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Activin A in asphyxiated full-term newborns with hypoxic ischemic encephalopathy

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1. ABSTRACT

Activin-A is a protein over-expressed and secreted by the brain after neuronal destruction. We evaluated whether serum activin-A increases in asphyxiated full-term newborns (AFTNs) at risk of hypoxic-ischemic-encephalopathy (HIE). 105 consecutive infants (35 affected by perinatal asphyxia due to acute fetal distress; 70 healthy gestational-age matched newborns) underwent cranial assessment and neurologic examination at 12, 24 and 72 hours after birth and, on discharge from the hospital and; activin-A and monitoring laboratory variables assessment at birth. According to the occurrence of HIE within 7-days after birth, AFTNs were subdivided in Group A (n= 20; no/mild HIE with good prognosis) and Group B (n= 15; moderate/severe HIE with a greater risk of neurological handicap). Activin-A was significantly (P less than 0.0001) higher in Groups A and B than controls and highest (P less than 0.001) in Group B. At 0.66 ng/L activin-A achieved a sensitivity of 93.33% and a specificity of 96.63%, respectively, as HIE diagnostic test. These findings show that activin A increased in AFTNs with HIE before the appearance of related signs.

2. INTRODUCTION

Activin A is a dimeric growth factor composed by two beta-A subunits, belonging to the transforming growth factor-beta superfamily of differentiation factors (1), and expressed in the Central Nervous System (CNS), where it exerts a number of biological actions, ranging from the regulation of the hypothalamus-pituitary-ovarian axis (1, 2) to synaptic plasticity (3). It has been shown that hypoxic/ischemic injury, mechanical irritation, and chemical damage of brain evoke a strong up-regulation of activin A and, because its induction occurs early after brain injury, it has been suggested that its measurement may provide a potential biochemical index of the presence, location, and extent of brain injury (4). Both *in vitro* and animal studies strongly implicated enhanced activin A expression as a common response to neuronal damage of various origins (4), and *in vivo* increased levels of activin A were reported in the urine (5) and the cerebrospinal fluid (CSF) (6) of asphyxiated full-term newborns developing Hypoxic Ischemic Encephalopathy (HIE).

Table 1. Neonatal clinical parameters at birth in asphyxiated newborns with absent/mild HIE (Group A) and moderate/severe HIE (Group B) and in healthy subjects (Group C)

	Group A	Group B	Group C
Gestational age >36 weeks (n°)	20	15	70
Male/Female	16/14	7/8	37/33
Birth weight (g)	3,326 +/- 232	3,284 +/- 330	3,353 +/- 416
Apgar score <3 at 1st min (n°)	20/20[1]	15/15[1]	0/70
Apgar score <3 at 5th min (n°)	20/20[1]	15/15[1]	0/70
RDS (yes/total)	8/20[1]	6/15[1]	2/70
Mechanical Ventilation Support (yes/total)	20/20	15/15	0/70
HIE according Sarnat's test			
- No or Mild (yes/total)	20/20[1]	0/15[1]	0/0
- Moderate or Severe (yes/total)	0/20[1]	15/15[1]	0/0
Cerebral US (normal/hyperechogenicity/CNS damage)			
- at birth	0/11[1]/0	0/9[1]/0	70/0/0
- after 12 hours	0/11[1]/0	0/9[1]/0	70/0/0
- after 24 hours	0/11[1]/0	0/9[1]/0	70/0/0
- after 72 hours	0/7[1]/0	0/4[1]/7	70/0/0
Prechtl's Test (normal/suspect/abnormal)			
- at birth	13/7/0[1]	4/7/4[1]	30/0/0
- after 12 hours	13/7/0[1]	4/7/4[1]	30/0/0
- after 24 hours	13/7/0[1]	4/7/4[1]	30/0/0
- after 72 hours	13/7/0[1]	4/7/4[1]	30/0/0

[1]P less than 0.0001 vs controls.

HIE is an important cause of mortality and morbidity in full-term newborns and long-term sequelae such as cerebral palsy, mental retardation, epilepsy, and learning disability have been shown in about 25- 28% of these infants (7). In term neonates, about 1-1.8% of infants suffer birth asphyxia and one third manifest significant neurologic deficits (7). Despite accurate postnatal monitoring procedures, the post-asphyxia period is crucial since, at a time when radiological pictures are still negative, brain damage may already be at a sub-clinical stage, with the symptoms being hidden by sedation (8). Nor is it yet clear how much time is available for pharmacological intervention: studies in perinatal animals suggest very rapid cellular destruction, while in adults neuronal death and apoptosis are slower and may require hours or several days (9, 10). At the present, the major diagnostic tools consist in the measurement of blood pH, uric acid and lactate levels, together with cerebral ultrasound and continuous Electroencephalography (EEG) recordings (8, 11, 12). The measurement of quantitative parameters, such as protein constituents of the nervous tissue, able to detect sub-clinical lesions at a stage when routine brain monitoring procedures are still silent could thus be particularly useful in the brain injury prevention and/or management (13).

In the present study we explored the hypothesis that activin A may be increased in serum collected immediately after birth from asphyxiated full-term newborns at risk of HIE, and evaluated whether measurement of its levels may constitute a useful tool for the early detection of post-asphyxia HIE.

3. MATERIALS AND METHODS

3.1. Patients

We carried out a case-control study at tertiary referral centers for Obstetrics and Neonatal Intensive Care units (NICUs) between April 2002 and August 2006, comprising 35 consecutive infants with perinatal asphyxia and 70 healthy infants as controls (1 asphyxia case vs 2 controls). Approval was obtained from the Human Investigations Committees of the participating institutions, parents of the subjects gave informed consent, and all procedures were performed according to the hospital's guidelines.

Case subjects were full term (gestational age more than 36 weeks) asphyxiated newborns delivered by emergency caesarean section due to acute fetal distress, defined according to the American College of Obstetricians and Gynecologists as non-reassuring fetal status (bradycardia, late deceleration of the fetal heart rate, severe and repetitive variable deceleration of the fetal heart rate, reduced beat-to-beat variability) (14). Asphyxia was defined according to an Apgar score less than 3 at the 5th minute, pH less than 7.0, or BE less than -12 in cord blood or venous blood taken from newborns within 60 min of birth, or the need for positive pressure ventilation (more than 3 minutes) (14). Infants that fulfilled 3 or more of the above clinical and biochemical criteria were included in the asphyxia group. Infants with any malformation, systemic infection, intrauterine growth retardation, or cardiac or hemolytic disease were excluded from the study. Other exclusion criteria were multiple pregnancies, congenital or perinatal infections, including chorioamnionitis, maternal drug addiction, maternal hypertension and diabetes.

Control subjects were 70 healthy term neonates matched for gestational age, delivered consecutively and in the same time period as the case subjects both by elective cesarean section (n= 23) or vaginally (n= 47).

Newborns in the asphyxiated group were mechanically ventilated and sedated using Fentanyl (Fentanest, Pharmacia and Upjohn, Milan, Italy) 0.5-2.5 microg/Kg/hr, and Midazolam (Ipnovel, Roche, Milan, Italy) 50-400 microg/Kg/hr. In all the asphyxiated newborns cerebral ultrasound scanning was recorded and neurological examination assessed at the time of urine collection by a single examiner who did not know the results of the urine test. Blood was drawn by means of a catheter inserted in the cubital vein at birth, in order to monitor clinical and laboratory parameters.

In all asphyxiated newborns, cranial assessment (by cerebral ultrasound scanning) and neurologic examination were performed at 12, 24 and 72 hours after birth (Table 1). In healthy controls, monitoring variables were recorded at birth and reviewed 12 hours later.

Arterial blood samples were obtained from umbilical cord in the first hour after birth to measure activin A and to monitor laboratory variables for the standard assessment (i.e. RBC count, glycaemia, urea, creatinine and ions concentration)

Table 2. Neonatal monitoring parameters at birth in asphyxiated newborns with absent/mild HIE (Group A) and moderate/severe HIE (Group B) and, in healthy subjects (Group C). Values are expressed as mean +/- SEM

	Group A (n=20)	Group B (n=15)	Group C (n=70)
Red blood cell count (10^6/mm^3)	3.92 +/- 0.2	3.88 +/- 0.1	3.9 +/- 0.05
Hemoglobin (g/dL)	13.5 +/- 0.03	13.6 +/- 0.08	13.6 +/- 0.06
Hematocrit rate %	40.2 +/- 0.4	40.4 +/- 0.6	41.1 +/- 0.5
Venous blood pH	7.03 +/- 0.01[1]	7.01 +/- 0.02[1]	7.36 +/- 0.06
Partial venous CO_2 pressure (mmHg)	69.6 +/- 1.8[1]	66.3 +/- 4.5[1]	41.3 +/- 0.6
Partial venous O_2 pressure (mmHg)	21.1 +/- 0.9[1]	19.6 +/- 1.7[1]	40.7 +/- 0.6
Base excess	-13.4 +/- 0.2[1]	-13.2 +/- 0.3[1]	-0.2 +/- 1.1
Na^+ (mmol/L)	139 +/- 0.5	139 +/- 1.2	140 +/- 0.6
K^+ (mmol/L)	4.2 +/- 0.1	4.3 +/- 0.2	4.1 +/- 0.2
Ca^{++} (mmol/L)	1.12 +/- 0.03	1.12 +/- 0.04	1.14 +/- 0.03
Plasma glucose (mmol/L)	4.2 +/- 0.3	4.2 +/- 0.2	4.3 +/- 0.1
Urea (mg/dL)	35.2 +/- 1.6	34.8 +/- 0.9	34.6 +/- 0.8
Creatinine (mg/dL)	0.86 +/- 0.09	0.87 +/- 0.12	0.80 +/- 0.36

[1]P less than 0.0001 vs controls.

3.1.1. Cranial Assessment

Standard cerebral ultrasound was performed by real-time ultrasound machine (Acuson 128SP5, Mountain View CA, USA) using a transducer frequency emission of 3.5 MHz) in the 12-24 hours after birth, at 72 hours from admission and on discharge from the hospital. In the controls cerebral ultrasound patterns were evaluated before discharge from the hospital 72 hours from birth.

3.1.2. Neurodevelopmental outcome

In the asphyxiated group, the presence within the first 7 days after birth of HIE was classified according to the criteria described by Sarnat (15). HIE was defined as mild if hyperexcitability or hypotonia persisted without seizures for at least 72-hours after birth; as moderate if the infant was lethargic and had hypotonia, weak primitive reflexes, and seizures; and as severe if the infant showed frequent seizures, apnea, flaccid weakness, or coma. EEG traces were recorded in the asphyxiated infants within 7-days from birth.

Neonatal neurological conditions were classified using a qualitative approach (16), by which each infant was assigned to one of three diagnostic groups: normal, suspect or abnormal. An infant was considered to be abnormal when one or more of the following neurological syndromes were unequivocally present: a) increased or decreased excitability (hyperexcitability syndrome, convulsions, apathy syndrome, coma); b) increased or decreased motility (hyperkinesia, hypokinesia); c) increased or decreased tonus (hypertonia or hypotonia); d) asymmetries (peripheral or central); e) defects of the central nervous system; f) any combination of the above. When indications of the presence of a syndrome were non-conclusive or if only isolated symptoms were present, e.g. mild hypotonia or only a slight tremor, the infant was classified as suspect.

3.2. Activin-A assay

Activin A measurement was blinded performed in duplicate using a specific two-site enzyme immunoassay purchased from Serotec (Oxford, Oxford, UK) as previously described (5, 6). The limit of detection for activin A was 10 pg/mL, and intra- and inter-assay coefficients of variation were 2.5% and 3.0% respectively. The activin A assay has no detectable cross-reaction with inhibin A, inhibin B, follistatin, activin B. Activin A plates were read at 490 nm on an automated ELISA plate reader.

3.3. Statistical analysis

The Kolmogorov-Smirnov test revealed that values had a Gaussian distribution, and therefore data were expressed as mean +/- SEM. Indexes were analyzed by ANOVA test followed by the Tukey's post-hoc test for parametric evaluations, and comparison between proportions was performed with the two-tail Fisher exact test. Data on neonatal outcomes and laboratory parameters were analyzed by Turkey one-way ANOVA and Mann-Whitney U test when not normally distributed.

We used the cut-off indicated by Receiver Operating Curve (ROC) analysis (17) to evaluate the positive and negative predictive values, specificity and sensitivity, and likelihood ratios (LR) with their respective 95% confidence bounds.

Statistical analysis was performed using the GraphPad Prism version 3.00 for Windows (GraphPad Software, Inc., San Diego, CA). A value of P less than 0.05 was considered statistically significant.

4. RESULTS

4.1. Clinical findings

At birth, no significant differences regarding weight, gestational age and gender distribution were found between asphyxiated and control groups, but according to the occurrence of HIE within the first 7-days after birth, asphyxiated infants were thus subdivided as follows: Group A (n= 20): no or mild HIE with good prognosis; Group B (n= 15): moderate or severe HIE with a greater risk of neurological handicap (Table 1).

Table 1 and Table 2 show the clinical and biochemical characteristics, respectively, recorded at birth in the three groups studied. As expected, Apgar scores at the 1st and 5th minutes, pH, PvCO2, base excess and the incidence of Acute Respiratory Distress Syndrome (ARDS) were significantly different in asphyxiated newborns compared with controls (P less than 0.001, for all) regardless of the severity of HIE, whilst no differences were found between the two asphyxiated sub-groups in the incidence of ARDS, clinical and laboratory monitoring parameters, or in cerebral ultrasound scans (P more than 0.05, for all).

At birth, periventricular hyperechogenicity was observed in 20 out of 35 asphyxiated infants (Group A: n= 11; Group B: n= 9; P more than 0.05), but no significant cerebral ultrasound pattern differences were found between the two asphyxiated sub-groups. The same ultrasound patterns were observed at 12, 24 and 48 hours monitoring time-points, respectively and no significant differences

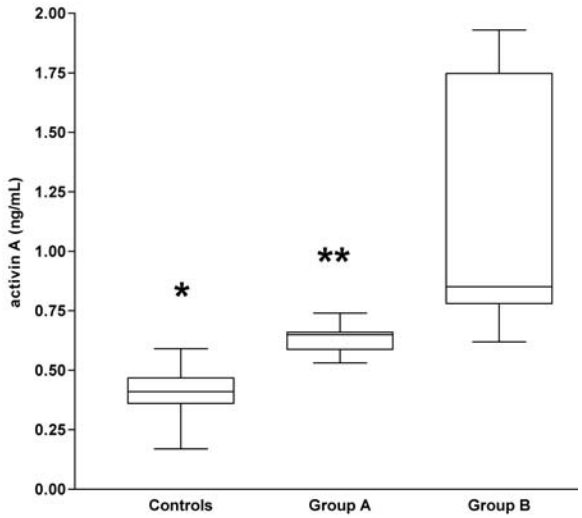

Figure 1. Serum activin A levels (ng/mL; expressed as mean +/- SE) at birth in healthy neonates (Controls, black circles), and in asphyxiated full term infants with absent/mild (Group A, open circles) and moderate/severe (Group B, triangles) HIE. Box plots represent medians and interquartile ranges *P less than 0.001 vs Group A and Group B; **P less than 0.001 vs Group A.

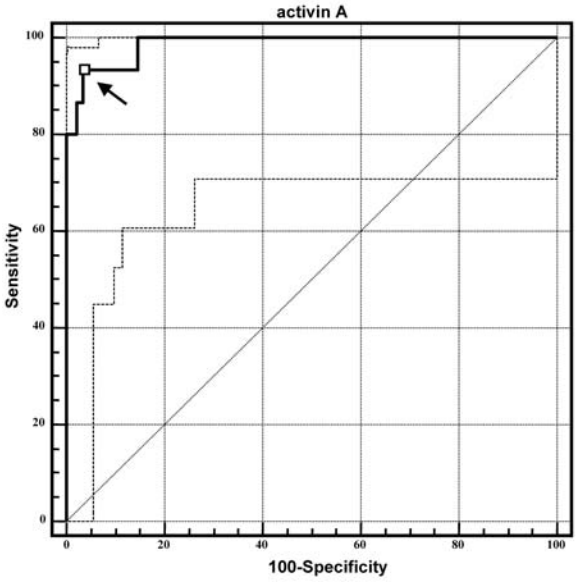

Figure 2. ROC curve analysis of serum concentrations of activin A for the prediction of HIE. Activin A at the cutoff of 0.66 ng/mL (indicated by the arrow) achieved the best accuracy for prediction of HIE in asphyxiated full term newborns (area under the ROC curve: 0.987; $CI_{95\%}$: 0.911 to 0.998%, P less than 0.0001, positive LR: 27.69; negative LR: 0.069).

were shown between asphyxiated sub-groups (P more than 0.05, for all, ns). At 72-hours cerebral ultrasound was negative for cerebral bleeding in all but 7 Group B infants (middle cerebral artery infarction: n= 2, intraventricular hemorrhage (IVH): n= 2; IVH with ventricular dilatation: n= 3). In the controls cerebral ultrasound patterns were negative for bleeding or other central nervous system diseases.

Fourteen of 35 asphyxiated infants were classified as suspect at neurological examination on admission (hypo-hypertonia: n= 9; hyperexcitability: n= 5). However, on account of their severe clinical conditions, the effects of sedative drugs and intervention by neonatal intensive care units, neurological examination was inconclusive, especially during the first 24-hours after asphyxia insult.

4.2. Serum activin A levels and the prediction of HIE

Serum activin A levels were detectable in all the samples measured. In details, concentrations were significantly higher in Group A (0.63 +/- 0.01 ng/mL; P less than 0.001) and Group B (1.13 +/- 0.12 nm; P less than 0.001) than in controls (0.41 +/- 0.01 ng/mL); and significantly (P less than 0.001) higher in Group B than Group A (Figure 1). At the best cut-off (activin A more than 0.66 nm) chosen by the ROC curve analysis (Figure 2) activin A achieved a sensitivity of 93.33% ($CI_{95\%}$: 68.0-98.9%) and a specificity of 96.63% ($CI_{95\%}$: 90.5-99.3%), respectively as a diagnostic test for HIE (ng/mL area under the curve: 0.987; $CI_{95\%}$: 0.911 to 0.998%) (Figure 3), with positive and negative LRs of 27.69 and 0.069, respectively.

5. DISCUSSION

Activin A is a dimeric glycoprotein composed by two betaA subunits, initially isolated from gonadal tissue (1, 2), but subsequent studies have shown the presence of betaA subunit mRNA and protein in non-genital sites and, mainly in the CNS where it is supposed to play important roles on neural development (1, 3, 18). On this regard, a large body of evidence (reviewed in 2) has shown that brain lesions up-regulate the expression of activin A and, that its temporal and spatial interplay appears crucial for orchestration of post-lesion restructuring: studies employing models of acute brain injury strongly favor the notion that enhanced activin A expression represents a common response to acute neuronal damage of various origin (2). With respect to human studies, we have already demonstrated high activin A concentrations into the cerebrospinal fluid (6) and the urine (5) of term infants affected by asphyxia and, on the basis of *in vitro* and animal studies (2), hypothesized that the increase of activin A may signal brain injury at birth. The present study is in line with those clinical findings in perinatal medicine, since it first reports that at birth full-term asphyxiated infants had increased serum activin A levels than healthy newborns and, that concentrations were higher in the asphyxiated infants who developed severe HIE than in those who did not or in controls. These data lead us to support again the notion that i) hypoxia/asphyxia may trigger activin A secretion and, ii) elevated activin A levels may be reasonably a direct expression of CNS increased production. Indeed, the increased release of activin A is a common feature not only in full-term infants with asphyxia and brain damage (5, 6 and present data), but also in newborns with IVH due to hypoxia (19, 20). Moreover, the association between the increase of activin A and the

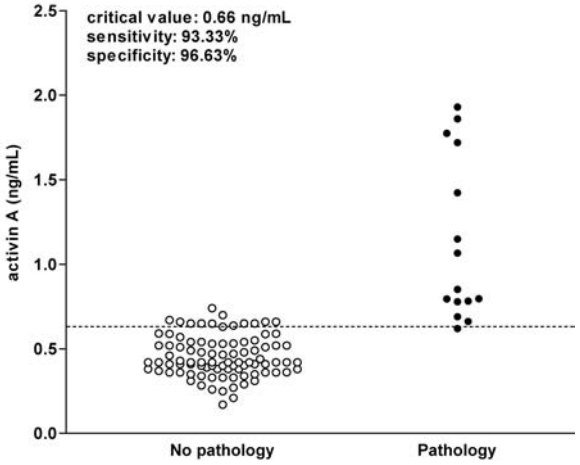

Figure 3. Data of both positive (HIE, pathology) and negative (no pathology) groups are displayed as dots on two vertical lines. The horizontal line indicates the cut-off point (critical value) with the best separation between the two groups.

degree of the cerebral distress in children underwent open heart surgery (21), a procedure associated with brain injury (22), would strongly support the concept that the raise of activin A may derive from an increased production into the CNS due to local injury. Due to its low molecular weight (about 28 kDa) (23), high activin A concentrations could also be due to increased intracranial pressure and cerebral edema (particularly in the white matter), which are known both to damage the CNS and alter brain-blood barrier permeability. Since activin A is also produced by the human placenta (24), increased levels of activin A in newborns may be due to increased placental expression and synthesis, however *in vitro* data revealed that hypoxia significantly reduced synthesis and secretion of activin A by the human placenta (25, 26).

In the present study we also found that serum activin A levels were higher in asphyxiated infants who developed severe HIE than in those who did not or in controls, but at a stage when ultrasound and other diagnostic procedures were still silent and of no avail, since on admission to our units standard monitoring procedures, laboratory parameters and neurological examination patterns were unable to indicate which infants would develop HIE. Conversely, already at this stage, newborns with serum activin A levels above the threshold defined by the ROC curve analysis had a great probability of developing HIE, since activin A measurement had a sensitivity and a specificity of 93.33% and 96.63%, respectively, as a diagnostic test for HIE, sharing values similar to those computed with the use of activin A determination into CSF (6) and urine (5). Moreover, an early increase in serum activin A levels has been reported in preterm newborns with signs of perinatal hypoxia (20) and in preterm newborns who later develop IVH (19). These data sustain the concept that the rise of activin A into bloodstream early occurs in brain injuries, and provide additional support for the expedience of monitoring activin A in the biological fluids for use it as a measurable parameter of brain lesion before routine monitoring procedures can be performed. Given that the half-life of the protein is about 45 minutes (23), this test also would offer the possibility of repeated monitoring to evaluate the timing as well as the duration of neuronal cells destruction.

The final point that merits discussion refers to the putative role of such an increased secretion of activin A. Indeed, it was proposed that the up-regulation of activin A in brain injury might serve as a neuroprotective factor, due to the fact that activin-A enhances the survival of midbrain and hippocampal neurons (27, 28); it decrease ischemic brain injury in infant rats (29); and it shields striatal and midbrain neurons against neurotoxic damage (27, 30). These evidences and those related to role of activin A in preventing apoptosis (31) and inhibiting caspase (32), two important pathways of neuronal death (33), together would propose the over-secretion of activin A as a rescue mechanism to reduce cell death after brain insults.

In conclusion, activin A levels are higher in serum of asphyxiated full-term newborns underwent to HIE, and may be useful to predict hypoxic-ischemic lesions before the appearance of related biophysical signs.

6. ACKNOWLEDGMENTS

All authors equally contributed to this article. All Authors have nothing to declare.

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Abbreviations: HIE: hypoxic ischemic encephalopathy; CNS: central nervous system; CSF: cerebrospinal fluid; NICUs: obstetrics and neonatal intensive care units; RBC: red blood cells; ARDS: acute respiratory distress syndrome; IVH: intraventricular hemorrhage

Key Words: Hypoxia, Brain Damage, Sensitivity, Specificity, Asphyxia

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