Early Biochemical Indicators of Hypoxic-Ischemic Encephalopathy after Birth Asphyxia

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ABSTRACT

Hypoxic-ischemic encephalopathy (HIE) after perinatal asphyxia is a condition in which serum concentrations of brain-specific biochemical markers may be elevated. Neuroprotective interventions in asphyxiated newborns require early indicators of brain damage to initiate therapy. We examined brain-specific creatine kinase (CK-BB), protein S-100, and neuron-specific enolase in cord blood and 2, 6, 12, and 24 h after birth in 29 asphyxiated and 20 control infants. At 2 h after birth, median (quartiles) serum CK-BB concentration was 10.0 U/L (6.0–13.0 U/L) in control infants, 16.0 U/L (13.0–23.5 U/L) in infants with no or mild HIE, and 46.5 U/L (21.4–83.0 U/L) in infants with moderate or severe HIE. Serum protein S-100 was 1.6 μg/L (1.4–2.5 μg/L) in control infants, 2.9 μg/L (1.8–4.7 μg/L) in asphyxiated infants with no or mild HIE, and 17.0 μg/L (3.2–34.1 μg/L) in infants with moderate or severe HIE 2 h after birth. No significant difference was detectable in serum neuron-specific enolase between infants with no or mild and moderate or severe HIE 2 and 6 h after birth. A combination of serum protein S-100 (cutoff value, 8.5 μg/L) and CK-BB (cutoff value, 18.8 U/L) 2 h after birth had the highest predictive value (83%) and specificity (95%) of predicting moderate and severe HIE. Cord blood pH (cutoff value, <6.9) and cord blood base deficit (cutoff value, >17 mM) increase the predictive values of protein S-100 and CK-BB. We conclude that elevated serum concentrations of protein S-100 and CK-BB reliably indicate moderate and severe HIE as early as 2 h after birth. (*Pediatr Res* **49: 502–506, 2001**)

Abbreviations:
HIE, hypoxic, ischemic encephalopathy
NSE, neuron-specific enolase
CK-BB, brain-specific creatine kinase (E.C. 2.7.3.2)
aEEG, amplitude-integrated continuous EEG

Perinatal asphyxia is a common cause of neonatal morbidity and mortality and neurologic disabilities among survivors. In addition to pulmonary, renal, and cardiac dysfunction, HIE develops in one third of asphyxiated newborns (1). Mild encephalopathy carries a good prognosis, although in moderate and severe encephalopathy the risk of death or neurologic sequelae increases greatly (2). Neuroprotective interventions are increasingly in the forefront of interest and have been shown to be effective in animal models. For clinical intervention, it is important to identify infants at a high risk for brain damage soon after birth and within the therapeutic window (3). Several indicators of brain damage have been investigated in the last decade (4–10). We examined the serum levels of three different brain proteins during the first day of life after birth asphyxia. Protein S-100 is a dimeric acidic calcium-binding protein constituting a major component of the cytosol of various cell types. Protein S-100B (ββ subunits) and S-100A1 (αβ subunits) are predominantly present in astrocytes and Schwann cells (11). NSE is the neuronal form of the intracytoplasmic glycolytic enolase. It is found in neuronal cell bodies, axons, and neuroendocrine cells and in neuroendocrine tumors (12). CK-BB is found in both neurons and astrocytes. CK-BB and NSE levels have been found to be elevated after birth asphyxia, but few data exist that concern the early time course of these factors after the hypoxic-ischemic event (13–17). Serum concentration of protein S-100 after birth asphyxia has not been assessed yet.

Our objectives were to investigate the postnatal time course of these markers in serum and to determine whether hypoxic-ischemic brain damage alters these markers and whether moderate or severe HIE can be predicted by elevated serum concentrations soon after birth.

METHODS

The study was performed from June 1998 to December 1999 and was approved by the Ethical Committee of the Medical Faculty, and written informed consent was given by the parents. We expected approximately one third of asphyxiated infants to exhibit moderate or severe HIE. Therefore, we decided to recruit approximately 30 infants in the asphyxia

group and 20 control infants. The protocol included two study groups.

Asphyxia group. Twenty-nine subsequently full-term newborn infants (gestational age, 37–42 wk) who fulfilled the following criteria were included in the study: arterial blood cord pH value <7.0, or arterial blood cord pH value between 7.01 and 7.1 and also an Apgar score after 5 min of <7. The asphyxia group was subdivided according to the clinical examination: no or mild HIE with a good prognosis, and moderate or severe HIE with a greater risk of neural handicap (18).

Control group. Twenty full-term infants who fulfilled all the following criteria were enrolled in the study: no maternal illness, arterial blood cord pH ≥7.2, after 5 min an Apgar score of >7, and an uneventful course during the first 3 d of life. Predefined exclusion criteria for both groups were congenital anomalies, tumors, maternal drug addiction, severe infections, and congenital metabolic disorders, but no infant was excluded. Clinical data are given in Table 1.

Study design. Blood samples were collected from cord blood and 2, 6, 12, and 24 h after birth. Standardized neurologic examinations were performed at 6, 12, 24, 48, and 72 h of age. Mild HIE was assumed according to Sarnat and Sarnat (19) if hyperexcitability or hypotonia persisted without seizures for ≥72 h after birth; moderate if the newborn was lethargic, had hypotonia, weak primitive reflexes, and seizures; and severe if the infant had apnea, flaccid weakness, frequent seizures, or coma.

Analysis of CK-BB, protein S-100, and NSE. Creatine kinase was determined at 25°C according to the optimized German standard method on Dax 72 (Bayer, Munich, Germany) or Modular PP (Roche, Mannheim, Germany) random assessment clinical analyzers. To quantify CK-BB, creatine kinase isoenzymes were fractionated electrophoretically on agarose gels, visualized by in-gel substrate reaction for fluorometric scanning using Helena (Greiner, Flacht, Germany) gel kits and rapid electrophoresis system. The area under the CK-BB curve was used to calculate its concentration.

Protein S-100 was measured manually with a sandwich type immunoluminometric assay kit (Byk Sangtec, Dietzenbach, Germany) that used MAb and an LB952 luminometer (Berthold, Wildbad, Germany). The assay uses three MAbs to detect the β chains in the $\beta\beta$ (S-100B) and $\alpha\beta$ (S-100A1) dimers. We have used the term protein S-100 for simplicity, which refers to both of these dimers.

NSE was measured on a Cobas Core II immunoanalyzer with the NSE ELISA II kit (Roche), a one-step sandwich type enzyme immunoassay that used two specific mouse MAbs. Free Hb, as an indicator of hemolysis, was quantified by bichromatic photometric measurement on Hitachi 911 or Modular PP analyzers (Roche). The manufacturer claims low detection limits of 0.1 μg/L for NSE and 0.02 μg/L for protein S-100. In 45 cases of low sample volumes, we reduced the amount of serum for protein S-100 assay from 100 μL to 25 μL, and 0.08 μg/L as the lower detection limit. Least square regression analysis for 18 samples were performed with both the original and the diluted protocol, and gave y (25 μL) = 1.019 × x (100 μL) − 0.04 with a regression coefficient of 0.98. Time required for the measurement of CK-BB, protein S-100, and NSE was 0.5, 3.5, and 0.75 h, respectively.

Data analysis. All values were presented as medians and interquartile ranges. Group comparisons were performed with the Mann-Whitney U test. Positive predictive value, negative predictive value, sensitivity, and specificity for development of moderate or severe asphyxia were obtained using optimal cutoff levels and were calculated on the material used in our study. Receiver operating characteristic curves were assessed using the areas under the curves. Correlations were calculated by the Spearman rank method. For statistical analysis, the SPSS 5.02 for Windows (SPSS, Chicago, IL, U.S.A.) was used. Probability values <0.05 were considered to be significant.

RESULTS

Birth weight, gestational age, sex, and mode of delivery were similar among the control infants, infants with asphyxia without or with mild HIE, and asphyxiated newborns with moderate or severe HIE (Table 1). Of the 29 newborns with asphyxia, seven did not develop HIE but 22 did. Fifteen newborns had mild, four moderate, and three severe HIE. Both

Table 1. *Characteristics of 29 infants with asphyxia and 20 control infants*

| Characteristics | Control group $n = 20$ | Infants with asphyxia | | p value |
		No or mild HIE $n = 22$	Moderate or severe HIE $n = 7$	
Birth weight (g)	3318 (2962–3886)	3540 (3070–3800)	3155 (2625–3640)	NS*
Gestational age (wk)	40 (38–42)	40 (39–41)	40 (38–40)	NS*
Sex (M/F)	6/14	13/9	2/5	NS*
Mode of delivery				NS*
Cesarean section	10	9	4	
Vaginal spontaneous	6	8	2	
Vacuum extraction	4	5	1	
Inborns/outborns	18/2	15/7	1/6	0.026†
Apgar score (1 min)	8 (7–9)	4 (2–7)	2 (1–2)	0.01†
Apgar score (5 min)	9 (8–10)	6 (5–8)	5 (3–5)	0.03†
Arterial cord blood pH	7.25 (7.22–7.28)	6.98 (6.87–7.01)	6.75 (6.62–6.90)	0.008†
Arterial cord blood base deficit	5 (3–6)	13 (11–16)	22 (17–28)	0.03†

Results are presented as medium (quartiles).

* p values are for comparison between control infants ($n = 20$) and infants with asphyxia ($n = 29$). † p values are for comparison between infants with no or mild HIE and moderate or severe HIE.

groups with asphyxia differed in Apgar scores at 1 and 5 min, arterial blood cord pH, and base deficit (Table 1). Two asphyxiated infants with severe HIE died; one within 12 h because of multiorgan failure, the other at d 26 with grade 4 intraventricular hemorrhage and renal and cardiac dysfunction. The third infant with severe HIE suffered from meconium aspiration and needed artificial respiration for 4 d. No artificial respiration was necessary in the four infants with moderate HIE, but one of them had renal failure.

CK-BB. At every time interval there was a significant difference ($p < 0.05$) in serum CK-BB concentrations between the asphyxia and the control groups (Table 2). Serum CK-BB was significantly higher in infants with moderate or severe HIE when compared with infants with no or mild HIE 2, 6, and 12 h after birth (Table 2). No difference was found between the two asphyxia groups in cord blood and 24 h after birth.

Protein S-100. At every time interval there was a significant difference ($p < 0.05$) in serum protein S-100 concentrations between the asphyxia group and the control group (Table 2). Serum protein S-100 was significantly higher in infants with moderate or severe HIE when compared with infants with no or mild HIE 2 and 6 h after birth (Table 2). No difference was observed between the two asphyxia groups at birth and 12 and 24 h after birth.

NSE. At any time interval we could not detect a significant difference in serum NSE concentrations between the control group and the asphyxiated infants (Table 2). However, serum NSE was higher in infants with moderate or severe HIE compared with infants with no or mild HIE 12 and 24 h after birth (Table 2). No difference was found between the two asphyxia groups at birth and 2 and 6 h after birth. Blood hemolysis increased the serum NSE levels, but no significant difference in hemolysis was detectable in the different groups.

The predictive capacities for serum CK-BB, protein S-100, NSE, pH, base deficit, and Apgar score (at 1 min) for HIE of moderate or severe degree are shown in Table 3. Combinations of biochemical measurements and combinations of biochemical markers with cord blood pH, cord blood base deficit, and Apgar score at 1 min are given in Table 4. The combination of serum protein S-100 (cutoff value, 8.5 μg/L) and CK-BB (cutoff value, 18.8 U/L) at 2 h had the highest positive predictive value and specificity (Table 4) for biochemical markers. A combination of serum protein S-100 at 2 h after birth with cord blood pH (cutoff value, <6.9) or cord blood base deficit (cutoff value, >17 mM/L) increased the positive predictive value and specificity. Correlation between the serum concentrations of protein S-100 and CK-BB at 2 h are given in Figure 1 ($\rho_s = 0.66$; $p < 0.001$).

DISCUSSION

In adults, neuronal necrosis and apoptosis after global ischemia are slow, and last for several hours to several days (20). Studies in perinatal animals suggest a quicker cellular destruction (3). It is not known how long the window of opportunity remains open for intervention, but any intervention will be more successful early after the insult (21). Energy substrates in the neonatal brain continue to run down for 12 to 48 h after hypoxia (22). Therefore an intervention might be effective 2 to 6 h after birth asphyxia. As neuroprotective interventions may be harmful (23), it is important to find early and reliable indicators of brain damage or of poor long-term prognosis to initiate or end neuroprotective treatment. Cranial tomography, somatosensory evoked potentials, and magnetic resonance tomography are useful for prognosis, but not in the first 24 h after birth (24, 25). Magnetic resonance spectroscopy reveals brain energy compromise (6), but is not practicable in most clinical situations. aEEG is a useful diagnostic tool for assessing encephalopathy. Previous reports (4, 5) established a high predictive value of postnatal aEEG for neurologic outcome.

Several studies measured biochemical factors in serum and cerebrospinal fluid glial fibrillary acidic protein after 12–48 h

Table 2. *Serum concentrations of CK-BB, protein S-100, and NSE at different times after asphyxia*

Biochemical markers (median/interquartiles)	Control group	Infants with asphyxia		*p* value*	*p* value†
		No or mild HIE	Moderate or severe HIE		
CK-BB (U/L)					
Cord blood	3.0 (1.75–11.5)	24.5 (7.5–48.5)	15.0 (14.0–40.0)	0.005	NS
2 h	10.0 (6.0–13.0)	16.0 (13.0–23.5)	46.5 (21.4–83.0)	<0.0001	0.003
6 h	7.0 (4.3–8.8)	10.4 (6.0–16.0)	27.0 (18.0–55.0)	0.002	0.002
12 h	5.0 (4.0–6.8)	12.5 (6.0–15.6)	33.3 (18.1–52.0)	<0.0001	0.012
24 h	5.0 (3.0–6.8)	6.5 (4.0–14.0)	9.5 (7.0–17.5)	0.028	NS
Protein S-100 (μg/L)					
Cord blood	0.8 (0.7–1.0)	1.5 (1.1–1.9)	2.5 (1.5–3.7)	<0.0001	NS
2 h	1.6 (1.4–2.5)	2.9 (1.8–4.7)	17.0 (3.2–34.1)	<0.0001	0.008
6 h	1.5 (1.2–2.3)	2.5 (1.6–3.8)	27.6 (2.6–52.3)	0.001	0.015
12 h	1.2 (1.1–1.5)	1.8 (1.5–2.3)	3.1 (1.3–23.1)	0.001	NS
24 h	1.0 (0.9–1.4)	1.6 (1.0–2.6)	3.9 (1.2–9.0)	0.04	NS
NSE (μg/L)					
Cord blood	29.6 (17.8–55.9)	48.9 (20.1–74.7)	106.8 (60.5–108.1)	NS	NS
2 h	30.3 (24.8–47.6)	32.5 (20.8–60.7)	60.8 (49.3–89.0)	NS	NS
6 h	37.1 (19.0–48.8)	35.6 (30.1–70.3)	52.7 (48.7–79.3)	NS	NS
12 h	28.7 (19.8–39.4)	34.0 (24.6–48.7)	54.3 (46.8–78.8)	NS	0.028
24 h	24.3 (17.2–39.5)	34.8 (21.4–44.4)	50.7 (41.8–70.9)	NS	0.033

* *p* values are for comparison between all asphyxiated infants and controls. † *p* values are for comparison between asphyxiated infants with no or mild HIE and infants with moderate or severe HIE.

Table 3. *Values for predicting moderate or severe HIE*

Variable	Cutoff value	PPV (%)	NPV (%)	Sens (%)	Spec (%)	AUC
Protein S-100						
2 h	8.5 μg/L	71	90	71	90	0.832
6 h	4.6 μg/L	63	90	71	86	0.805
CK-BB						
2 h	18.8 U/L	46	100	100	65	0.879
6 h	17.0 U/L	55	94	86	77	0.877
NSE						
2 h	44 μg/L	46	93	83	68	0.768
6 h	46 μg/L	42	93	83	65	0.763
Arterial cord blood pH	<6.9	46	89	71	73	0.838
Cord blood base deficit	>17 mM/L	50	94	83	77	0.905
Apgar score (1 min)	<3	50	94	73	86	0.825

Abbreviations: PPV, positive predictive value; NPV, negative predictive value; Sens, sensitivity, Spec, specificity; AUC, area under the curve. It is important that markers demonstrate high specificity and high positive predictive value.

Table 4. *Combination of factors for predicting moderate or severe HIE*

Variable	PPV (%)	NPV (%)	Sens (%)	Spec (%)
Protein S-100 and CK-BB	83	91	71	95
CK-BB and cord blood pH	71	91	71	91
CK-BB and cord blood base deficit	67	91	67	91
CK-BB and Apgar score (1 min)	83	96	83	95
Protein S-100 and cord blood pH	100	88	57	100
Protein S-100 and cord blood base deficit	100	92	67	100
Protein S-100 and Apgar score (1 min)	80	88	57	96

Abbreviations: PPV, positive predictive value; NPV, negative predictive value; Sens, sensitivity; Spec, specificity. Values of serum factors are at 2 h after birth. Cutoff values are given in Table 3.
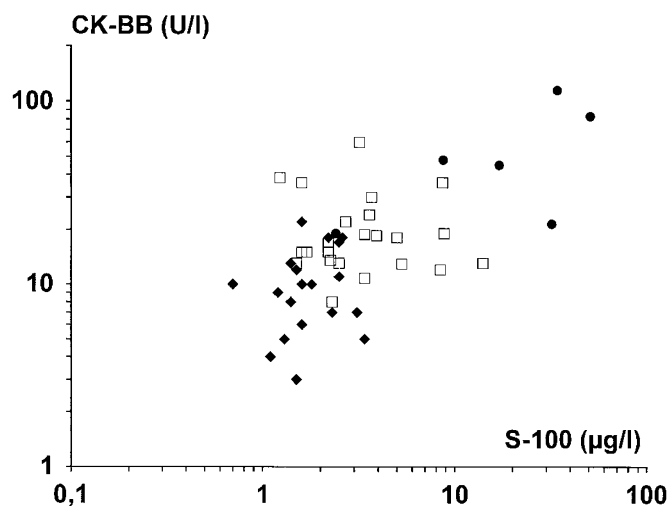

Fig. 1. Correlation of serum CK-BB and protein S-100 in 45 infants at an age of 2 h. Measurements for control infants (♦), asphyxiated infants with no or mild HIE (□), and asphyxiated infants with moderate or severe HIE (●) are shown.

(7); excitatory amino acids after 18–66 h (8); and IL-6 12 h after the hypoxic-ischemic event (9). Urinary lactate:creatine ratio predicts HIE within 6 h with ^{1}H nuclear magnetic resonance spectroscopy (10), but a useful indicator for HIE should be specific even earlier, and requires a rapid and readily available laboratory technique. Besides this, infants with asphyxia often have oliguria, and urine sampling may not be possible (26). Our results do not confirm serum NSE as an early predictor of HIE. As late as 12 h after birth, serum concentrations of NSE increased significantly in the infants with moderate and severe HIE when compared with infants with no or mild HIE. Results of other studies (16, 17) with serum NSE in asphyxiated newborns are in concordance with our data, whereas NSE in cerebrospinal fluid seems to be more favorable.

Concerning CK-BB, these results are in accordance with studies where the increase was within the first 4–15 h of life (13–15). Our serum CK-BB activities were lower than in the studies of Walsh *et al.* (14) and Fernandez *et al.* (15), which was probably related to the measurement of total creatine kinase activity at different temperatures. CK-BB is also expressed in placenta, lungs, gastrointestinal tract, and kidneys, all of which might be involved in the sequelae of asphyxia. Although the placenta is rich in CK-BB activity, it seems that its contribution to serum CK-BB levels in the neonate is negligible (27). Cuestas (13) found that the blood CK-BB levels were not increased in neonates with renal or gastrointestinal tract disorders. Therefore, we assume that the increased levels originate mainly from the brain of the asphyxiated infants.

Protein S-100 is also known to be present in the striated muscle, heart, kidneys, adipocytes, and thymus of newborns (28, 29). Until now, no study has examined the serum values of protein S-100 in asphyxiated and age-related control infants. In adults, protein S-100 is not detectable in serum under normal conditions (30); however, in cerebral diseases it ranged from zero (31) to highly predictive values (32). Additionally, clinical studies in adults evaluated different time patterns for the increase of serum protein S-100, such as transient increase after cardiac operations (33), peak level within 24 h after cerebral hemorrhage (34) or global cerebral ischemia (32), and a peak level at d 3 after an acute ischemic stroke (34, 35). In the serum of infants, protein S-100 has been determined after cardiac

operations and extracorporeal circulation, demonstrating age-related concentrations that were highest in neonates and infants with Down's syndrome with a pattern of transient increase similar to adult patients (36). In asphyxia, serum protein S-100 release follows a pattern similar to the transient increase observed after cardiac operations. We postulate that in case of asphyxia, elevated levels in serum are caused by leakage of protein S-100 into the extracellular fluid after hypoxic damage of glial cell and neuronal membranes and by lower renal excretion. The integrity of the blood–brain barrier seems to influence its levels in serum, which explains why serum protein S-100 concentration is lower in adults than in infants (36). Recent studies detected transient serum elevations of protein S-100 without any relationship to permanent neuronal damage, and the question has arisen as to whether protein S-100 arises from noncerebral sources (37, 38).

We could not exclude the possibility that protein S-100 and CK-BB release comes only from the brain. In regard to this, it might be a simple epiphenomenon of general ischemia related to asphyxia. Asphyxia may involve the whole body, and the release of proteins into the blood might be a general sign of change in cell membrane integrity and vascular permeability caused by the whole body ischemic-reperfusion injury. In our opinion, no single diagnostic marker should form the basis for decisions on neuro-protective therapy. But the decision as to which infants could be candidates for postasphyxial treatment should probably be based on several findings, which include aEEG, cord blood pH, cord blood base deficit, Apgar score, and serum protein S-100 and CK-BB. These biochemical markers may be helpful in deciding whether an early initiated neuroprotective therapy should be continued or stopped. However, our data, obtained from a small number of infants, refer to HIE and not permanent brain damage. Neurodevelopment follow-up studies in our infants in the next few years will show whether elevated serum protein S-100 and CK-BB will also predict developmental delay.

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