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Original article

Preliminary case control study to establish the correlation between novel peroxidation biomarkers in cord serum and the severity of hypoxic ischemic encephalopathy

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ABSTRACT

Background: Hypoxic-ischemic encephalopathy (HIE) has deleterious neurological consequences. To identify patients at risk of neuronal damage deserving implementation of neuroprotective strategies clinicians have relied on prenatal sentinel events, postnatal clinical assessment (Apgar score), and blood gas analysis.

This feasibility study aimed to assess if lipid peroxidation byproducts associated with neuronal damage correlated with cord blood metabolic acidemia in patients with HIE.

Population and Methods: This is a case/control study in which cases were newborn infants with severe acidemia (pH < 7.00; base excess ≥ 12 mmol/L) while control babies exhibited normal gases (pH = 7.20–7.40; base excess = −4 to +4 mmol/L) in the first cord blood analysis performed immediately after birth. Concomitantly, lipid peroxidation byproducts were determined using ultra performance liquid chromatography coupled to mass spectrometry in the same cord blood sample.

Results: A total of 19 controls and 20 cases were recruited. No differences in gestational characteristics were present. However, cases exhibited profound metabolic alterations as compared to controls (Cases vs. Control: pH = 6.90 ± 0.1 vs. 7.33 ± 0.03; base excess = −15 ± 3 vs. −1 ± 2 mmol/L), 85% were admitted to the NICU, and 50% developed symptoms of HIE. 8-iso-15(R)-PGF2α (P = 0.01) and total isoprostanes (P = 0.045) presented statistically significant differences between cases and control groups and correlated with level of HIE.

Conclusions: The 8-iso-15(R)-PGF2α and isoprostanes reflecting oxidative damage are significantly increased in severe postnatal acidemia. Follow up studies with adequate power are necessary to confirm if these biomarkers measured in cord blood serum could be predictive of neonatal encephalopathy.

1. Introduction

Impaired gas exchange during labor leads to hypoxemia, hypercapnia, and metabolic acidosis that if severe enough may cause neonatal encephalopathy [1]. Brain cells are highly dependent on aerobic metabolism to obtain energy for ATP powered ion pumps, synaptic activity, and redox status. Intense hypoxia and re-oxygenation with high oxygen concentrations may lead to cell swelling, hyperexcitability and oxidative stress [2]. Altogether these circumstances may cause neuronal necrosis or activate pro-apoptotic pathways that lead to amplification of the initial damage evolving for hours, days or even weeks [3]. Injury can occur as intracranial hemorrhage or infarction, or the more global injury of hypoxic-ischemic encephalopathy (HIE). HIE occurs in 1–8 per 1000 live births in developed countries. Notably, around 30% of the affected newborns will die in the postnatal period, and a significant number of survivors will develop severe and permanent neurocognitive, motor or sensorial sequel [4].

In perinatal asphyxia, blood gas analysis of arterial cord samples is characterized by a profound metabolic acidemia with increased lactate concentration. However, lactate production can be induced by non-hypoxic conditions such as glycogenolysis, alkalosis, or catecholamine infusion. Therefore, the reliability of the analysis of cord

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blood gases is of limited value in predicting HIE [5–7]. Moreover, the clinical methods employed to assess HIE which include Apgar score, amplitude integrated electroencephalography, and magnetic resonance imaging are useful for predicting some outcome of neonatal hypoxia but lack the sensitivity needed to implement effective therapies such as hypothermia [8].

For these reasons, there has been a protracted search for biomarkers assessing brain damage and predicting neurocognitive outcome in the neonatal population. The aim of these markers would be to early detect and monitor neuronal damage, to study the pathogenesis of brain damaging disorders, and finally being reliable in predicting outcome [9]. To date the most widely employed encompass among others S100B, neuron-specific enolase, activin A, adrenomedullin, Interleukin 1β, and IL6. However, increased levels in different biofluids of these biomarkers do not always correspond to brain damage. Moreover, these metabolites are not brain specific and may be released by tissues different than brain [10]. Recently, highly specific proteins such as glial fibrillary acidic protein (GFAP) and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), markers of gliosis and neuronal injury, respectively, have been employed to assess neuronal damage in HIE. Notably, while these biomarkers have shown to be useful in adult brain conditions, findings in the neonatal patients have been somewhat limited. Although some studies shed promising results for both biomarkers for assessment of the degree of brain damage and later neurodevelopmental outcomes [11], more recently it has been confirmed that there is no correlation between cord blood samples and HIE staging and later neurodevelopmental outcomes. Thus, GFAP and UCH-L1 plasma levels correlate with the severity of HIE and with neurocognitive outcome when determinations are made between 6 and 12 h after birth [12,13].

The role of oxidative stress biomarkers has been reviewed [14]. In the experimental setting metabolomic studies performed in animal models of hypoxia reoxygenation have shown that oxidative stress associated metabolites strongly correlate with brain injury. The most specific and reliable biomarkers identified have been isoprostanes, neuroprostanes, non-protein bound iron, and 4-HNE proteins adducts [15–17]. F2-isoprostanes have been determined to be both biomarkers and mediators of oxidative stress in numerous diseases [18]. Actually, a previous study revealed that F2-isoprostanes increased after ischemia-reperfusion injury in humans [19]. In a rat model of global perinatal asphyxia, increased brain levels of F2-isoprostanes were found in neonatal rats after 20-min asphyxia, as well as alterations in behavioral patterns [20]. Also, high 8-isoprostane levels were found in cerebrospinal fluid from preterm infants with evidence of white matter injury on magnetic resonance imaging at term [21].

The aim of this study was to identify if markers of lipid peroxidation validated in the newborn period significantly correlated with severe metabolic acidosis determined in cord blood serum immediately after birth using ultra performance liquid chromatography-mass spectrometry methodology.

2. Material and methods

2.1. Study design and participants

This is a prospective case-control observational study performed at the Division of Obstetrics and Gynecology of the Helsinki University Central Hospital (HUCH) during a 12-month period. The Ethics Committee of the HUH approved the study protocol and informed consent was obtained from the parents of all recruited newborn infants.

Eligible participants were inborn newly born infants with gestational age between 37 and 42 weeks. Patients were classified in two groups according to the umbilical cord arterial blood pH, base excess and pCO_2 obtained immediately after birth: control group (pH = 7.20 to 7.40, base excess = 4 to −4 mEq/L, pCO_2 = 5–6.9 kPa) and case group (pH ≤ 7.05, base excess = ≤ 12 mEq/L, pCO_2 > 8 kPa).

2.2. Sample collection and storage

Serum samples were obtained from cord umbilical artery immediately after cord clamping and before resuscitation maneuvers were initiated. Collected blood was immediately put into a dry cryotube. The tube was held in vertical position and at room temperature to improve the coagulation during 30 min after the extraction. Then, the sample was centrifuged at 2000 × *g* for 10 min at room temperature, and the supernatant (serum) was separated and aliquoted. Thereafter, samples were stored at −80 °C until analysis.

2.3. Reagents

Isoprostanes' standards of 8-iso-15(R)-$PGF_{2\alpha}$, 1a,1b-dihomo-$PGF_{2\alpha}$, 2,3-dinor-$iPF_{2\alpha}$-III, 8-iso-15-keto-PGE_2, 8-iso-15-keto-$PGF_{2\alpha}$, 8-iso-PGE_2, 5-$iPF_{2\alpha}$-VI, 8-iso-$PGF_{2\alpha}$, the prostaglandins PGE_2 and $PGF_{2\alpha}$, as well as the deuterated internal standard (IS) $PGF_{2\alpha}$-D_4 were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). Potassium hydroxide was from Sigma Aldrich Química SA (Madrid, Spain). Methanol (LC-MS grade) and n-heptane (analytical grade) were obtained from J.T. Baker (Avantor Performance Materials B.V., The Netherlands). Formic acid (98%) and ethyl acetate (analytical grade) were from Panreac (Barcelona, Spain).

2.4. Materials

SPE-96 well plates (Discovery® DSC-18, 100 mg) were used for sample solid-phase extraction (SPE) (Sigma-Aldrich, St. Louis, MO, USA). Ultrasonic bath (BandelinSonorexDigitec, Berlin, Germany) was used to improve the hydrolysis efficiency. Vortex mixer was from VelpScientifica (Usmate, Italy). Centrifuge Biocen22R was from OrtoAlresa (Madrid, Spain). Thermomixer HLC from Ditabis (Pforzheim, Germany). Speed vacuum concentrator (mi Vac) was from Genevac LTD (Ipswich, United Kingdom). 96-well sample plates (Acquity UPLC 700 μL) from Waters (Barcelona, Spain).

2.5. Ultra high performance liquid chromatography-mass spectrometry chromatographic system (UPLC-MS/MS)

The chromatographic system used consisted of a Waters Acquity UPLC-Xevo TQD system (Milford, MA, USA). The conditions employed were established in a recently published work [30]. Briefly, negative electrospray ionization (ESI^-), capillary voltage 3.5 kV, source temperature 120 °C, desolvation temperature 300 °C, nitrogen cone and desolvation gas flows were 25 and 680 Lh^{-1}, respectively, and dwell time was 5 ms. An Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm) and pre-column (2.1 × 5 mm) from Waters, and binary mobile phase CH_3OH (0.1% v/v HCOOH):H_2O (0.1% v/v HCOOH) with gradient elution were employed. The flow rate was 0.4 mL min^{-1}, the column temperature was 37 °C and the injection volume was 10 μL. Mass spectrometric detection was carried out by multiple reaction monitoring (MRM).

2.6. Sample treatment

The sample treatment was optimized and assayed as described before [30]. Briefly, serum samples were thawed on ice, and 100 μL