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NSE and S100 after Hypoxia in the Newborn Pig

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ABSTRACT

Perinatal asphyxia is an important cause of neonatal morbidity and mortality. There is the potential to halt cerebral damage if neural rescue strategies are applied within a short period of time after an insult. It is therefore important to be able to accurately identify neonates who may benefit from neural rescue therapies. Recent studies in asphyxiated neonates have correlated S100B and NSE with outcome; however, interpretation of these studies were difficult, as the timing of the measurements were not consistent. We measured NSE and S100 in 1-d-old piglets after a mild or severe hypoxic insult. Measurements were performed at 6–72 h after the insult and correlated with histologic outcome. There were no differences of the NSE or S100 concentrations between controls and the mild hypoxia group. After 24 h, there was a significant difference of NSE between the control/mild insult group and severe insult group. After 48 h, the S100 concentrations were significantly different between the control/mild insult group and the severe insult group. Both proteins showed good correlation at these time points with outcome as measured by histology score at 72 h. In conclusion, NSE and S100B measured in the serum of piglets after hypoxia increased significantly and correlated with outcome. This increase occurs too late to be used within the first 24 h but might be helpful for the clinician in determining the timing of an insult. **(*Pediatr Res* 58: 953–957, 2005)**

Abbreviations

CFM, cerebral function monitor
CSF, cerebrospinal fluid
IRMA, immunoradiometric assay
NSE, neuron-specific enolase

Perinatal asphyxia occurs in 1 in 500 deliveries and is an important cause of neonatal morbidity and mortality. There is the potential of neural rescue therapies to halt cerebral damage when applied within a "window of opportunity" of limited duration. It is therefore especially important to be able to accurately identify those neonates who may benefit from such interventions (1). The early and accurate prediction of the severity of brain damage remains difficult.

Several brain-specific proteins have been measured in CSF and serum and correlated with outcome after hypoxic/ischemic reperfusion injury (1–4). S100 is an astroglial specific protein with calcium binding capacity. It is a homo- or heterodimer consisting of two subunits, alpha and beta. S100$\beta\beta$ is present in high concentrations in glial and Schwann cells, S100$\alpha\alpha$ in glial cells, and S100$\alpha\beta$ in striated muscle, heart, and kidney (5). NSE is an isoenzyme of the glycolytic enzyme enolase and is present in high concentrations in neuronal cell bodies, axons, and in cells of neuroendocrine origin (6). After neuronal injury, S100B (S100$\beta\beta$) and NSE pass into the CSF and across the blood-brain-barrier (6,7). Recent studies in asphyxiated neonates have correlated S100B and NSE with outcome (3,4,8,9). The results correlated with outcome but measurements had been taken at differing and unknown times after insult and from different bodily fluids (serum or CSF). As the probable beginning and duration of the insult in neonates is often uncertain, correlations with outcome are difficult to interpret as the timeline of the increase of NSE or S100 after an insult is not known.

The aims of the current study were to measure NSE and S100B at various times in serum of piglets subjected to hypoxia and to determine whether NSE and S100B are early predictors of outcome.

METHODS

Animal preparation. The study was approved by the University of Queensland Animal Experimentation Ethics Committee. Experiments were carried out in accordance with the Australian National Health and Medical Research Council Guidelines. Adequate measures were taken to minimize pain or discomfort during the experimental procedures. Seventeen term piglets (Large White × Landrace) were studied on d 1 after birth, after a period of suckling.

Anesthesia was induced with 1–2% halothane (Rhône Mérieux, Melbourne, Australia) in air. A 24-gauge catheter was inserted into an ear vein and an induction dose of propofol (5 mg/kg) administered. A mixed infusion of propofol (10 mg/kg/h) and alfentanil (55 μg/kg/h) was used to maintain anesthesia until the piglet was intubated with a size-3 cuffed endotracheal tube.

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The piglet was then ventilated with a neonatal ventilator (Bearcub, Bourns, CA) to maintain oxygen saturation above 95% and arterial Pco_2 at 30–50 mm Hg. The infusion rate of the intravenous anesthesia was then increased to deliver propofol at 20 mg/kg/h for 15 min, 15 mg/kg/h for 15 min, then 10 mg/kg/h for the remainder of the anesthetic. A 24-gauge cannula was inserted into a mammary vein and 10% glucose infused at 3 mL/kg/h. The umbilical artery was cannulated using a 3.5F neonatal umbilical catheter (Argyle, Sherwood Medical Co., St. Louis, MO). Temperature was measured rectally and maintained at 39.0 ± 0.5°C, which is the normal temperature for piglets. EEG was monitored using a cerebral function monitor (CFM; Lectromed Devices Ltd., Letchworth, Hertfordshire, UK).

After a standardized stabilisation period (120 min after induction of anesthesia), the oxygen intake was decreased to 3–7% as required to suppress the EEG to $<5\mu V$. A mild insult was defined as 20 min of suppressed EEG and a severe insult was defined as 40 min of suppressed EEG. The piglets subjected to a severe insult also had a minimum of 10 min of hypotension (mean arterial blood pressure <70% of prehypoxia). The mean arterial blood pressure was controlled by manipulating the inspired oxygen within the range of 3–7%. A brief increase in Fio_2 prevented hypotension, a decrease resulted in hypotension. Hypotension was not caused by blood withdrawal but was a direct result of the hypoxic insult.

Piglets were then reoxygenated with 100% oxygen, and the metabolic acidosis half corrected with 8.4% sodium bicarbonate given intravenously. The anesthesia was discontinued, the ventilation weaned according to blood gas analysis, and the piglet extubated. The arterial and venous lines removed because the piglets were housed together in a cage and more active animals would bite through the indwelling their catheters as well as those of the other piglets. Piglets were then nursed in a warmed cage, fed *via* an orogastric tube every 2–3 h with piglet milk formula (Survive, Pig Milk Replacer, Aus Vac, Bendigo, Australia). A carer observed the piglets for 72 h. The piglets were assessed every 8 h for 24 h and at 48 and 72 h using a neurology score as described by Thoresen *et al.* (10). The scoring system consists of nine neurologic items that are scored from 0 (definitely pathologic) to 2 (normal) and added up to a maximum score of 18 for a normal piglet. Piglets were also monitored at these time points using a CFM. Seizures were defined as rhythmic pathologic movement of the limbs or repetitive spike wave activity on the CFM. Clinical and electrographic seizures were treated with diazepam (DBL, Mulgrave, Australia) and phenobarbitone (DBL) if they occurred. Control animals were subjected to the same procedures and length of anesthesia as the hypoxic piglets except for the hypoxic insult.

Blood for measurement of NSE and S100B was taken before to the insult and at 6, 12, 24, 48, and 72 h after the insult. The piglets were anesthetized with halothane for the purpose of blood sampling and CFM measurement. The blood was allowed to clot for 30 min at room temperature, centrifuged at 3000 rpm for 10 min, and the serum frozen at −20°C until the assay was performed. Commercially available radioimmunoassays for S100B (Sangtec S100 IRMA, Sangtec Medical, Bromma, Sweden) and NSE (Prolifigen NSE IRMA, Sangtec Medical) were used. Each measurement was performed in duplicate according to the manufacturer's recommendations, and the averages are reported. The sensitivities of the assays are reported by the manufacturers to be 0.1 and 0.5 μg/L, respectively.

In this study, histopathological changes visible 72 h after the insult were used as the outcome measurement. At 72 h posthypoxia, the piglets were killed and the brains perfused *via* both carotid arteries with warmed heparinized saline and then 4% paraformaldehyde. After this, the brains were removed, cut at 3–4 mm intervals into coronal sections, embedded in paraffin, and then cut for histologic examination into 8-μm sections. The first, 50th and 100th section from either hemisphere from the cortex, hippocampus, basal ganglia, thalamus, cerebellum, and medulla were stained with hematoxylin/eosin. The extent of damage for each brain region was graded as previously described (11) at 72-h post insult. Cell death was graded from 0 to 9 in each brain region. Each grade reflected the percentage of damage in three ascending categories (Table 1). The categories were neuronal necrosis, laminar/focal necrosis, and confluent infarct. The highest possible score for the five brain regions was 45, representing maximal damage (confluent necrosis in all areas).

Statistics. All values are presented as mean and SD as they were normally distributed unless otherwise indicated. Comparisons between groups were performed using one-way ANOVA with Bonferroni correction for repeated testing or Kruskal-Wallis test if not normally distributed. Pearson's correlation coefficient was used to test for correlation between variables. Probability values <0.05 were considered to be significant.

RESULTS

Five control animals, five subjected to a mild insult, and seven subjected to a severe insult were studied. There were no differences in the birth weights or postnatal ages of the groups. Table 2 outlines the physiologic parameters of all piglets before and after the insult, *i.e.* at the beginning of resuscitation. One piglet died from presumed septicemia 60 h after the insult, four in the severe insult group had clinical seizures, and no piglet had electrographical seizures that were not detected clinically. All of the control animals and the animals exposed to a mild insult recovered quickly and demonstrated normal behavior 24 h post hypoxia. Five of the animals that underwent a severe insult were neurologically abnormal, *i.e.* were not able to walk or feed within the first 12 h (Table 3).

Histology. All control animals and the animals exposed to a mild insult had normal histology 72 h after the insult. In contrast, none of the animals exposed to a severe insult had normal histology at that time point. All demonstrated moderate-to-severe changes such as necrosis and apoptosis. The cortex showed the most severe changes with confluent infarction. The hippocampus was affected consistently by neuronal necrosis and the basal ganglia and thalamus showed focal areas of neuronal necrosis. The combined histology score of the in the severe insult group was significantly higher than that of the control or mild insult group (Fig. 1) The mean (SD) histology score for the control group was 0 (0), for the mild insult group

Table 1. *Grading scale for histopathological changes 72 hours after a hypoxic insult in neonatal piglets*

Changes in morphology	% of area affected	Grade
No damage	0	0
Neuronal necrosis	<20	1
	20–50	2
	>50	3
Laminar necrosis	<20	4
	20–50	5
	>50	6
Confluent infarct	<20	7
	20–50	8
	>50	9

Table 2. *Physiologic parameters of piglets before and at the end of insult*

	Controls (n = 5)	Mild insult (n = 5)	Severe insult (n = 7)
age (hours)	16.6 (10.9)	10 (4.0)	12.8 (7.6)
weight (kg)	1.2 (0.26)	1.6 (0.2)	1.6 (0.3)
pCO_2 before insult (mmHg)	35.7 (0.8)	36.8 (8.4)	39.7 (8.2)
BP before insult (mmHg)	59.3 (1.7)	57.6 (7.5)	58.1 (4.1)
BP at end of insult (mmHg)	62.9 (2.3)	56.9 (8.5)	46.3 (5.9)*,§
min BP at end of insult	55.6 (3.2)	35.5 (4.2)	29.2 (4.4)*,§
HR before insult	180.8 (43.8)	170.15 (43.5)	170.6 (22.2)
HR at end of insult	182.7 (32.7)	202.5 (25.6)	183.3 (17.9)
min HR at end of insult	138.2 (36.9)	107.7 (19.5)	125.8 (21.9)
SaO_2 at end of insult	86.6 (14.9)	42.2 (10.7)*	37.0 (8.7)*,§
pO_2 at end of insult	121.9 (21.9)	42.13 (44.5)*	17.56 (3.9)*,§
pH at end of insult	7.41 (0.2)	7.07 (0.4)*	6.9 (0.16)*
BE at end of insult	5.6 (7.5)	−12.8 (4.9)*	−19.3 (3.5)*,§

numbers are mean (SD)

BP: blood pressure, HR: heart rate in beats per minute, SaO_2: oxygen saturation, BE: base excess

*: $p < 0.05$ vs control group

§: $p < 0.05$ vs mild insult group

Table 3. *Results of the neurology and histology scores in piglets up to 72 hours after a hypoxic insult*

	Controls (n = 5)	Mild insult (n = 5)	Severe insult (n = 7)
neuroscore at 12 hours	16.0 (4)	13.5 (1.3)	9.67 (3.2)
neuroscore at 24 hours	18.0 (1.7)	17.3 (1.5)	12.33 (5.3)*
neuroscore at 72 hours	18.0 (2.2)	17.2 (1.5)	14.5 (5.2)
histology score at 72 hours	0 (0)	0.5 (1)	13.63 (6.8)*,§

numbers are mean (SD)
*: $p < 0.05$ vs control group
§: $p < 0.05$ vs mild insult group

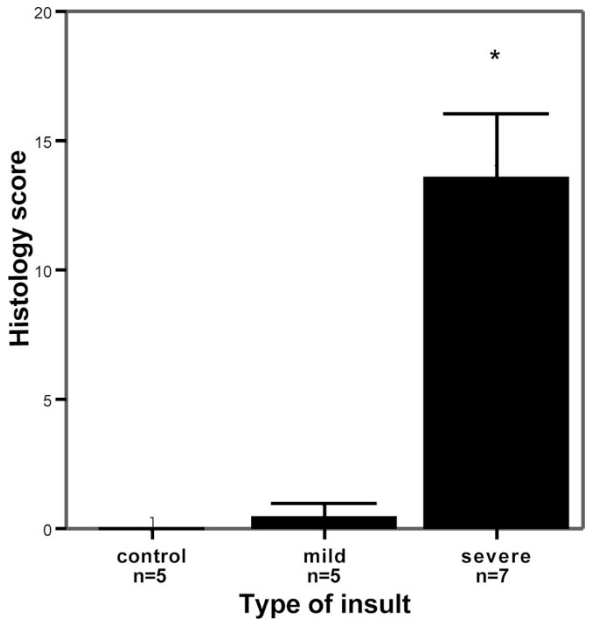

Figure 1. Histology score 72 h after hypoxia in newborn piglets. Numbers are given as median ± SEM. *$p < 0.01$ compared with control and mild hypoxia.

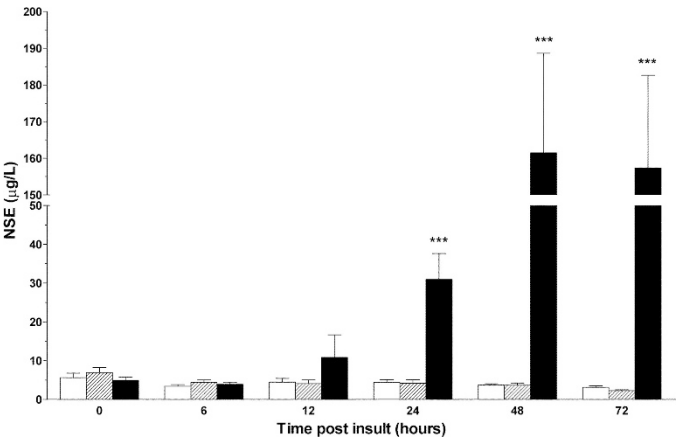

Figure 2. Serum concentrations of S100 measured in newborn piglets after hypoxia. Numbers are given as median ± SEM. **$p < 0.001$ compared with control and mild hypoxia. Control animals (*white bars*, $n = 5$); animals subjected to mild hypoxia (*shaded bars*, $n = 5$); animals subjected to severe hypoxia (*black bars*, $n = 7$).

0.5 (1), and for the severe insult group 13.6 (6.8), $p < 0.05$ (control *versus* severe and mild *versus* severe).

NSE. Concentrations before the insult were not different among the three groups. There were no statistically significant differences in the concentrations of the control animals when compared with the mild hypoxia group at any time point (Fig. 2). After 24 h, however, there was a significant difference between the concentrations of the control/mild insult group and severe insult group. This difference persisted up to 72 h.

The correlation of the histology score with NSE concentrations at the different time points supported above results. At 24 h the correlation coefficient (r) was 0.89, the coefficient of determination (r^2) was 0.8; at 48 h, $r = 0.95$ and $r^2 = 0.9$; and at 72 h, $r = 0.97$ and $r^2 = 0.94$ (Fig. 3).

In the group of piglets subjected to a severe insult there were no differences in the NSE concentrations between the piglets with seizures and those that did not have seizures. In that group there were also no differences in the NSE concentrations when comparing the piglets with higher neurology scores to those with lower, very abnormal neurology scores.

S100B. Concentrations before the insult were not different among the three groups. There were no statistically significant differences in the concentrations of the control animals when compared with the mild hypoxia group at any time point (Fig. 4). From 48 h after insult, there was a significant difference between the concentrations of the control/mild insult group when compared with severe insult group. This difference was still present at 72 h.

Correlating the histology score with 100B concentrations gave the following results: At 48 h the correlation coefficient (r) was 0.9, the coefficient of determination (r^2) was 0.8; and at 72 h, $r = 0.81$ and $r^2 = 0.64$ (Fig. 5).

Comparing the piglets subjected to a severe insult with seizures to those subjected to a severe insult without seizures, there were no differences the S100B concentrations. There were also no differences in the S100B concentrations when comparing the piglets with higher neurology scores to those with lower, very abnormal neurology scores in that severe insult group.

DISCUSSION

This study outlines the time course of NSE and S100B in serum after a hypoxic period in a newborn animal model. The piglet was chosen, as it is similar to the term human infant in size, development, and cerebral maturation (12). The amino acid sequences of the porcine NSE and S100B are not known, but the protein is well preserved between species (13–15). The baseline values of NSE and S100β in the piglets were comparable to the values found in term neonates in cord blood (3). This suggests sufficient similarity between the human and the porcine proteins for the RIA to measure porcine proteins accurately.

Elevations of NSE and S100B have been found in various forms of acute brain damage in adult patients. Measured in CSF, they are sensitive markers for brain damage after head trauma (2), ischemic stroke, and cerebral hypoxia (7,16). When measured in serum, both show good correlation with outcome after ischemic stroke and cardiac arrest (17–20).

NSE measured in amniotic fluid of women in preterm labor has been found to correlate with intraventricular hemorrhage or

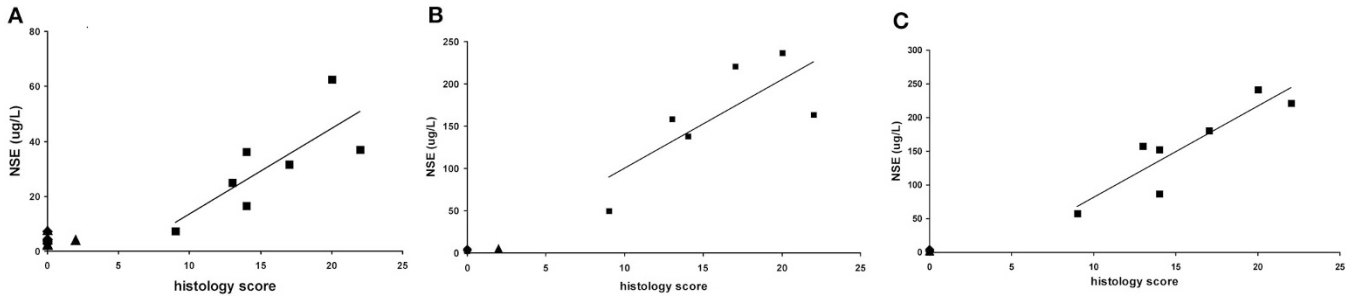

Figure 3. Correlation between serum concentrations of NSE and histology score in newborn piglets: (*A*) 24 h after a hypoxic insult, $R^2 = 0.8$, (*B*) 48 h after a hypoxic insult, $R^2 = 0.9$, and (*C*) 72 h after a hypoxic insult, $R^2 = 0.94$. Control animals (*diamonds*, $n = 5$); animals subjected to mild hypoxia (*triangles*, $n = 5$); animals subjected to severe hypoxia (*squares*, $n = 7$).

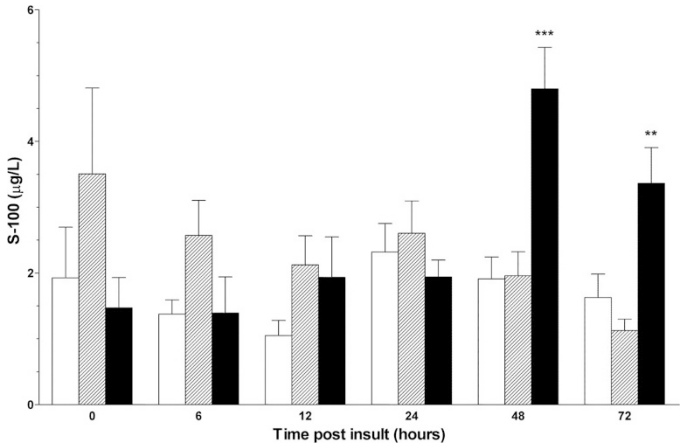

Figure 4. Serum concentrations of S100B measured in newborn piglets after hypoxia. Numbers are given as median ± SEM. $*p < 0.01$ compared with control and mild hypoxia; $**p < 0.001$ compared with controls and mild hypoxia. Control animals (*white bars*, $n = 5$); animals subjected to mild hypoxia (*shaded bars*, $n = 5$); animals subjected to severe hypoxia (*black bars*, $n = 7$).

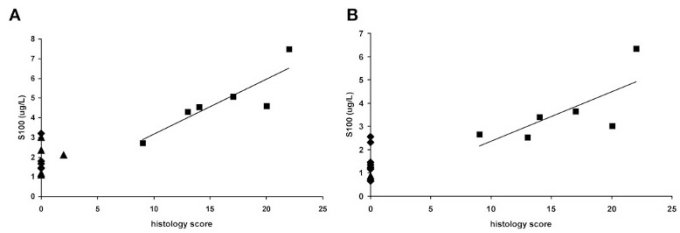

Figure 5. Correlation between serum concentrations of S100B and histology score in newborn piglets: (*A*) 48 h after a hypoxic insult, $R^2 = 0.8$ and (*B*) 72 h after a hypoxic insult, $R^2 = 0.64$. Control animals (*diamonds*, $n = 5$); animals subjected to mild hypoxia (*triangles*, $n = 5$); animals subjected to severe hypoxia (*squares*, $n = 7$).

periventricular leukomalacia (21). S100 measured in preterm infants, may be an indicator of white matter lesions (22). In the pediatric population, S100 has been measured after cardiac surgery and correlated with cerebral injury (23).

Concentrations of S100B and NSE have been measured in the CSF of neonates following perinatal asphyxia in three studies. Garcia-Alix *et al.* measured NSE in CSF from 69 asphyxiated term newborn infants 12 and 72 h after birth (8). NSE at both time points correlated well with degree of encephalopathy and neurodevelopmental outcome at 1 y of age. Thornberg *et al.* (1) used CFM and measured NSE in CSF of 22 asphyxiated term infants between 2 and 96 h after birth. In 15 of these infants, NSE was also measured in serum between 2 and 64 h after birth, and in both CSF and serum simultaneously in 10 infants. NSE in CSF correlated well with degree of encephalopathy and CFM pattern. Blennow *et al.* (4) recently measured NSE and S100B in CSF of 22 asphyxiated infants between 6 and 89 h after birth. Both proteins correlated with degree of encephalopathy as well as outcome at 4 y of age.

However, collecting CSF samples by lumbar puncture in a sick neonate may be technically difficult and may lead to hemodynamic compromise. Urine or blood sampling is associated with less risk to the infant and allows more frequent testing to monitor the course of the injury. Gazzolo *et al.* (24) measured S100B in the urine of 44 asphyxiated infants at 4–72 h of birth and compared with healthy controls. S100B concentrations in moderately/severely asphyxiated neonates were significantly higher than in controls and mildly asphyxiated infants. Verdu *et al.* (9) determined NSE in serum of 25 asphyxiated infants between 24 and 72 h after birth. Median follow-up time of these infants was 3.5 y. NSE levels were significantly higher in infants with encephalopathy who developed neurologic sequelae when compared with infants with normal outcome. Nagdyman *et al.* (3) assessed NSE and S100B in serum of 29 asphyxiated infants in cord blood and 2, 6, 12, and 24 h after birth. Serum S100B was found to be significantly higher in neonates with severe or moderate encephalopathy at 2 and 6 h after birth when compared with infants with no or mild encephalopathy. Serum NSE was significantly higher in infants with moderate or severe encephalopathy at 12 and 24 h when compared with infants with no or mild encephalopathy. However, the NSE and S100B levels did not correlate with long-term neurologic outcome. In the present study, the outcome measure used was histology. This is an objective tool to assess cerebral damage. The NSE and S100B levels showed good correlation with histology, indicating that the significant increase in the piglets subjected to a severe hypoxic period is caused by severe neuronal damage with subsequent release of these proteins. The levels measured were comparable to those reported by other authors in asphyxiated neonates (3,8,9) but were lower than those reported in adult patients following stroke (17) or cardiac arrest (18,19). There are several possible reasons for this: Firstly, the immature, newborn brain contains less glial and axonal mass and myeli-

nation (25), which may account for the observed lower serum concentrations of NSE and S100. Secondly, in ischemic adult stroke, damage generally develops quickly and significant cell death occurs within 12–24 h with slower cell death, between 2 and 3 d occurring to a lesser extent (26). In global ischemia of the immature brain, it may be that cell death occurs more slowly, with apoptosis being a major and delayed component, resulting in an attenuated release of proteins from damaged cells into the CSF and serum (27,28).

Neonates included in previous studies fulfilled varying definitions of perinatal asphyxia and the timing and nature of the insult was unknown. Antenatal and intrapartum monitoring techniques such as cardiotocography are unreliable in assessing the timing of an insult. Biochemical markers may be more accurate in defining the onset of a hypoxic insult. However, to be able to be used as predictors of outcome after perinatal asphyxia, the relationship between insult time and time to peak, as well as relationship between the peak level and outcome has to be known. That means multiple blood samples need to be taken to identify the peak level and determine the timing of the insult. Only one study reliably evaluated the time course of NSE in serum. In the study by Schorkhueber *et al.* (18), NSE was measured in 56 adult patients at 6, 12, 24, 48, and 72 h after cardiorespiratory arrest. Neurologic outcome was assessed 6 mo after the arrest. Patients with a poor neurologic outcome had significantly higher NSE levels than those with a good neurologic outcome at 12, 24, 48, and 72 h. The NSE value at 72 h was the best predictor of neurologic outcome. In addition to this, it was found that patients with good neurologic outcome had decreasing levels of NSE, whereas patients with adverse neurologic outcome had rising levels of NSE after 6 h, presumably indicating ongoing neuronal damage. The time course of NSE in our animal model correlates well with Schorkhueber's study.

In conclusion, NSE and S100B measured in serum of hypoxic piglets increased significantly after 24 and 48 h, respectively, and correlated with histologic outcome. NSE and S100B may be useful predictors of outcome after hypoxic/ischemic reperfusion injury but cannot be used as predictors within the "window of opportunity" for the initiation of neural rescue therapy. However, they may be useful to establish the timing of an insult.

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