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Association between decreased cord blood inter-alpha inhibitor levels and neonatal encephalopathy at birth

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Abstract

Background: Inter-alpha inhibitor proteins (IAIPs) are structurally related proteins found in the systemic circulation with immunomodulatory anti-inflammatory properties. Reduced levels are found in inflammatory related conditions including sepsis and necrotizing enterocolitis, and in neonatal rodents after exposure to hypoxia ischemia. In the current study, cord blood IAIP levels were measured in neonates with and without exposure to hypoxic-ischemic encephalopathy (HIE).

Methods: This is a prospective cohort study including infants born ≥36 weeks over a one-year period. Term pregnancies were divided into two groups: a “reference control” (uncomplicated term deliveries), and “moderate to severe HIE” (qualifying for therapeutic hypothermia). IAIPs were quantified using a sensitive ELISA on the cord blood samples.

Results: The study included 57 newborns: Reference control group (n = 13) and moderate/severe HIE group (n = 44). Measurement of IAIP cord blood concentrations in moderate to severe HIE

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CRediT authorship contribution statement

Lynn Bitar: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Barbara S. Stone-street:** Visualization, Validation, Investigation, Funding acquisition, Conceptualization. **Yow-Pin Lim:** Supervision, Software, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation. **Joseph Qiu:** Supervision, Software, Resources, Project administration. **Xiaodi Chen:** Visualization, Supervision, Project administration, Methodology, Conceptualization. **Imran N. Mir:** Writing – review & editing, Visualization, Validation, Supervision. **Lina F. Chalak:** Writing – review & editing, Visualization, Validation, Supervision, Funding acquisition, Data curation, Conceptualization.

Consent statement

Before delivery, each mother granted written informed consent.

Declaration of competing interest

Yow-Pin Lim and Joseph Qiu are employees of ProThera Biologics that develops the IAIP-related products. All other authors have no conflicts or any competing interest to disclose.

group [278.2 (138.0, 366.0) μg/ml] revealed significantly lower IAIP concentrations compared with the control group [418.6 (384.5, 445.0) μg/ml] ($p = 0.002$).

Conclusions: These findings suggest a potential role for IAIPs as indicators of neonates at risk for HIE. IAIP levels could have diagnostic implications in the management of HIE. Future research is required to explore the relationship between HIE and IAIPs as biomarkers for disease severity.

Category of study: Translational.

Keywords

Inter-alpha inhibitor proteins; Hypoxic-ischemic encephalopathy

1. Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is one of the most prevalent and severe conditions affecting approximately two out of every 1000 live births [1]. It results from reduced blood flow, which restricts nutrient and energy supply to the fetal brain, primarily as a result of perinatal asphyxia before or around the time of delivery. This suggests a shift towards anaerobic metabolism, triggering both systemic and brain parenchymal inflammatory responses [2–4].

Therapeutic hypothermia (TH) remains the only approved therapy for moderate to severe HIE. It is effective only when initiated within a narrow 6-h window after birth [1,5]. However, TH offers only partial protection [6], emphasizing the critical need for complementary or alternative treatments to improve outcomes after HIE-related insults. There have been no approved pharmaceutical agents as yet to treat HIE [2,7]. Immune-mediated modulation of inflammation could potentially attenuate brain injury and offer an additional neuroprotective strategy [8].

Inter-alpha inhibitor proteins (IAIPs) are a family of structurally related anti-inflammatory immunomodulatory proteins, which have received increasing attention as a result of their contribution to many disease states including HI related brain injury in newborns, and stroke in adult subjects [9–12]. In addition, significant reductions in IAIPs have been observed in neonates with sepsis [13], and in those who develop necrotizing enterocolitis [14]. Reduced IAIP levels have also been demonstrated in pre-clinical studies after exposure of neonatal rodents and fetal sheep to hypoxia ischemia (HI) [8,15]. Furthermore, extensive preclinical studies in rodents have demonstrated the potential beneficial effects of IAIPs to attenuate neuropathological injury, neuronal loss, enhance anti-inflammatory effects, attenuate inflammatory mediated blood-brain barrier disruption, and to provide beneficial behavioral effects [2,6,9,11,16–21]. Although we have previously identified the presence of IAIPs in the human brain in autopsy samples, studies have not as yet examined potential changes in blood levels of IAIPs in the cord blood of human neonates, who develop HIE.

The primary objective of the current study was to address this gap in knowledge, by (1) determining arterial cord blood levels of IAIPs in neonates diagnosed with HIE and (2) comparing IAIP levels between moderate/severe HIE cases and controls.We hypothesized

that systemic inflammation and parenchymal brain injury in neonates with HIE could be associated with reductions in cord blood concentrations of IAIPs when compared with control infants who were not exposed to HIE.

2. Materials and methods

2.1. Study design

This single-center prospective cohort study was conducted at Parkland Hospital from June 2010 to June 2011. We screened newborns who were admitted to the neonatal intensive care unit (NICU), with gestational ages of ≥36 weeks or more and a birth weight ≥ 1800 g for eligibility. Patients with congenital anomalies or those referred for comfort care were excluded.

We divided the neonates into two groups after this screening:

- The “reference control” group included uncomplicated full-term births delivered by repeat cesarean-section without labor. Control neonates were recruited on every Wednesday of the study period (designated control recruitment day).
- The “moderate/severe HIE” group, included all neonates with HIE qualifying for therapeutic hypothermia (those neonates were included during the entire study period interval). Criteria for qualification for therapeutic hypothermia included an acidotic umbilical cord arterial blood (pH ≤ 7.00 or base deficit ≥16 mEq/L), a history of an acute perinatal event (in the absence of blood gas reading, or a pH of 7.01 to 7.15, or a base deficit of 10 to 15.9 mEq/L in cord arterial blood), a 10-min Apgar score ≤ 5 or the need for assisted ventilation at birth.

We relied upon the availability of eligible control infants (convenience sampling) during the pre-specified control recruitment days. A small subset of the designated control infants was found to have had an acute perinatal event (perinatal acidemia with evidence of meconium aspiration and/or chorioamnionitis) and were analyzed separately.

2.2. Blood collection

Immediately after birth and before delivery of the placenta, umbilical arterial cord blood was routinely sampled from double-clamped sections of the umbilical cord to identify perinatal acidemia [22]. The umbilical arterial blood samples were obtained within 1–2 min of birth, with arterial 5-ml sterile plastic syringes. Thereafter, the blood samples were centrifuged for 10 min at 10,000 rpm and the resulting serum samples were stored at −80 °C in the laboratory until analysis. As per routine hospital policy, 0.5 ml of the umbilical cord arterial blood was used for blood gas analysis.

2.3. Enzyme-linked immunosorbent assay (ELISA)

IAIPs were quantitatively measured with a competitive ELISA using a human monoclonal antibody specific for the light chain of human IAIP (MAb 69.26), as previously described by Lim et al. [23] and Shah et al. [24]. Briefly, 200 ng of purified IAIP in phosphate-buffered saline (PBS) was immobilized on Microlon 600 High Binding 96-well microplate (Greiner BioOne, Monroe, NC) for 1 h at room temperature (RT). Serial dilutions of purified IAIPs

in PBS containing 1 % serum were used to establish a standard curve. Fifty μl of serum samples diluted 1:100 in PBS or serially diluted IAIPs were added to individual wells of a 96 well plate to quantify the IAIP levels in serum. Subsequently, 50 μl of biotinylated MAb 69.26 was added to each well, and the plates were incubated on a shaker for 30 min at room temperature, followed by three washes with PBS and 0.05 Tween 20 (PBS-T). The bound biotinylated-MAb 69.26 was detected by adding HRP-conjugated streptavidin (1:5000 dilution, Innova Biosciences, Cambridge, UK) for 30 min at RT. After three washes with PBS-T, 100 μl Enhanced K-Blue TMB substrate (Neogen, Lexington, KY) was added, and the reaction was then stopped by adding 100 μl 1 M HCl. The absorbance at 450 nm was measured using SpectraMax reader (Molecular Devices, Freemont, CA). Each serum sample was assayed in triplicate and the mean value was calculated. The linear range of the assay was between 50 and 1000 μg/ml and the inter and intra-assay variations were determined to be <5 %.

2.4. Statistical analyses

A convenience sample was used for this pilot study. We summarized continuous variables such as the amount of IAIPs in cord blood (measured via ELISA), as mean ± standard deviation and median with interquartile range (IQR).

To compare competitive-ELISA values between the groups (control and moderate/severe HIE), a two group unpaired *t*-test was used. To measure of the test's overall ability to discriminate between affected and unaffected neonates, we used a receiver operating curve (ROC) and calculated the area under the curve (AUC). Significance was defined as a two-tailed p value of <0.05. All statistical analyses were conducted using R, version 4.1.0.

2.5. Study approval

The experimental procedures in this study were performed after approval from the Institutional Review Board of the University of Texas Southwestern Medical Center. Written informed consent was obtained from each mother before delivery.

3. Results

The study included a total of 57 neonates: Group 1 "reference control" (n = 9), and Group 2: "moderate/severe HIE" (n = 44) along with a subset of 4 infants originating from the reference control cohort with evidence of perinatal acidemia, meconium aspiration, and/or chorioamnionitis.

The demographic characteristics of the control and moderate/severe HIE groups are summarized in Table 1. Fourty-one % of the moderate to severe HIE infants were female, and the racial distribution included 57 % white, 23 % black, 14 % Latin, and 7 % Asian within this group. Seventy-five percent were delivered by cesarean-section and their Apgar scores were 3.0 ± 2.0 and 4.0 ± 3.0 at 1 and 5 min, respectively. The mean age of the mothers was 29.0 ± 7.0 years, and around a quarter of them experienced pregnancy related complications, including gestational hypertension (34 %), diabetes (25 %), clinical chorioamnionitis (23 %), or placental abruption (25 %). In contrast, infants within the reference control group were all delivered by cesarean-section without labor and had Apgar

scores of 8.0 ± 1.0 and 9.0 ± 1.0 at 1 and 5 min, respectively. The mean age of the mothers was 29.0 ± 5.0 years, and they did not experience any pregnancy related complications.

IAIP levels are reported as a median with interquartile range (IQR), with a high value observed in the control group [418.6 (384.5, 445.0) μg/ml]. The moderate/severe HIE group exhibited significantly lower values of IAIPs [278.2 (138.0, 366.0) μg/ml] compared with the control group ($p = 0.002$) (Fig. 1). The circled value* in Fig. 1 corresponds to the single infant diagnosed with both HIE and sepsis; this infant exhibited the lowest IAIP value among neonates in the moderate/severe HIE group. In addition, the IAIP levels in the small group of infants experiencing the adverse perinatal events were not significantly different from the levels in the control group [365.8 (327.3, 404.4) μg/ml]. We compared the cord blood serum IAIP levels between moderate/severe HIE neonates and controls using receiver operator curve characteristics (ROC) analysis to more fully understand the predictive power of IAIP levels for neonates with HIE as shown in Fig. 2. The ROC curve IAIP levels demonstrated a predictive capability for moderate to severe HIE with an area under the curve (AUC) of 0.838. This value is indicative of a strong diagnostic potential, as evidenced by a 95 % confidence interval ranging from 0.721 to 0.955. Notably, a lower IAIP level correlates with a higher probability of a positive test result, emphasizing its relevance in clinical settings for predicting HIE severity.

4. Discussion

In this prospective cohort study, we examined cord blood IAIP levels in two groups of neonates. The results of the competitive ELISA assay revealed that the neonates with moderate/severe HIE exhibited lower IAIP cord blood values compared with those in the control group. Although we have previously shown that levels of IAIPs were lower after exposure of neonatal rodents to hypoxic-ischemia [15], the current study is the first to show that cord blood concentrations of IAIPs could be a useful marker of exposure to HIE in human neonates. Our findings support the contention that reductions in the anti-inflammatory endogenous proteoglycans (IAIPs) are associated with the development of HIE. These preliminary findings further suggests that determination of IAIPs could be a useful indicator/biomarker in neonates at risk for the development of HIE.

Neuroinflammation is an important component in the evolution of HIE, which results in the release of pro-inflammatory cytokines and cytotoxic factors by glial and immune cells. This cascade triggers neuroinflammatory responses that predispose to brain injury and subsequent neuronal death [25,26]. IAIPs are recognized as potent immunomodulatory molecules with strong anti-inflammatory properties, capable of attenuating sepsis-related increases in pro-inflammatory cytokines and improving survival from sepis in neonatal rodents [10,27]. IAIP levels are reduced in neonatal inflammatory conditions, including sepsis and necrotizing enterocolitis (NEC) [13,14], which increase the susceptibility of premature neonates to brain injury [24,28]. These findings suggest that IAIPs could be affected by inflammatory conditions in newborn infants. The reductions in IAIP levels in the neonates with moderate/severe HIE could be attributed to a series of interconnected events, including inflammation, oxidative stress, and damage to brain tissue. Importantly, IAIPs are recognized for their role in modulating neuroinflammatory biomarkers, reducing

the generation of reactive oxygen species (ROS), and preventing neutrophil entrapment within microcapillaries [16]. These functions of IAIPs emphasize their potential importance as crucial protective factors against deleterious processes occurring in neonates exposed to inflammatory conditions [13,14]. These findings also suggest that the significant differences in the levels between the control group and the moderate/severe HIE group could underscore a crucial role of anti-inflammatory molecules potentially to mitigate some aspects of HIE.

Levels of IAIPs in the small acute perinatal event group did not differ from those of the control group. Therefore, a larger group of infants exposed to acute adverse perinatal events is required before conclusions can be made regarding the effects of other adverse events on cord blood IAIP levels. Nonetheless, we speculate that the effects of HIE and other acute perinatal conditions could potentially have differing effects on the magnitude of changes in cord blood IAIP levels that could result from distinct pathophysiological mechanisms inherent in both HIE and other acute perinatal conditions. Acute perinatal events, such as chorioamnionitis, appear to be a poor predictor for the development of neonatal encephalopathy, even in the presence of increased serum cytokines, such as IL-6 levels [29]. Consequently, the reduced IAIP levels observed in the cord blood of infants who subsequently developed HIE suggest a more complex relationship, indicating that reductions could be directly linked to the presence of neonatal encephalopathy and/or the attendant systemic events. This emphasizes the complexity of the factors influencing neonatal brain injury.

The timing of the insult and the duration of exposure to critical conditions could also influence the IAIP levels and result in variations in IAIP responses. The acute insult and resulting inflammation attendant to HIE could trigger a response that results in the consumption of IAIPs. Additional research is required in order to investigate the effects of other adverse perinatal events on cord blood IAIP levels and to determine the effects of specific perinatal complications on IAIP levels.

In this study, the control group consisted of neonates delivered by c-section without labor, whereas the HIE group comprised neonates who underwent labor and were mostly delivered by c-section. Given that labor represents a potential inflammatory state, this may also represent a confounding factor, and that could have contributed to the decreased IAIP levels in the HIE group.

It is also worth emphasizing that only one newborn in the moderate/severe HIE group was diagnosed with both HIE and sepsis, and that infant exhibited the lowest IAIP value within the group. Sepsis can have a profound impact on the immune system, and the presence of both conditions could compromise antioxidant defense mechanisms of the infant, exacerbating the reduction in IAIPs. The combination of HIE and sepsis may result in an increased release of inflammatory mediators, accentuating the overall inflammatory response.

It is crucial to consider alternative or additional therapies to hypothermia to improve outcomes of both full-term and premature neonates exposed to HIE [30]. In animal experiments, Chen et al. demonstrated in preclinical rodent studies that treatment with

that IAIPs reduced cell death and white matter injury after exposure to HI [9]. Moreover, Logsdon et al. showed that IAIPs administration could attenuate inflammation mediated disruption of the blood-brain barrier in adult male mice and neonatal male and female mice [21]. Furthermore, this treatment is durable, as young adult rats at P42 also exhibited less parenchymal brain damage even 35 days after they had been exposed to HI as neonates, suggesting that not only are IAIPs neuroprotective in the neonates, but they also maintain durable neuroprotection in the young adult rats after the neonatal exposure to HI [6].

These findings suggest that further exploration into the potential of IAIPs as a neuroprotective agent is required. Although the relatively large molecular size of IAIPs may pose a challenge for traversing the neonatal blood-brain barrier under normal conditions, there is a potential for IAIPs to cross the barrier in the context of HIE and/or act by its beneficial systemic effects [31]. Alternatively, elevated concentrations of IAIPs in the circulating blood of the brain could also affect the brain vasculature endothelium thereby altering or reducing other inflammatory substances that could also penetrate and/or affect the brain [9,10,12,14,16].

Given the role of IAIPs in immunomodulation and based on previous findings in the preclinical studies, there is a potential for IAIPs to be explored as a biomarker for HIE-related brain injury. There is an critical need to further elucidate mechanisms fundamental to the development of HIE and to establish the utility of IAIP levels to distinguish between infants with and without HIE. The validity of IAIPs as a biomarker will be evaluated in future research to discern its efficacy in the stratification of HIE by severity levels in order to gain insight into the relationship between IAIPs and neonatal encephalopathy severity in the clinical setting. We speculate also that in future work, administrating IAIPs could help restore depleted IAIP levels in the systemic circulation. This, in turn, might enhance natural immune defense mechanisms against oxidative stress and inflammation, possibly leading to a reduction in the severity of brain injury.

There are several limitations to our study and opportunities for future research. The number of neonates included in the study is relatively small, and a larger sample size could increase the generalizability of the findings. The small sample size (n = 4) in the “acute perinatal event” group was the result of convenience sampling. There was reliance upon the availability of eligible infants (convenience sampling) during the pre-specified control recruitment days, which could limit the generalizability of the findings to a broader population. Performing a power analysis or formal sample size calculations was not feasible because previous studies have not determined IAIP levels in human neonates.

The measures obtained in this study represented values at birth. IAIP levels most likely vary over time, in response to treatments including hypothermia, as a function of disease severity and the evolution of disease. The cord blood samples in this study represent a limitation, as cord blood composition may differ significantly from the values of postnatal sample as a result of changes in the immune status of the neonates, making it difficult to obtain precise conclusions regarding the long term neurological outcomes. Therefore, variations in the levels of IAIPs during the evolution of HIE and treatment with therapeutic hypothermia remains to be determined by future research. A subset of infants was examined at 2 years of

age, and small numbers and a type 2 error limit ability to test for significance and are only exploratory so they were not included in the main analysis. In addition, this research was conducted at a single medical center and needs to be replicated.

Nonetheless, the strength of the current study is that this is the first clinical study to examine the potential for IAIPs as a possible biomarker for HIE in neonates, suggesting a novel and promising avenue for research with implications for diagnosis and treatment.

5. Conclusion

In conclusion, the lower IAIP levels in infants with moderate/severe HIE compared with the control group, suggest a potential relevance of IAIPs in inflammation, particularly in the context of HIE. Based upon the positive findings from preclinical studies, and the reduced levels of IAIPs in infants with HIE, we speculate that further investigation into their potential diagnostic and therapeutic roles in the diagnosis and treatment of HIE is warranted.

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Data availability

Request for access to the data should be directed to the corresponding author with a reasonable request, and the data will be shared in accordance with applicable data protection and confidentiality guidelines.

Abbreviations:

ELISA	Enzyme Linked Immunosorbent Assay
HI	hypoxia ischemia
HIE	hypoxic-ischemic encephalopathy
IAIPs	inter-alpha inhibitor proteins
IQR	interquartile range
NEC	necrotizing enterocolitis
NICU	neonatal intensive care unit
ROS	reactive oxygen species
TH	therapeutic hypothermia

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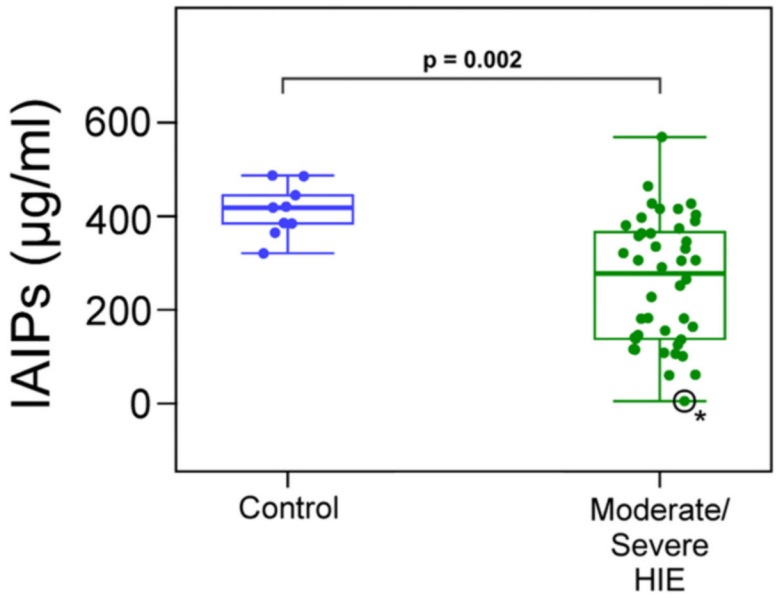
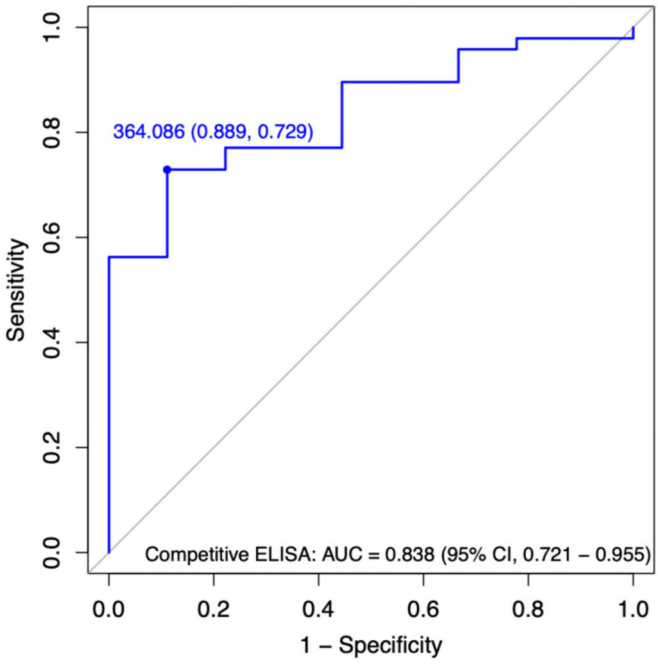


Fig. 1.
Cord blood IAIPs concentrations in control and moderate/severe HIE groups (concentrations in µg/ml).



Threshold of competitive ELISA	Sensitivity	Specificity	PPV	NPV
364.086	0.729	0.889	0.972	0.381

Fig. 2.

Comparison of IAIP levels of moderate/severe HIE neonates against control neonates using a ROC (receiver operator characteristics) curve analysis.

Table 1

Demographic and clinicopathological characteristics of mothers and their neonates diagnosed with moderate or severe HIE.

Maternal characteristics	**Reference control (n = 9)**	**Moderate/severe HIE (n = 44)**
Mode of delivery, No. (%)		
Cesarean	9 (100%)	33 (75%)
Vaginal	0 (0%)	11 (25%)
Age (years), mean ± SD	29 ± 5	29 ± 7
Gravida, mean ± SD	3 ± 1	3 ± 1
Gestational hypertension, No. (%)	0 (0%)	15 (34%)
Diabetes Mellitus, No. (%)	0 (0%)	11 (25%)
Clinical chorioamnionitis (>38 C), No. (%)	0 (0%)	10 (23%)
Placental abruption, No. (%)	0 (0%)	11 (25%)
Neonatal characteristics	**Reference control (n = 9)**	**Moderate/severe HIE (n = 44)**
Weight (g), mean ± SD	3464.0 ± 407.0	3250.8 ± 637.1
Gestational age (weeks), mean ± SD	39.3 ± 0.5	38.0 ± 2.0 (min = 36; max = 42)
Race, No. (%)		
White	7 (78%)	25 (57%)
Black	0 (0%)	10 (23%)
Asian	2 (22%)	3 (7%)
Latin	0 (0%)	6 (14%)
Sex, No. (%)		
Male	6 (67%)	26 (59%)
Female	3 (33%)	18 (41%)
Apgar score 1 min, mean ± SD	8.0 ± 1.0	3.0 ± 2.0
Apgar score 5 min, mean ± SD	9.0 ± 1.0	4.0 ± 3.0
Cord arterial pH, mean ± SD	7.23 ± 0.04	7.00 ± 0.10
Base Deficit (mmol/L), mean ± SD	6.0 ± 2.0	20.4 ± 10.1
Days in the hospital, mean ± SD	3 ± 1	25 ± 16