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Oxidative stress markers at birth: Analyses of a neonatal population

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ABSTRACT

In order to further understand neonatal stress and, thus, control it efficaciously, there is a need for more information on the manifestations of stress at the molecular level in the newborn, with particular regard to oxidants, and anti-oxidant and anti-stress mechanisms, including mitochondrial heat shock protein-chaperones such as Hsp60. We investigated patterns of anti-oxidants, biomarkers of oxidative stress, and Hsp60 levels in sera from newborns and found significant associations between glutathione (GSH) levels and gestational age, delivery modality, and lipid hydroperoxydes (LOOH) level. LOOH levels and spontaneous (vaginal) delivery were independently associated with increased GSH levels when these were above the median. Hsp60 and LOOH levels were positively correlated whereas Hsp60 and GSH levels were inversely correlated in spontaneously delivered newborns; in contrast, Hsp60 and GSH levels were positively correlated in newborns delivered by cesarea. Our results point to new directions in the search for definite patterns of GSH, LOOH, and Hsp60 in the newborn's serum that might have functional and diagnostic significance and that could help in the monitoring of newborn health during and after delivery. In addition, the data provide a starting basis for investigating the precise roles and interplay of GSH and Hsp60 in the maintenance of an optimal redox balance at birth to cope with the stress inherent to delivery, and also for investigating the predictive value of any given pattern of GSH, LOOH, and Hsp60 at birth with regard to health status and risk of disease in adult life.

Introduction

Birth is a very complex event and is associated with an elevated risk of unfavorable outcomes, even in at-term pregnancies of healthy women with no significant clinical history. Transition from intra- to extra-uterine life is a dramatic and stressful event. At the end of gestation, many physiological changes in various organs, such as those of the cardiovascular, respiratory, and urinary systems, occur in order to allow neonatal survival after birth. The antioxidant enzyme system and the non-enzymatic antioxidants are up regulated during the last period of gestation (Auten and Davis, 2009). Antioxidant enzyme expression generally increases in most fetal compartments throughout the progression of pregnancy. It has been found that superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase activities increase with gestational age, whereas lipid peroxidation decreases in human placental and fetal tissues (Ahamed et al., 2009). Besides oxygen, other factors, such as labor in spontaneous delivery or anesthesia and surgical operation in cesarean section, may also contribute to maternal and neonatal stress at the time of delivery (Pence et al., 2002). Furthermore, neonatal redox balance has been shown to be gender dependent (Hamon et al., 2011), and prenatal glucocorticoid exposure is known to influence oxidative balance in both fetus and placenta (Stark et al., 2011).

Oxidative stress occurs either through a free radical process or enzymatically in the course of arachidonic acid metabolism. Lipid peroxidation is potentially harmful because this uncontrolled

Abbreviations: AGA, gestational age; GSH, reduced glutathione; GSSG, oxidized glutathione; GPx, glutathione peroxidase; Hsp, heat shock proteins; LOOH, lipid hydroperoxides; ROS, reactive oxygen species; SOD, superoxide dismutase.

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self-enhancing mechanism causes disruption of membrane lipids and other cell components (Paamoni-Keren et al., 2007). High levels of malondialdehyde, isoprostanes, and lipid hydroperoxides (LOOH) have been detected in the perinatal period in various biological fluids of both complicated and uncomplicated pregnancies. To counteract lipid peroxidation, there exist various defensive mechanisms consisting of antioxidant free-radical scavenging molecules such as glutathione (GSH, a tripeptide consisting of glutamic acid–cysteine–glycine), which serves as substrate for the enzyme glutathione peroxidase. In addition to GSH, several other non-proteinic (e.g., vitamin E) and proteinic (e.g., heat shock proteins or Hsp) systems play a major role in the maintenance of redox balance.

Among the Hsp (David et al., 2013), the chaperonin Hsp60 is typically an intra-mitochondrial chaperone with a key role in the maintenance of the functional conformation of the intra-organellar proteins, including those involved in the respiratory chain (Ostermann et al., 1989), which is necessary for organism survival. For example, a case of congenital deficiency of Hsp60 in a female newborn who presented facial dysmorphic features, hypotonia, and breathing difficulties and developed metabolic acidosis, died of heart failure 2 days after birth (Agsteribbe et al., 1993).

Hsp60 levels may increase either during stress or under pathologic conditions such as chronic inflammatory (Cappello et al., 2011), autoimmune (Rodolico et al., 2010), and cardiovascular (Novo et al., 2011) diseases. Extracellular Hsp60 levels have been found significantly higher in children with septic shock compared to critically ill children without septic shock and to healthy controls (Wheeler et al., 2007). However, to the best of our knowledge, no data are so far available on Hsp60 levels in healthy at term newborns. Altogether, data on the molecular patterns of the newborn stress are scarce. To fill this gap, the present study aimed to estimate the impact of a series of maternal, fetal, and environmental variables on GSH, LOOH and Hsp60 levels in healthy at term newborns.

Materials and methods

Subjects and samples

A total of 80 healthy at term newborns were recruited at random, applying the following criteria: born in house in our University Clinic; absence of congenital malformations, severe congenital illness or any other condition requiring neonatal intensive care; and no history of maternal congenital heart disease or chronic inflammatory disease.

Cord blood samples were collected at birth in sterile tubes and centrifuged at 1500 × *g* at 24 °C for 10 min, and plasma and serum were separately collected and stored at −80 °C for subsequent determinations. Main anamnestic (maternal illness, prophylaxis, gestational age), anthropometric (weight, length, head circumference) and clinical (Apgar score, amniotic fluid characteristics, ventilation performances) data for each newborn were collected according to routine institutional guidelines and stored in an electronic datasheet (Microsoft Excel) for subsequent analyses. A serial identification code was given to each newborn for both, biological samples and clinical information, to honor personal privacy and evaluate the data without bias.

The study was approved by an institutional review committee and the parents of the newborns gave informed consent. All procedures were in accordance with institutional guidelines and did not cause any additional stress or risk to mothers or newborns.

Determination of lipid hydroperoxide (LOOH) levels

Plasmatic levels of LOOH were evaluated in a subset of 57 randomized samples by assessing the oxidation of Fe^{2+} to Fe^{3+} in the presence of xylenol orange at $\lambda = 560$ nm (Novo et al., 2011). The assay mixture contained, in a total volume of 1 mL/100 μL of plasma, 100 μM xylenol orange, 250 μM ammonium ferrous sulfate, 90% methanol, 4 mM butylated hydroxytoluene, and 25 mM H_2SO_4. After a 30-min incubation at 27 °C, the absorbance was measured using a U2000 Hitachi spectrophotometer. Calibration was done using 0.2–20 μM hydrogen peroxide. Experiments were performed in duplicate.

Determination of glutathione (GSH) levels

Plasmatic levels of GSH were measured in a subset of 52 randomized samples in 200 μL of plasma, using a spectrophotometric assay based on the reaction of thiol groups with 2,2-dithio-bis-nitrobenzoic acid (DTNB) at $\lambda = 412$ nm ($\epsilon_M = 13{,}600\ M^{-1}\ cm^{-1}$, where ϵ_M is a wavelength-dependent molar absorptivity coefficient). Experiments were performed in duplicate.

Determination of Hsp60 levels

Hsp60 determination by ELISA was performed in a subset of 19 randomized serum samples as described (Rizzo et al., 2012; Parenti et al., 2010), and a commercial kit (Hsp60 human EIA kit, Enzo Life Science, Cat No. ADI-EKS-600, Enzo Life Science AG, Lausen, Switzerland). All reagents were brought at room temperature and the Hsp60 standard was diluted to generate a standard curve with six points, ranging from 3.125 to 100 ng/mL according to the manufacturer's instructions. First, 100 μL of prepared standards and serum (undiluted) was added in duplicate to each wells and incubated at room temperature for 1 h. After washing six times with 1× washing buffer included in the kit, 100 μL of diluted anti-Hsp60 goat polyclonal antibody was added to each well and incubated at 24 °C for 1 h. Then, 100 μL of diluted horseradish peroxidase-conjugate anti-goat IgG was added to the wells and incubated at 24 °C for 30 min. After washing, 100 μL of 3,3′,5,5′-Tetramethylbenzidine substrate was added and incubated for 15 min in the dark. Lastly, 100 μL of acid stop solution was added and absorbance was measured at 450 nm, using a microplate photometric reader (DV990BV4, GDV, Milan, Italy). Sample concentration was calculated by interpolating the measured values in the standard curve. The sensitivity of the Hsp60 (human), Enzyme Immuno Assay (EIA) we utilized has been determined to be 3.125 ng/mL. The Intra-Assay Coefficient of variation of Hsp60 (human), EIA has been determined to be <10% and the inter-assay coefficient of variation has been determined to be <10%. Hsp60 (human) EIA is specific for Hsp60 and has been certified for the detection of human Hsp60.

Statistical analysis

Statistical analyses were performed using the StatView® 5.0 software (SAS Institute Inc. Cary, NC, USA). Potential differences in clinical and laboratory characteristics were assessed by univariate comparisons using the χ^2 test for nominal variables and the unpaired Student's *t*-test for numeric variables. ANOVA was used to test the differences in biochemical parameters in relation to delivery options. Correlation analysis was performed using the Spearman rank correlation method in order to assess potential correlations between GSH levels and gestational age as well as lipid hydroperoxides. Multivariate analysis was performed by logistic