher in the normothermia group that did not receive TH, whereas IL-10 was higher in the hypothermia group that received TH. In order to examine the changes in cytokines during TH, repeat measures multivariate analysis was performed on serum cytokines from 12 and 48 h after birth to determine whether the changes in the values were significant changes. The hypothermia group showed statistically significant changes in IL-6 at 12 and 48 h after birth ($P = 0.029$). Therefore, it was determined that TH was able to reduce IL-6, a proinflammatory cytokine, significantly (Figure 2).

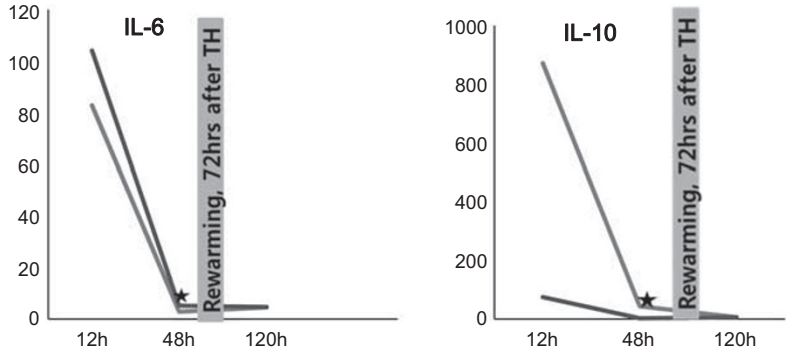
Table 3. Serum cytokine changes at postnatal 12 h, and then 48 and 120 h

Serum levels, pg ml^{-1}	*Hypothermia* (n = *12*)	*Normothermia* (n = *8*)	P-*value*
IL-6			
12 h	82.77 ± 32.6	104.06 ± 44.4	0.672
48 h	2.58 ± .0.7	5.17 ± 1.1	0.010*
120 h	4.54 ± 1.3	4.69 ± 2.19	0.946
IL-10			
12 h	871.66 ± 400.3	74.94 ± 32.1	0.072
48 h	43.45 ± 14.8	2.42 ± 0.12	0.038*
120 h	5.88 ± 3.7	5.90 ± 2.60	0.998
IL-1β			
12 h	3.73 ± 0.8	3.14 ± 0.3	0.523
48 h	2.89 ± 0.9	2.97 ± 0.2	0.634
120 h	114.92 ± 110.7	81.45 ± 78.6	0.808
IL-4			
12 h	30.93 ± 11.9	27.93 ± 9.1	0.843
48 h	15.33 ± 5.6	9.65 ± 5.0	0.460
120 h	15.98 ± 9.0	11.70 ± 9.5	0.747
IL-8			
12 h	244.60 ± 75.6	315.32 ± 147.8	0.681
48 h	120.58 ± 36.7	551.21 ± 429.4	0.350
120 h	889.13 ± 798.1	932.62 ± 893.7	0.978

Abbreviation: IL, interleukin. Values are expressed as mean ± s.e. **P*-value < 0.05 comparing hypothermia group with normothermia group.

DISCUSSION

HIE causes injuries to the brain though various mechanisms, one of which is the inflammatory response. When ischemic insult occurs, the human body releases various cytokines and chemokines. This causes progression of the inflammatory response that in turn causes neutrophils and monocytes to migrate to the injured site to cause neuronal cell damage.[16,19–21] High levels of IL-6, a proinflammatory cytokine, are known to negatively affect neurologic outcomes,[22,23] whereas IL-10, an anti-inflammatory cytokine, has a neuroprotective effect.[24–26] As described above, serum cytokines levels are elevated when hypoxic ischemic insult occurs, reaching a peak concentration at ~12–24 h after hypoxic insult.[21] In this study, blood sampling was performed a total of 3 times. In order to determine cytokine levels during secondary energy failure when serum cytokines are at their highest levels following hypoxia, the first test was performed at 12 h after birth. Moreover, serum cytokines were analyzed at 48 h after birth to examine the cytokine changes in neonates during TH, and serum



IL-6	hypothermia	normothermia	P-value
12h	82.77 ± 32.6	104.06 ± 44.4	0.672
48h	2.58 ± 0.7	5.17 ± 1.1	0.010*
120h	4.54 ± 1.3	4.69 ± 2.19	0.946
Δ(12h-48h cytokines) P values	0.029¶	0.059	

IL-10	hypothermia	normothermia	P-value
12h	871.66 ± 400.3	74.94 ± 32.1	0.072
48h	43.45 ± 14.8	2.42 ± 0.12	0.038*
120h	5.88 ± 3.7	5.90 ± 2.60	0.998
Δ(12h-48h cytokines) P values	0.057	0.059	

Figure 2. Mean serum interleukin (IL-6) and IL-10 concentrations at postnatal hours. **P*-value < 0.05 comparing hypothermia group with normothermia group (statistics: nonparametric Wilcoxon test). ¶*P*-value < 0.05 comparing 12 h serum cytokine with 48 h serum cytokine (statistics: repeated measure analysis of variance (ANOVA)).

cytokines were also analyzed at 120 h after birth to examine serum cytokine concentrations after the completion of TH and when their body temperatures returned to normal.

The levels of anti-inflammatory cytokine IL-10 decreased over time. IL-10 levels at 12 h after birth showed no difference between the two groups. The mean IL-10 value in the hypothermia group was higher than that in the normothermia group (871.66 ± 400.3 vs 74.94 ± 32.1 pg ml^{-1}, respectively); however, as the differences in IL-10 values between individuals were so large, the two groups did not show a statistically significant difference from each other ($P = 0.072$). Inflammatory responses according to hypoxia insult showed individual-based differences and the level of inflammatory responses varied according to HIE severity, such as mild, moderate and severe HIE. Thus, the testing of initial serum cytokine levels is expected to vary in different individuals. Therefore, we focused more on serum cytokine levels at 48 h after birth when TH was being performed, rather than at 12 h after birth when serum cytokine levels were at their highest. Serum cytokine levels tested at 48 h after birth showed lower IL-6 in the hypothermia group, whereas IL-10 was higher in the hypothermia group. These results indicate that TH inhibits proinflammatory cytokine IL-6 and activates anti-inflammatory cytokine IL-10, through which inflammatory responses are mitigated in the body. Although serum cytokines decreased over time, a significant change in serum cytokines occurred in only one interval. Only the change in IL-6 concentrations from 12 to 48 h after birth in the hypothermia group was significant; thus, this study showed that TH can significantly reduce the level of the proinflammatory cytokine IL-6. Moreover, TH can help improve future neurologic outcomes in HIE neonates by effectively inhibiting and reducing IL-6 that has a negative effect on neurologic outcomes.

There were some limitations to our study. The basic characteristics of patients in the hypothermia group receiving TH and the normothermia group not receiving TH were not perfectly matched, and the study included a small sample size of only 20 neonates. All neonates in the normothermia group were outborns. Neonates in the normothermia group were diagnosed with HIE within 6 h from birth, but because they were not transferred in time to a tertiary hospital capable of performing TH and were admitted to our NICU at more than 6 h after birth. Analysis of the Apgar score at 1 and 5 min after birth in newborns in the Normothermia group was based on a transfer note written by doctors at a secondary medical institution, and not on scores given by the doctors who participated in the study. The neurologic examination of HIE newborns was conducted by the doctors who participated in the study, and was performed immediately after arrival at the NICU. The neurologic examination results indicated moderate HIE. Apgar score of inborn babies in the hypothermia group were performed by doctors who participated in the study. However, Apgar score of outborn babies in the normothermia group were performed by different doctors. Therefore, the reliability of Apgar score is considered rather low. This led to small differences among patient characteristics in the Apgar score.

In this study, we reconfirmed that TH can mitigate the inflammatory response by reducing cytokine levels, particularly IL-6. Future studies should include a greater number of HIE neonates, as well as greater variety of serum cytokines, to more clearly determine the effects of TH on inflammation within the body.

CONCLUSION

Our study confirmed that TH was able to maintain proinflammatory cytokine IL-6 at low levels and anti-inflammatory cytokine IL-10 at high levels. We also confirmed that IL-6 was significantly reduced during TH, enabling inhibition of the inflammatory response in the body.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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