Maternal Nitric Oxide Supplementation Decreases Cord Blood S100B in Intrauterine Growth-retarded Fetuses, Diego Gazzolo,1 Matteo Bruschettini,2 Romolo Di Iorio,2 Emanuela Marinoni,3 Mario Lituani,1 Mauro Marras,2 Rossana Sarli,3 Pier Luigi Bruschettini,1 and Fabrizio Michetti4

Intrauterine growth retardation (IUGR) is thought to reflect suppression of genetic growth potential by decreased supplies of oxygen and substrate (1). NO supplementation may be useful in IUGR to increase uteroplacental circulation (2–5). The use of biochemical markers to assess the extent of brain distress associated with NO treatment could be appropriate. S100B is an acidic calcium-binding protein of the EF-hand family concentrated in the nervous system (6). The appearance of S100B in biological fluids is an indicator of brain distress in both infants and adults (7–10). Recently, blood S100B concentrations in the perinatal period have been shown to correlate with brain maturation and damage (11–14).

We investigated the effect of maternal NO supplementation on brain distress in IUGR fetuses as assessed by cord blood S100B. We selected 51 pregnant women (gestational age, 27–35 weeks) with IUGR fetuses and impaired uteroplacental blood flow. Exclusion criteria included multiple pregnancies, gestational and type 1 diabetes, maternal infections and fever, fetal malformations and chromosomal abnormalities, metabolic diseases, and maternal diseases of the central nervous system. Patients were assigned, by use of computer-generated random numbers, to receive either placebo (n = 25) or transdermal glyceryl trinitrate (Nitroderm TTS; Ciba-Geigy) at a dose of 5 mg/16 h daily until delivery (n = 26). Placebo and glyceryl trinitrate patches were indistinguishable and were numbered and contained in identical envelopes so that patients did not know the group to which they were recruited. Similarly, neither the physicians nor the investigators who analyzed the samples knew which patients were treated with placebo and which with glyceryl trinitrate patches. The local ethics committee approved the study, and written informed consent was obtained from all participants.

Two patients in the glyceryl trinitrate group declined to accept administration of the drug, and one did not follow correctly the indications for patch application and was removed from the data analysis.

Gestational age at the time of enrollment did not differ between the two groups. All fetuses were delivered by elective cesarean section for obstetrical reasons and did not experience uterine contractility. Gestational ages were determined by clinical data and by a first-trimester ultrasound scan. IUGR was defined by the presence of ultrasonographic signs (biparietal diameter below the 10th centile and abdominal circumference below the 5th percentile) according to the normograms of Campbell and Thoms (15) and by a fall in the centile of fetal size recorded between the first scan after referral and subsequent scans. The flow velocity waveforms (FVWs) of the main branch of the uterine artery bilaterally, the umbilical artery (UA), and fetal middle cerebral artery (MCA) were recorded by means of a duplex pulsed color Doppler ultrasound (SSD-2000; Aloka). A reading >2 SD above the mean for the gestational age in uncomplicated pregnancies was defined as a pathologic resistance index (RI) for the uterine artery and as a pathologic pulsatility index (PI) for the UA. An abnormal PI in the FVWs of the fetal MCA was defined as a pathologic RI for –2 SD from the mean for that particular gestational age in uncomplicated pregnancies. A UA/MCA PI ratio >1 was considered patho-

References
logic. The control group consisted of 20 apparently healthy fetuses matched for gestational age at sampling and with birth weights between the 10th and 90th percentiles, delivered by elective cesarean section, and not experiencing uterine contractility. In these infants, the FVWs of the MCAs were not recorded because they had normal velocimetry waveforms in the uterine and umbilical arteries.

Maternal blood samples were collected from the groups studied before and after the administration of a patch (glyceryl trinitrate or placebo). In the control group, blood was drawn before the induction of anesthesia. Immediately after delivery, the umbilical cord was clamped before any signs of breathing were detected, and blood was drawn from the vein.

Heparin-treated blood samples taken from the umbilical cord at birth were immediately centrifuged at 900g for 10 min, and the supernatants were stored at −70 °C. The S100B concentration was measured in all samples by a commercially available IRMA (Sangtec® 100; AD Sangtec Medical) specific to the β subunit of the protein, which is known to predominate (80–96%) in the human brain (16, 17). Each measurement was performed in duplicate, and the means were reported. The limit of detection was 0.2 μg/L.

Neonatal neurologic outcome was assessed on the 7th day from birth by a qualitative approach similar to that described by Prechtl (18). Each infant was assigned to one of three diagnostic groups: normal, suspect, or abnormal. The same examiner, who did not know the neurologic condition at birth, tested all infants.

NO metabolites (NOx) and S100B concentrations were expressed as mean values ± SD. Statistical analysis was performed using the Spearman rank-order correlation. Groups were compared using the Kruskal–Wallis one-way ANOVA, with the Mann–Whitney U-test used when data were not normally distributed. Comparison between the incidences of neonatal neurologic outcome and of acute respiratory distress syndrome was performed with the Fisher exact test. Maternal NOx and S100B values before and after treatment were compared using the Student t-test for paired data. The clinical characteristics of the women are expressed as means ± SD and were compared by unpaired data. Statistical significance was set at P < 0.05.

Uteroplacental and fetal Doppler findings in the groups studied are shown in Table 1. Transdermal glyceryl trinitrate administration was associated with a significant decrease in uterine artery RI and the UA PI (P < 0.05), whereas no significant difference was found in MCA PI (P > 0.05). Because of the reduction in the UA PI, the UA/MCA PI ratio decreased significantly (P < 0.05) after NO administration. In the placebo group, FVWs worsened with a significant increase in the uterine artery RI (P < 0.05), leading to elective cesarean section. This produced a higher mean gestational age at delivery in the treated than in the placebo group. NOx and S100B concentrations in maternal plasma before and after treatment were not significantly different (P > 0.05). Maternal S100B and NOx measurements did not correlate with UA PI in both the NO-treated and the untreated group (P > 0.05). NOx and S100B protein in maternal plasma measured at the time of elective cesarean section did not differ between the IUGR and control groups (P > 0.05).

In fetal plasma, NOx concentrations were similar in the three groups: 27.5 ± 5.2 mol/L in controls, 22.6 ± 5.5 mol/L in the placebo group, and 21.6 ± 2.9 mol/L in the treated group (P > 0.05). No correlation was found between maternal and fetal concentrations in any group (P > 0.05). Fetal S100B concentrations were significantly higher in the placebo IUGR group (3.73 ± 1.63 μg/L) when compared with the NO-treated IUGR group (0.91 ± 0.43 μg/L) and controls (1.54 ± 0.72 μg/L; P < 0.05), whereas no differences were observed between the NO-treated IUGR group and controls (P > 0.05; Fig. 1). S100B concentrations correlated with the MCA PI in untreated growth-retarded fetuses (r = 0.64; P < 0.01), but not in the NO-treated IUGR group (P > 0.05). We found no significant correlations between maternal or fetal S100B and NOx concentrations in any group (P > 0.05).

At birth, as expected, gestational age, placental weight, and birth weight were significantly higher in the control group (P < 0.05). Apgar scores evaluated at 1 and 5 min were similar in the three groups. No significant differences between placebo and treated groups were observed in birth weight or Apgar scores at 1 or 5 min (P > 0.05), but gestational age was significantly higher in the treated group (P < 0.05). The incidences of adverse neonatal neurologic outcome and respiratory distress syndrome were significantly lower in the treated groups (P < 0.05).

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<th>Table 1. Characteristics of pregnant women studied.</th>
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<td>Maternal age, years</td>
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<td>Fetal plasma NOx, mol/L</td>
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a Mean ± SD

b P < 0.05.
The clinical and monitoring parameters evaluated in the present study are consistent with previous observations indicating that transdermal glyceryl trinitrate administration improves uteroplacental perfusion, the quality of the pregnancy, and short-term neonatal outcome (2–5). As reported for other NO donors, long-term administration of NO donors to the mother does not affect maternal plasma NO concentrations (5, 19). Nitroglycerin has a low molecular weight and rapidly crosses the placenta, directly affecting placental and fetal compartments.

The present research shows that S100B, a well-established biochemical marker of brain distress, is significantly decreased in the cord blood of IUGR fetuses after maternal treatment with a NO donor. The concentrations of S100B detected in the NO-treated group in this study were essentially superimposable on those of preterm healthy fetuses in a previous study (11). The significantly higher S100B concentrations in IUGR fetuses of the placebo group compared with preterm controls support the view that the increase did not reflect a lower gestational age. The high cord blood S100B concentrations observed in IUGR fetuses whose mothers were not treated with NO offer laboratory support for the notion that fetal brain distress may lead to neonatal brain damage even when fetal hemodynamic adaptive mechanisms are present. This finding is relevant because redistribution in fetal circulation during IUGR is commonly regarded as neuroprotective (2–5, 20).

S100B may be released in the nervous system as a cytokine with a neurotropic role at low concentrations and a neurotoxic role at high concentrations (6). Changes in S100B concentrations measured in the cord blood of IUGR fetuses that have been treated with NO may reflect, at least in part, a relevant role of S100B in the cascade of events accompanying brain distress. S100B may also be released from other sites in which S100B is concentrated, such as adipose tissue, although data on the presence of the protein in adipose tissue at this age have not been published.

In conclusion, these findings suggest a simple and valuable tool to assess, at birth, both fetal brain distress accompanying IUGR and the positive effects of NO administration.

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References
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Fetal DNA in plasma and serum of pregnant women has been reported to be significantly increased in preeclampsia (1, 2). This increase may even precede clinical diagnosis (3). We hypothesized that subsequent development of hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome will further increase fetal DNA in maternal plasma and serum. In addition, total cell-free DNA may be increased in plasma as a result of tissue destruction, which generally occurs in HELLP syndrome.

To confirm our hypothesis, we recruited three groups of pregnant women with informed consent and matched for maternal age [MA], in years] and gestational age [GA], in weeks + days] at the time of blood drawing; the median ages (and ranges) were as follows: normotensive women (n = 10), MA, 30 (24–37) and GA, 32 + 6 (27 + 0–34 + 3); preeclamptic women without HELLP syndrome (n = 7), MA, 28 (24–36) and GA, 33 + 0 (27 + 6–34 + 3); and preeclamptic women with HELLP syndrome (n = 10), MA, 31 (26–38) and GA, 26 + 6–33 + 6. All women in the latter group had HELLP syndrome at the time of the blood drawing, except two who developed HELLP only after delivery. All participants were carrying single male fetuses. Preeclampsia was defined as a diastolic blood pressure > 90 mmHg on two or more consecutive occasions after 20 weeks of pregnancy in previously normotensive women, with proteinuria > 0.3 g/L in a 24-h collection period. HELLP syndrome was defined as the simultaneous occurrence of hemolysis (lactate dehydrogenase, > 600 U/L), increased liver enzymes (serum aspartate aminotransferase and alanine aminotransferase > 70 U/L), and low platelets (< 100 × 10^9/L) (4). Diastolic blood pressure [median (ranges)] in mmHg] was 70 (60–80), 120 (110–130), and 118 (110–140) for women with normotensive and preeclamptic pregnancies without and with HELLP syndrome, respectively.

Blood was collected in a plain and an EDTA-containing Vacutainer Tube from all pregnant women (n = 27). Serum and plasma were obtained by centrifugation for 10 min at 1200g and stored at −30°C. We isolated DNA from 500 μL of thawed plasma or serum using the PureGene and QIAquick protocols and eluted the DNA in 50 μL of 10 mmol/L Tris-HCl (pH 8.5), as described previously (5). Real-time quantitative PCR (ABI Prism 7700 Sequence Detection System; Applied Biosystems) was used for gene quantification. Copy numbers of the SRY gene (GenBank Accession No. X57772) and albumin gene (GenBank Accession No. M12523) were quantified separately in split samples to determine the amount of fetal DNA and total DNA, respectively. PCR primers and probe for SRY were as follows: SRY-106F, 5′-AATGCGGATAGTCAATCG-3′; SRY-192R, 5′-TAACGTGACTGCTCCGCTCTAGTTCCT-3′; and SRY-168VIC, 5′-VIC-ATCTGACCTGACTGCTGATCTACCT-3′. For each amplification reaction (50 μL), we used 300 nM each primer, 160 nM probe, 25 μL of TaqMan Universal Master Mix (Applied Biosystems), and 5 μL of DNA sample. After 10 min at 95°C, 40 two-step cycles were performed (30 s at 95°C and 1 min at 60°C). Primer and probe sequences for albumin were as follows: ALB-15659F, 5′-GAGTACCTGCTCCGCTCTAGTTCCT-3′; ALB-15739R, 5′-CCCTGACTGCTGATCTACCT-3′; and ALB-TET, 5′-TET-TGCTGACCTGCTCTGCTGATCTACCT-3′. Reaction conditions were identical to SRY amplification, except that 120 nM of the ALB-TET probe was used. Cycle threshold values for SRY and ALB were presented by the computer and were transformed to copy numbers with calibration curves of known concentrations of male genomic DNA (Sequence Detection Systems 1.7.1; 6 pg of DNA = 1 SRY copy = 2 ALB copies = 1 cell-equivalent). Finally, copy numbers of SRY and ALB were expressed in cell-equivalents/mL of maternal plasma or serum.

DNA from each plasma sample was isolated and quantified on two different occasions: day 1 and day 2. DNA from serum was analyzed once on day 2. Therefore, SRY and albumin gene copy numbers could be quantified in 27 × 3 (× 2 plasma and × 1 serum) = 81 samples. Because of insufficient material, however, only 73 samples were available, and in 1 sample, quantification of the SRY gene failed because of an erroneous laboratory procedure. As a result, 27 pregnant women provided 73 DNA samples, leading to 73 albumin and 72 SRY measurements, respectively.

Both fetal and total DNA in plasma increased markedly with the severity of the hypertensive disorder of pregnancy (Fig. 1). Only plasma DNA values of day 1 were presented because these were quantified in all 27 individuals. Note that the two preeclamptic women who developed HELLP syndrome after delivery had fetal cell-free DNA concentrations that were higher than those of