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Nonprotein-bound Iron in Postasphyxial Reperfusion Injury of the Newborn

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ABSTRACT. Objective. To investigate if the availability of nonprotein-bound iron after birth asphyxia is related to the severity of the postasphyxial injury and neurodevelopmental outcome.

Methods. Nonprotein-bound iron (bleomycin assay) and thiobarbituric-acid-reactive species, an index of oxidative lipid damage, were measured in plasma of 50 newborn infants (gestational age >34 weeks) between 0 to 8 hours, 8 to 16 hours, and 16 to 24 hours after birth. Three groups were compared: healthy infants (n = 20), moderately asphyxiated infants (n = 15), who were neurologically normal during the first 24 hours after birth and severely asphyxiated infants (n = 15), who developed abnormal neurologic signs in the first 24 hours after birth.

Results. In the severely asphyxiated infants, liver enzymes, creatinine, urea, and uric acid concentrations were significantly elevated. Eleven severely asphyxiated infants were brain-damaged, 9 of them died during the neonatal period. Nonprotein-bound iron was detectable in 30% of the control, 60% of the moderately asphyxiated, and 80% of the severely asphyxiated infants. During the whole study period nonprotein-bound iron concentration was significantly elevated in severely asphyxiated infants as compared with controls. Three of the four severely asphyxiated infants who had a normal outcome at 1 year of age, had no detectable nonprotein-bound iron during the study period. Stepwise logistic regression analysis with neurodevelopmental outcome at 1 year of age (normal versus adverse/death) as dependent variable and all the measured parameters for organ damage as independent variables revealed that the nonprotein-bound iron concentration at 0 to 8 hours after birth was the most significant variable and at the same time the only variable that entered the model, in relation to neurodevelopmental outcome. Thiobarbituric-acid-reactive species tended to be higher in severely asphyxiated infants, suggesting oxidative lipid damage.

Conclusion. Nonprotein-bound iron may play an important role in oxidative damage-mediated postasphyxial brain injury and subsequent neurodevelopmental outcome.

METHODS

The study population consisted of 50 infants with a gestational age of 35 weeks or more, who consecutively were admitted to our neonatal unit for birth asphyxia or for observation (see also below). Thirty patients suffered from birth asphyxia, defined as fetal distress (abnormal heart rate pattern, meconium-stained amniotic fluid, and a cord or first capillary pH of less than 7.1), requiring immediate assisted ventilation for more than 2 minutes. These infants were divided into a moderately asphyxiated group (n = 15), without neurologic abnormalities during the first 24 hours after birth and a severely asphyxiated group (n = 15), who subsequently developed neurologic abnormalities during the first 24 hours after birth (eg, disturbances in consciousness, hypotonia, hypo-, or areflexia including weak or absent suck or Moro reflexes) and/or convulsions. Transient hyperalertness or hyperreflexia were not considered to be neurologic abnormalities. Twenty healthy infants served as a control group. Assignment to the moderately or severely asphyxiated group was done within the

ABBREVIATIONS. H2O2, hydrogen peroxide; LDH, lactic acid dehydrogenase; O2·−, superoxide; OH·, hydroxyl radical; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; TBARS, thiobarbituric-acid-reactive species.

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first 24 hours of life and before the results of the various blood samples (ie, nonprotein-bound iron, thiobarbituric-acid-reactive species, [TBARS], liver and renal function tests, see also below) were available. Most of these control infants were admitted for observation for possible infection (premature rupture of membranes, maternal fever), which was eventually excluded, or for observation for hypoglycemia because of maternal diabetes. None of them became hypoglycemic during the study period. All infants were admitted to our neonatal unit within 2 hours after birth. None of them had congenital malformations. The study was approved by the scientific board of the Department of Pediatrics and the Ethical Committee of the University Hospital of Leiden. Informed parental consent was obtained in all cases.

Determination of Nonprotein-bound Iron and Lipid Peroxidation

Blood was collected into heparinized glass tubes and immediately centrifuged (750 × g, 10 minutes); the plasma was stored under argon at −70°C until analysis. Plasma samples that showed pink discoloration (hemolysis) were excluded from the study. Nonprotein-bound iron in plasma was measured by the bleomycin assay. Using this assay, the absence of nonprotein-bound iron, ie, the presence of iron binding capacity, can be measured as well as the presence of nonprotein-bound iron, ie, the lack of iron-binding capacity. If nonprotein-bound iron is present, the lower detection limit is 0.6 μM. The glass tubes used to collect the blood did not contain detectable amounts of iron. The intra- and interassay coefficients of variation of the bleomycin assay are 6.6% and 7.4%, respectively. Lipid peroxidation was detected by measuring the concentration of TBARS according to the method of Asakawa and Matsushita.4 Because bilirubin is known to react with thiobarbituric acid reagent8 interference was checked by adding bilirubin in high concentrations to the reaction medium. To eliminate the effect of bilirubin, a correction factor was introduced following the method of Thornham et al.15 Measured TBARS (μM) = 0.034 bilirubin (μM) = corrected TBARS (μM). All TBARS values in this study are expressed as corrected TBARS. Preliminary studies showed no effect of storage on nonprotein-bound iron or TBARS concentration.

Study Design

When blood was withdrawn from the patients for clinical purposes, a small additional sample was taken to determine nonprotein-bound iron and TBARS. These samples were collected during the following time-periods: 0 to 8, 8 to 16, and 16 to 24 hours after birth. Mean time (± 1 SD) of blood sample collection during the subsequent time periods was 4.0 ± 1.2, 11.5 ± 2.5, and 21.8 ± 2.0 hours for control infants; 4.4 ± 2.2, 11.7 ± 1.5, and 20.2 ± 2.1 hours for moderately asphyxiated infants; and 4.4 ± 1.0, 11.6 ± 1.7, and 18.8 ± 2.0 hours for severely asphyxiated infants, respectively. Additional blood samples were taken 24 to 36 hours after birth to determine liver enzymes: serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and lactate dehydrogenase (LDH), renal function (creatinine, urea), and uric acid levels. Neurologic examinations were performed by the attending neonatologist. When the neurologic observations were abnormal, the newborn was also examined by a pediatric neurologist. Brain tissue damage and abnormal electrocortical brain activity were assessed by cranial two-dimensional ultrasound, computed tomography, and/or electroencephalagram registrations. Follow-up at the age of 1 year was evaluated by the Van Wiechen neurodevelopmental assessment test, which is used in Dutch child health care for children from 4 weeks to 5 years of age. It is based on milestones and warning symptoms, such as asymmetry, dystonia, persistence of primitive reflexes, and hearing or visual disturbances as defined by Touwen,24 and on five to eight items covering the five fields of development as described by Gesell and Amatruda.25 (The items were chosen in such a way that at least 90% of a healthy population will achieve them by the age at examination).

Statistical Analysis

Differences between the perinatal and laboratory data of the three groups were assessed by one-way factorial analysis of variance. When a significant difference was found, analysis of variance was followed by the Scheffe's procedure for comparison between the groups. Because of a skewed distribution of the nonprotein-bound iron and TBARS data, nonparametric statistics were used to analyze these data. Only those infants with observations during at least two time periods were included in this statistical analysis (see “Results”). Differences between the three groups within one time period were assessed by the Kruskal-Wallis test, followed by the Mann-Whitney U test, to compare between each two groups, when a significant difference was found. Differences between the three time periods within one group were analyzed by the Friedman test, followed by the Wilcoxon signed ranks test to compare between each two time periods, when a significant difference was found.

To investigate if there was an association between the long-term outcome of the infants in this study and one or more of the parameters for organ damage, a stepwise forward logistic regression (P value to enter = .05; P value to remove = .10) was performed with outcome at 1 year of age (good [normal] or adverse [abnormal, death]) as dependent variable and cord/first pH, SGOT, SGPT, LDH, creatinine, urea, uric acid and nonprotein-bound iron, and TBARS at 0 to 8 hours, 8 to 16 hours, and 16 to 24 hours as independent variables. Adding or removing variables was based on the likelihood criteria.

Results in the text and figures are expressed as mean (± 1 SD) or as median with ranges where appropriate. P < .05 were considered statistically significant.

RESULTS

Patients Characteristics and Laboratory Data

Patients characteristics are shown in Table 1. There were no significant differences between the three groups regarding birth weight or gestational age, although the infants of the control group tended to have a lower gestational age. Presumptive causes of the asphyxiated infants were abruptio placenta (n = 3), meconium aspiration (n = 6), strangulation of the umbilical cord around the neck (n = 3), insufficient progression of delivery, resulting in cesarean section or vacuum extraction (n = 16), and unknown (n = 2). Cord or first capillary pH (within 10 minutes of birth) and 5-minute Apgar scores of the severely asphyxiated infants were significantly lower than those of the moderately asphyxiated infants, whose values were significantly lower than those of the control infants. Individual and mean laboratory data of all the infants are shown in Fig 1. The SGOT, SGPT, LDH, and creatinine concentrations of the severely asphyxiated infants were significantly higher than those of the moderately asphyxiated and control infants. The urea and uric acid concentrations of the moderately and severely asphyxiated infants were significantly higher than those of the control infants.

<table>
<thead>
<tr>
<th>TABLE 1. Patient Characteristics of the Three Study Groups (Means ± 1 SD)*</th>
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<td>CONT</td>
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<td>Birth weight (g)</td>
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<td>Cord/first pH</td>
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<td>Median Apgar at 5 min (range)</td>
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*CONT = control group [n = 20]; MA = moderately asphyxiated group [n = 15]; SA = severely asphyxiated group [n = 15]; GA = gestational age.
† P < .05 vs CONT.‡ P < .05 vs MA.
Nonprotein-bound Iron

Figure 2 and Table 2 show the individual concentrations of nonprotein-bound iron (medians indicated by the little dash) in the three groups during the subsequent postnatal time periods. Nonprotein-bound iron was detected in 6/20 (30%) of the control, 9/15 (60%) of the moderately asphyxiated, and 12/15 (80%) of the severely asphyxiated infants in at least one of the three time periods. The nine control infants without detectable nonprotein-bound iron in plasma were not different for any patient characteristic as compared with the remaining six control infants with detectable nonprotein-bound iron. Moreover, no reason was found in these six patients for the occurrence of nonprotein-bound iron.

In this clinical study it was not always possible to obtain a blood sample from each patient during each of the time periods (eg, rejection of blood sample because of hemolysis, death [severely asphyxiated infants] or discharge): 14 control infants (n = 14, n = 12, and n = 13 at 0 to 8 hours, 8 to 16 hours, and 16 to 24 hours, respectively), 13 moderately asphyxiated infants (n = 10, n = 9, and n = 11 at 0 to 8 hours, 8 to 16 hours, and 16 to 24 hours, respectively), and 13 severely asphyxiated infants (n = 12, n = 12, and n = 11 at 0 to 8 hours, 8 to 16 hours, and 16 to 24 hours, respectively).
severely asphyxiated infants \((n = 12, n = 12, \text{and } n = 11 \text{ at 0 to 8 hours, 8 to 16 hours, and 16 to 24 hours, respectively})\) had at least nonprotein-bound iron determinations during two time periods. Patient characteristics and laboratory data of these infants were not different from the original groups. For statistical analysis of the data shown in Fig 2 only those infants with observations during at least two time periods were included. In the severely asphyxiated infants, the plasma concentration of nonprotein-bound iron was significantly elevated during the whole study period as compared with the control infants, and from 0 to 8 hours as compared with the moderately asphyxiated infants. No significant changes of nonprotein-bound iron concentration were seen between the three time periods within one group.

**TBARS**

The median (range) plasma concentration of TBARS for the three groups were as follows: 6.79 (4.43 through 15.01), 6.36 (4.74 through 11.30), and 6.35 (4.72 through 17.29) \(\mu M\) at 0 to 8 hours; 7.81 (5.53 through 10.9), 9.87 (3.60 through 13.89), and 9.50 (4.27 through 27.54) \(\mu M\) at 8 to 16 hours; and 7.99 (6.21 through 11.3), 9.22 (6.01 through 12.32), and 9.43 (6.32 through 16.58) \(\mu M\) at 16 to 24 hours for control, moderately asphyxiated, and severely asphyxiated infants, respectively. TBARS tended to be higher in the asphyxiated infants as compared with the control infants at 8 to 16 hours and 16 to 24 hours, but this difference never reached significance. No significant changes of TBARS concentration were seen between the three time periods within one group. Mean values (± 1 SD) for maximal total bilirubin plasma concentrations during the first 24 hours of life were 94.8 ± 43.8, 61.8 ± 14.9, and 62.5 ± 41.4 \(\mu M\) for control, moderately asphyxiated, and severely asphyxiated infants, respectively, and did not differ between groups.

**Short-term Outcome**

Fourteen of the 15 severely asphyxiated infants needed assisted ventilation beyond the resuscitation period, 9 infants had convulsions requiring anticonvulsive therapy, 1 infant received an erythrocyte transfusion because of a low hemoglobin and two needed dopamine medication to treat hypotension during the study period. Nine of the 15 severely asphyxiated infants developed major neurologic abnormalities. All died during the early neonatal period after supportive treatment was withdrawn because of their deteriorating neurologic condition (eg, coma, extensive cerebral damage), determined by computer tomography and/or a virtually flat electroencephalogram. Among them were those two severely asphyxiated infants excluded from the statistical analysis because of only one blood sample (both died in the first 8 hours after birth and had rather high nonprotein-bound iron concentrations: 82.9 \(\mu M\) and 97.6 \(\mu M\)). At discharge two of the six surviving severely asphyxiated infants had abnormal neurologic examinations (hypotonia and little spontaneous movement). All the control and moderately asphyxiated infants were neurologically normal at discharge.
Long-term Outcome

All the control and moderately asphyxiated infants were neurologically normal at 1 year of age. Of the six surviving severely asphyxiated infants, two showed a delayed neurodevelopmental outcome at 1 year of age. The other four survivors were neurologically normal at 1 year of age. Three of these infants had no detectable nonprotein-bound iron during the study period. Stepwise logistic regression analysis with outcome at 1 year of age (good [normal] or adverse [abnormal, death]) as dependent variable and cord/first pH, SGOT, SGPT, LDH, creatinine, urea, uric acid, and nonprotein-bound iron and TBARS at 0 to 8 hours, 8 to 16 hours, and 16 to 24 hours as independent variables, revealed that the nonprotein-bound iron concentration at 0 to 8 hours was the most significant variable ($P < .001$) and at the same time the only variable that entered the model. An increase of the nonprotein-bound iron plasma concentrations at 0 to 8 hours was associated with an increased risk for an adverse outcome at 1 year of age (odds ratio = 1.12/μM nonprotein-bound iron; 95% confidence interval: 1.00 through 1.26). Figure 3 shows the individual nonprotein-bound iron concentrations (medians indicated by small dash) at 0 to 8 hours after birth in the infants with a good (n = 26) and an adverse outcome (n = 10).

**DISCUSSION**

To our knowledge, this is the first clinical study that has investigated the relation between severity of birth asphyxia, plasma concentration of nonprotein-bound iron and TBARS, and subsequent neurologic outcome. There appeared to be an association between elevated plasma concentrations of nonprotein-bound iron at 0 to 8 hours after birth and an adverse outcome after severe birth asphyxia. Moreover, the severely asphyxiated infants with no detectable nonprotein-bound iron in their plasma all had a normal neurodevelopmental outcome at 1 year of age. These findings may imply that nonprotein-bound iron plays an important role in the pathogenesis of postasphyxial reperfusion/reoxygenation injury. Interestingly, the association between nonprotein-bound iron in plasma and adverse outcome appeared to be lost after 8 hours of age, despite sometimes high concentrations of nonprotein-bound iron (Fig 2). Possibly the reoxygenation of the brain upon early reperfusion is an important cofactor to generate reactive oxygen species such as $O_2^·$ and hydrogen oxide, leading to formation of the highly toxic $OH^·$ radical in the presence of nonprotein-bound iron and to brain cell damage.

The ability of plasma of newborn infants to inhibit nonprotein-bound iron-induced lipid peroxidation in vitro is significantly less compared with that of adult plasma. Moreover, healthy newborn infants often have detectable nonprotein-bound iron in their plasma and this seems to be inversely related to their gestational age. In a study from Moison et al, none of the adults had detectable nonprotein-bound iron in their plasma, but 6/24 term and 10/21 preterm infants had detectable concentrations, which makes newborn infants more susceptible to nonprotein-bound iron-induced oxidative damage. The values for nonprotein-bound iron in some of the control infants of our study were higher than those previously reported by us. This may be related to their somewhat lower gestational age (11 of the 20 control infants had gestational ages between 35.0 and 37.0 weeks), because preterm infants do have higher nonprotein-bound iron values than term infants. With respect to the three outlying values of nonprotein-bound iron in plasma in the control group (two values in the 8 to 16 hours period [25.3 and 42.0 μM]; one value in the 16 to 24 hours period [28.7 μM], see also Fig 2) we must admit that we did not measure plasma hemoglobin and have to consider the possibility that the key source of these nonprotein-bound iron concentrations could be associated with heme or hemoglobin, despite our attempts to clinically exclude this by excluding plasma samples showing pink discoloration. However, the role of heme or hemoglobin in detecting nonprotein-bound iron is not likely to be of great importance in our study. In a previous study we showed that despite similar hemoglobin concentrations, very different levels of nonprotein-bound iron were detected. Furthermore, it has been demonstrated that addition of up to 3 mg hemoglobin per milliliter plasma (ie, 46.5 μmol hemoglobin per liter plasma) did not influence the measurement of nonprotein-bound iron. This amount of hemoglobin results in a discoloration of plasma that can easily be identified. Because we excluded samples showing pink discoloration, the amounts of hemoglobin in the remaining samples must have been <46.5 μmol.

The very high nonprotein-bound iron levels in the asphyxiated infants could be related to injury-induced iron release into the plasma. In the normal situation, iron is required for several important processes: oxygen transport (hemoglobin), mitochondrial respiration, proper function of several important enzymes, and antibacterial defense and is usually firmly bound to transferrin in plasma or intracellularly to ferritin. These forms of iron are not capable of inducing free radical production by catalyzing the Haber-Weiss reaction and therefore provide safe iron transport and storage systems. However,
ever, lowering of the plasma pH, as occurs during ischemia, enables transferrin to liberate its iron, inducing free radical production. These free radicals are capable of releasing even more iron by mobilizing it from ferritin. By these mechanisms a cascade of iron release and free radical production can be activated and lead to extensive cell damage. The injured cells may release their intracellular iron into the surrounding environment, thereby further increasing the plasma concentration of nonprotein-bound iron. Nonprotein-bound iron levels as high as 21.5 \( \mu \text{mol/L} \) were reported in an adult treated for leukemia despite the fact that plasma transferrin was only 50% saturated before therapy. The authors suggest that chemotherapy-induced destruction of leukemic cells released sufficient iron to saturate the transferrin and produce high nonprotein-bound iron levels. Iron released after anoxic-ischemic damage may produce even higher nonprotein-bound iron concentrations: the latent iron-binding capacity of plasma is very limited in the newborn and much less of the released iron can be bound. We therefore suggest that the increased plasma concentration of nonprotein-bound iron in our asphyxiated infants reflects the increased liberation of nonprotein-bound iron from iron-binding proteins and/or damaged cells within the organ systems.

The involvement of nonprotein-bound iron in free radical mediated damage is well established. Halat et al showed the essential role of nonprotein-bound iron in the promotion of postischemic lipid peroxidation. Others found that local cerebral injection of iron salts increased lipid peroxidation, whereas lipid peroxidation was inhibited by the iron chelator deferoxamine. We have recently demonstrated in newborn lambs, subjected to severe hypoxic-ischemic reperfusion injury, that there was a very rapid increase in nonprotein-bound iron concentration within 15 minutes after the completion of the hypoxic-ischemic insult. Highest values were reached at 60 minutes after completion of the hypoxic-ischemia.

These processes can occur in all important organ systems, which may contribute to the elevated liver enzymes and abnormal renal function tests especially in the severely asphyxiated infants. However, the brain may be especially at risk for free radical-mediated injury because neuronal membranes are very rich in polyunsaturated fatty acids (sensitive to free radical attack) and several areas of the human brain are especially rich in iron. Moreover, the iron-binding capacity of cerebrospinal fluid is low (low concentration of transferrin) and most of the iron will be in its active ferrous form because of a high concentration of vitamin C and a low concentration of ceruloplasmin in cerebrospinal fluid.

It can be questioned whether the increased plasma concentration of nonprotein-bound iron in our severely asphyxiated infants is merely a marker of an increased leakage from injured cells, or if it is also actively inducing peroxidative damage itself (eg, endothelial damage). Lesnfsky et al found that intracellular, but not extracellular, iron administration augmented myocardial reperfusion injury. However, recent studies showed that chelating agents confined to the intravascular space improved outcome after head injury or cardiac arrest. Another important question is whether the iron in this study will be in the active ferrous or less reactive ferric form. Because of the high concentration of vitamin C and low concentration of ceruloplasmin in the newborn, the nonprotein-bound iron in these infants is likely to be in the highly active ferrous form. We therefore suggest that the increased plasma concentration of nonprotein-bound iron in this study is more than only a marker indicating multi-organ damage and may have played an important role in the extent of the postasphyxial reperfusion injury and the generally poor neurologic outcome of the severely asphyxiated infants. In this respect, studies concerning the prevention of free radical-mediated reperfusion injury, which especially focus on scavenging the nonprotein-bound iron, showed that deferoxamine, the prototype chelating agent, has been used successfully to diminish oxidative damage in various studies. However, caution is required, because deferoxamine has shown to be toxic to premature baboons. Other promising therapies may be the administration of fresh adult plasma (high content of unsaturated transferrin) and/or an exchange transfusion. In newborns, exchange transfusions have been shown to lower iron, ferritin, and vitamin C levels and raise the concentration of transferrin, thereby increasing the latent iron-binding capacity. However, further clinical studies are indicated to evaluate the effect of these therapies and to search for new agents, which can prevent free radical production, scavenge nonprotein-bound iron, and can be used safely in newborn infants.

Although the plasma concentration of TBARS in the severely asphyxiated infants tended to be higher, suggesting an increased lipid peroxidation, these differences did not reach significance. However, it is important to realize that TBARS, although they are the most frequently quoted evidence for the involvement of free radicals in human disease, do have their limitations. They only measure lipid peroxidation, although proteins and DNA are more often the targets of oxidative damage than are lipids. Moreover, lipid peroxidation often occurs late in the oxidative injury process. Therefore, we suggest that oxidative damage may play a role in the observed association between elevated plasma concentrations of nonprotein-bound iron and an adverse outcome after severe birth asphyxia.

In summary, the results of this study suggest a relation between elevated plasma concentrations of nonprotein-bound iron and an adverse outcome at 1 year of age after severe asphyxia. Although nonprotein-bound iron may play an important role in the pathogenesis of postasphyxial reperfusion injury and subsequent neurologic outcome, further study is warranted to evaluate the exact mechanisms of this relationship.

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