Urinary Thiobarbituric Acid–Reacting Substances as Potential Biomarkers of Intrauterine Hypoxia

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Background: Currently available clinical tools cannot accurately identify the extent of perinatal hypoxic injuries. During hypoxia, reactive oxygen species cause lipid peroxidation of cell membranes, yielding oxidation products that constitute thiobarbituric acid–reacting substances (TBARS).

Objective: To see if the concentrations of TBARS excreted in urine would be elevated during the first day of life in term and preterm infants following chronic hypoxia or acute asphyxia.

Design: Thiobarbituric acid–reacting substances levels were measured by a spectrophotometric assay in urine samples collected from term and near-term (≥ 34 weeks gestation, n=22), and preterm (<34 weeks gestation, n=52) infants on the first day of life.

Patients: Infants were admitted to the St Peter’s University Hospital (New Brunswick, NJ) neonatal intensive care unit from July 1997 to January 1999. Acute asphyxia was defined as umbilical cord blood pH values less than 7.05, or Apgar scores of less than 5 at 5 minutes. Chronic hypoxia was defined as intrauterine growth retardation or low birth weight (small for gestational age) associated with pregnancy-induced hypertension or reversal of umbilical arterial blood flow.

Results: Among term infants, urinary TBARS levels were significantly increased following acute asphyxia (P=.02). Levels of TBARS also tended to be elevated following chronic hypoxia. Urinary TBARS levels in term infants tended to be increased in those requiring mechanical ventilation (P=.05) or delivery room resuscitation (P=.15), as well as in those passing intrauterine meconium (P=.13) or having clinical evidence of hypoxic-ischemic encephalopathy (P=.24).

Conclusions: The results show a correlation between elevated urinary TBARS levels in term and near-term infants, and perinatal hypoxia (as determined by low Apgar scores or umbilical cord blood acidosis). We speculate that TBARS concentrations may be useful as a biomarker for perinatal hypoxic injury in newborns. Further studies are needed to determine whether elevations in TBARS levels are better predictors of the extent of hypoxic injury than existing markers.


Intrauterine hypoxia is a major risk factor for an abnormal outcome in the neonatal period. Approximately 700 neonatal deaths following intrauterine hypoxia are reported in the United States each year, comprising more than 2% of total infant mortality. The neurodevelopmental sequelae exhibited by survivors can be severe, constituting a large part of pediatric health care expenditures. Despite the potential severity and prevalence of this condition, the diagnosis is usually based on nonspecific clinical criteria, since no reliable markers have previously been demonstrated to correlate with the extent of intrauterine hypoxic insult. Such biomarkers, if available, would be particularly helpful in identifying infants exposed to chronic hypoxia in utero. Examination of these infants has not often revealed pathognomonic findings, and the identification of such findings is necessary to provide postnatal medical management that optimizes the medical and neurologic outcome. In addition, several specific interventions are currently under investigation to limit neurologic injury resulting from hypoxic-ischemic insult, including cerebral hypothermia and antioxidant therapies. These appear to be most effective when applied early in the course of this process. Therefore, the identification of a rapid and early marker of the extent of perinatal asphyxia is an important step in identifying patients eligible for prospective clinical trials and, ultimately, in devising specific preventive interventions.

Clinical and laboratory criteria that are currently used to identify asphyxi-
PATIENTS AND METHODS

PATIENT CHARACTERISTICS

All inborn infants admitted to the neonatal intensive care unit at St Peter’s University Hospital (New Brunswick, NJ) during the period between July 1997 and January 1999 were screened for eligibility, excluding those from multiple gestations or with major congenital anomalies (1471 term and 602 preterm infants were screened). Of these, 6 term infants (34 or more weeks’ gestation) and no surviving preterm infants met the criteria for acute asphyxia. Six term infants and 15 preterm infants met the criteria for chronic hypoxia. All of these infants were enrolled. At the time that each eligible term infant was enrolled, a newborn infant of comparable gestational age but with no signs or symptoms of hypoxia, asphyxia, or growth retardation was selected as a control. Two potential term control infants were not included because consent could not be obtained. Therefore, 10 term infants served as controls. At the time of enrollment of each preterm infant, 2 to 3 infants of comparable gestational age were selected as controls. More preterm controls per subject were selected because of anticipated difficulties in obtaining consent and urine samples within 24 hours in this group. Therefore, 40 infants were recruited as controls. Of these infants, consent was not obtained from 2, urine was not available from 1, and 37 served as controls for the analysis. Study personnel obtained demographic and medical information from maternal and infant medical records.

For analysis, infants were divided into 3 groups. Infants with acute asphyxia were defined as those with umbilical cord blood pH values less than 7.05, or 3-minute Apgar scores of less than 5. Chronic hypoxia was defined as intrauterine growth retardation or low birth weight (small for gestational age) associated with either pregnancy-induced hypertension (PIH) or reversal of umbilical arterial blood flow. Control infants were those of appropriate size for gestational age who did not exhibit any signs of fetal or neonatal hypoxia. Preterm infants (<34 weeks gestation) and term or near-term infants (≥34 weeks gestation) were analyzed separately.

DETERMINATION OF URINARY TBARS

Urine samples were obtained from study infants on the first day of life. Informed consent was obtained from parents for the acquisition of samples, and these studies were approved by the Committee for the Protection of Human Subjects in Research at St Peter’s University Hospital. All specimens were collected into sterile containers and stored at -70°C for batched analysis. Thiothiburic acid–reacting substances levels were measured as previously described.13 Briefly, 200 µL of urine was combined with 10 µL of 5% butylated hydroxytoluene (BHT, in glacial acetic acid) and 300 µL of a 0.3% aqueous thiothiburic acid (TBA) solution. The samples were then vortexed and incubated at 100°C for 30 minutes. After cooling to room temperature, the absorbance of samples at 532 nm was measured using a Lambda 3B spectrophotometer (Perkin Elmer Corp, Baden See, Germany). Samples were blanked against reference cuvettes containing reagents without urine. The concentration of TBARS was calculated from the absorbance at 532 nm, using 156000 as the molar extinction coefficient. The quantity of TBARS is proportionate to the amount of MDA, a lipid peroxidation product generated by the oxidation of membrane lipids by ROS. Malondialdehyde reacts with TBA to form a 1:2 MDA-TBA adduct, which absorbs at 532 nm. In the present study, MDA was confirmed to be the predominant TBA-reacting adduct by high-performance liquid chromatography analysis of representative samples.

To control for urine concentration, data were normalized to urine creatinine concentrations. Urinary creatinine was measured using the revised Jaffe method.15 Briefly, alkaline picrate (formed by combining picric acid and 10% sodium hydroxide in a ratio of 5:1) was added to serial dilutions of urine. Samples were incubated at room temperature for 15 minutes, and the absorbance at 500 nm was measured.

DATA ANALYSIS

Results are expressed as means ± SDs. As urinary TBARS concentrations were not normally distributed, we transformed them to their natural logs, which were normally distributed. Group means were compared using unpaired t tests. A P value less than .05 was considered significant.

Umbilical cord blood pH might be expected to be predictive of injury associated with perinatal hypoxic insult. When gas exchange across the placenta is compromised, tissue hypoxia leads to anaerobic metabolism and lactic acidosis. In addition, carbon dioxide accumulation further reduces fetal arterial pH. Nevertheless, Winkler et al10 found no differences between infants with cord pH values above or below 7.20 with respect to the development of clinical signs of asphyxia (seizures, persistent hypotonia, and renal or cardiac dysfunction). Fee et al11 noted that among 110 term infants with a cord blood pH less than 7.05 and a base deficit greater than 10 mEq/L, 73% were admitted to regular nurseries and were discharged as normal. Seven of 9 infants who had abnormal neurological features at birth had no abnormal characteristics at follow-up. In another study,9 no infant with an umbilical cord blood pH less than 7.00, but with a 1-minute Apgar score greater than 3 had seizures or hy-


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Potonia. Dennis et al found no association between uterine or perinatal hypoxia. In order to quantify ROS generated in the fetus can serve as a biomarker of intrauterine level. Therefore, we hypothesized that ROS that are increased following chronic intrauterine hypoxia, rather than direct measurements of physiologic phenomena that may follow hypoxic insult, as determined by the patient history and clinical criteria.

Among term and near-term infants (n=22), those with chronic hypoxia (n=6) had a lower mean gestational age than those with acute asphyxia (n=6) or control infants (n=12), and they tended to have lower birth weights (Table 1). Infants with acute asphyxia had significantly lower 1-minute and 5-minute Apgar scores than controls. The log concentration of urinary TBARS in term infants with acute asphyxia (9.06±0.60) was significantly greater than that of controls (7.97±0.88) (P<.01), but were not further increased following chronic intrauterine hypoxia.

**Table 1. Demographic Variables and Urinary TBARS Levels in Term and Near-Term Infants***

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control (n=10)</th>
<th>Chronic Hypoxia (n=6)</th>
<th>Acute Asphyxia (n=6)</th>
</tr>
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<tbody>
<tr>
<td>Birthweight, g</td>
<td>2786 ± 926</td>
<td>1987 ± 569</td>
<td>3418 ± 661</td>
</tr>
<tr>
<td>Gestational age, wk</td>
<td>37.4 ± 1.6</td>
<td>35.2 ± 1.0†</td>
<td>38.3 ± 2.7</td>
</tr>
<tr>
<td>Apgar scores</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1 min</td>
<td>7.7 ± 2.4</td>
<td>8.8 ± 0.4</td>
<td>1.0 ± 0.6†</td>
</tr>
<tr>
<td>5 min</td>
<td>8.6 ± 1.3</td>
<td>9.2 ± 0.4</td>
<td>3.3 ± 2.0†</td>
</tr>
<tr>
<td>TBARS, ng/mg of creatinine</td>
<td>7.97 ± 0.87</td>
<td>8.33 ± 1.38</td>
<td>9.06 ± 0.60†</td>
</tr>
</tbody>
</table>

*Values are expressed as means ± SDs. TBARS indicates thiobarbituric acid–reacting substances.
†Values significantly differ from control values; P<.05.

**Table 2. Association of Urinary TBARS Levels With Clinical Indicators in Term and Near-Term Infants***

<table>
<thead>
<tr>
<th>Indicators</th>
<th>TBARS (ng/mg Creatinine)</th>
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<tbody>
<tr>
<td></td>
<td>Yes (n=6)</td>
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<tr>
<td>Delivery room resuscitation</td>
<td>8.75 ± 0.61</td>
</tr>
<tr>
<td>Meconium passage</td>
<td>9.09 ± 0.81 (4)</td>
</tr>
<tr>
<td>Hypoxic-ischemic encephalopathy</td>
<td>9.21 ± 0.91 (2)</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>9.07 ± 0.61 (6)</td>
</tr>
</tbody>
</table>

*Values are expressed as means ± SDs (number of patients requiring treatment). TBARS indicates thiobarbituric acid–reacting substances.

Among term and near-term infants (n=37), those with chronic hypoxia (n=15) had a lower mean gestational age than those with acute asphyxia (n=12) and control infants (n=14). Only the most severe base deficits (>20 mEq/L) in severely affected infants have been associated with adverse neurological outcomes. These data indicate that umbilical cord blood pH values are neither sensitive nor specific in identifying birth asphyxia and its sequelae. Similarly, Apgar scores have been used to identify infants with birth asphyxia. However, the 1-minute Apgar score has poor sensitivity and positive predictive value in detecting acidosis, and 25% to 75% of infants with acidosis are assigned normal Apgar scores. Therefore, current methods have been inadequate in identifying infants with hypoxic injury. This is most likely because all of these assessments are indirect reflections of physiologic phenomena that may follow hypoxic insult, rather than direct measurements of the pathophysiologic progression of asphyxia at the cellular level.

Hypoxia induces cellular damage, in part by the generation of reactive oxygen species (ROS), including hydrogen peroxide, singlet oxygen, superoxide anion radical, hydroxyl radical, and lipid hydroperoxides in affected tissues. Therefore, we hypothesized that ROS that are generated in the fetus can serve as a biomarker of intrauterine or perinatal hypoxia. In order to quantify ROS in...
Acute asphyxia could not be studied in preterm infants because of insufficient numbers.

In this study, we have shown that urinary TBARS, measured during the first day of life, are elevated in term and near-term infants following acute asphyxia, as defined by low Apgar scores or acidemia. Full-term infants also tend to exhibit higher urinary excretion of TBARS following chronic low-grade oxygen deprivation, as indicated by intrauterine growth retardation in the presence of PIH or reversed umbilical arterial blood flow. Urinary TBARS were not directly correlated with PIH (defined by maternal blood pressure), suggesting that the association of TBARS levels with chronic hypoxia occurs primarily in infants with the most severely compromised uteroplacental blood flow (reversed umbilical arterial blood flow or severe PIH). We also found that preterm infants exhibit elevated urinary TBARS levels relative to term infants, possibly reflecting stresses inherent in preterm delivery.

This study is limited by the small sample size (term subjects, n=12), a consequence of the low incidence of perinatal hypoxia and asphyxia during this period at our neonatal intensive care unit. Nevertheless, the physiology of hypoxic-ischemic injury supports the potential role of early measurement of TBARS levels in the identification of affected infants. The generation of free radicals during tissue hypoxia plays a major role in the pathogenesis of tissue injury. Newborns are particularly susceptible to such injury because they exhibit an imbalance between antioxidant and oxidant-generating systems. Free radicals, in excess, inactivate proteins, disrupt DNA, and oxidize lipids.\(^{16,17}\) The oxidation of lipids by radicals has been long regarded as a critical event leading to cellular injury. Cell membranes contain a high proportion of polyunsaturated lipids and are susceptible to peroxidation, resulting in the formation of hydroperoxides, the most abundant product being MDA.\(^ {18,19}\) Malondialdehyde has a long half-life (\textgreater{}20 days) in neutral or acidic solutions, but is metabolised in vivo by reaction with tissue proteins and nucleic acids.\(^ {20}\) Although its biological half-life is likely to be variable and has not been well described, MDA and TBARS have been used in animals and humans as reliable indicators of the response to pro-oxidant provocations.\(^ {21}\) Conditions initiating lipid peroxidation in the perinatal period (eg, uteroplacental restriction) are likely to be ongoing (acute or chronic) in the presence of unlimited substrate.

Therefore, TBARS measurements, which are proportional to MDA content, may provide a direct assessment of the progression of hypoxic injury at the cellular level. Consistent with our data, several previous studies have demonstrated that serum MDA levels in healthy full-term infants are low at birth and rise postnatally in response to the stress of abdominal delivery and/or to the events of respiratory transition during the first days of life.\(^ {22,23}\) Umbilical cord blood MDA levels are increased following fetal hypoxia, suggesting that MDA reflects the extent of lipid peroxidation in vivo.\(^ {24,25}\) Hasegawa et al\(^ {25}\) observed that the content of TBARS in the brains of newborn mice increased during reoxygenation after 20 minutes of hypoxia. Malondialdehyde levels are also elevated in preterm infants, particularly those receiving oxygen and mechanical ventilation.\(^ {27}\) Buonocore et al\(^ {28}\) showed that umbilical cord blood total lipid hydroperoxides are increased in preterm infants following fetal hypoxia. Furthermore, high umbilical cord blood hydroperoxide levels in preterm infants have been correlated with adverse outcomes in the newborn period that are reflective of ROS-mediated oxidative injury (death, severe intraventricular hemorrhage, necrotizing enterocolitis, or pulmonary hemorrhage).\(^ {29}\)

In this study, we have defined acute and chronic perinatal asphyxia using existing standards that are available at the time of birth (ie, Apgar scores, umbilical cord blood pH, and intrauterine growth). Although these measures are known to be unreliable in determining the severity of hypoxic injury, no other early parameters have been established as gold standards for diagnosing hypoxic injury in newborns. Further studies will be required to validate TBARS as a true biomarker for perinatal hypoxic-ischemic injury. To be useful, such a biomarker must do the following: (1) be scientifically sound, (2) be available rapidly during the early hours of life, (3) concur with more easily obtained or established clinical and laboratory indicators, and (4) provide further information or precision not provided by the other methods of predicting outcomes of interest. Previous studies have validated the first criterion by establishing that MDA (measured by TBARS) is produced as a direct product of lipid peroxidative damage to cell membranes, and that hypoxia induces free radical generation, oxidative stress, and lipid peroxidation. In this study, we found that early TBARS measurements are correlated with perinatal stress and conventional measures of hypoxia, suggesting that the second and third criteria are valid. Further studies will be required to validate the final criterion by establishing the predictive value of elevated urinary TBARS with regard to long-term medical and neurologic prognoses. As we enter an era during which specific therapies may become available to avert the course of hypoxic-ischemic encephalopathy, the early identification of patients at risk will gain urgent importance.

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REFERENCES


