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Cord Blood Genomic Analysis Highlights the Role of Redox Balance

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Abstract

Neonates are exposed to elevated levels of reactive oxygen species as they transition from a hypoxic intrauterine to a normoxic extrauterine environment at birth. This increased oxidative stress is associated with neonatal morbidity. Current antioxidant supplementation treatment strategies have yet to translate into improved neonatal outcomes. Our understanding of a newborn's intricate redox balance, particularly at a genomic level, remains limited. Here, we performed genomic microarray analyses (~14,500 genes) on extracted mRNA from umbilical cord whole blood at term gestation (n=10). Bioinformatic analyses identified 282 genes (2.0%) that were consistently present within the highest quintile of expressed genes. These genes were highly associated with oxidant stress and included superoxide dismutase 1, catalase, peroxiredoxins, and uncoupling proteins. Pathway analyses identified statistically significantly over-represented functional pathways including 'oxidative stress', 'oxidative stress response mediated by nuclear factor-erythroid 2-related factor', 'hypoxia-inducible factor signaling', and 'mitochondrial dysfunction' (p < 0.05). These results suggest that neonates require high levels of antioxidants and an intricate cellular redox balance to ensure a successful transition to the extrauterine environment. Understanding the genes necessary to maintain this delicate redox balance may lead to the development of alternative treatment strategies.

Keywords

Oxidative Stress; Neonate; Redox Balance; Genomics; Umbilical Cord Blood

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Introduction

In the neonatal period, an increase in oxidative stress occurs in response to the transition from a hypoxic intrauterine environment (PaO₂ of 20-25 mm Hg) to a normoxic extrauterine environment (PaO₂ of 100 mm Hg) [1,2]. With their first breath, newborn infants are directly exposed to increased oxygen concentrations that result in the generation of reactive oxygen species (ROS) [3]. An impaired oxidant/antioxidant balance may also exist in the neonate, due to a decreased activity of superoxide dismutase (SOD) in fetal erythrocytes, high levels of free iron, and reduced antioxidant levels (*i.e.* vitamins) [4]. In 1988, Saugstad was the first to suggest a link between oxidative stress and neonatal morbidities, coining the phrase 'oxygen radical disease of neonatology' [5]. Since that time, multiple investigators have studied levels of antioxidants and their substrates in premature, growth restricted, and full-term neonates [6-8]. They have identified causative links between oxidation-reduction (redox) balance *in vivo* and unique disease sequelae of prematurity, including bronchopulmonary dysplasia [9], retinopathy of prematurity [10], necrotizing enterocolitis [11] and periventricular leukomalacia [12]. Despite an appreciation for the role of oxidative stress in neonatal disease, treatment strategies providing exogenous antioxidant enzymes, substrates, and/or vitamins have yet to fully demonstrate a reduction in neonatal morbidities [13].

Reactive oxygen species are now known to be important cell signaling molecules. Antioxidants do not function solely as free radical scavenging systems, but instead they regulate critical signal transduction pathways [14], are implicated as second messengers activating protein cascades [15], and ultimately control gene expression. However, it is still unclear what genes and pathways are active at birth and which systems are upregulated in response to the relative hyperoxia that exists immediately following the neonate's first breath. The purpose of this study was to examine gene expression in healthy full-term newborn umbilical cord blood to understand the complex redox balance that exists at birth. We hypothesized that a genomic approach would potentially identify essential antioxidant pathways necessary for successful neonatal transitioning, and suggest innovative and alternative treatment strategies for this patient population.

Materials and Methods

This study was approved by the Tufts Medical Center Institutional Review Board (IRB). Informed parental consent was obtained for all enrolled neonatal subjects (n=10). Pertinent clinical information about the subjects is provided in Table 1. Following elective cesarean delivery at term (36 5/7 to 39 5/7 weeks' gestation), 7.5 mL of umbilical cord whole blood from each infant was directly placed into PAXgene™ Vacutainer whole blood RNA tubes. Samples were stored at room temperature for a minimum of six hours prior to total RNA extraction with the QIAamp RNA Blood Mini Kit per the manufacturer's instructions (Qiagen). On column DNase digestion was performed to eliminate DNA contamination. The quantity and quality of extracted total RNA was assessed with the Agilent Bioanalyzer 2100 to ensure that each sample had a minimum starting concentration of 1 µg, and clear 18s and 28s ribosomal peaks prior to amplification, respectively.

Total RNA was amplified in accordance to the Eberwine protocol [16] with the commercially available Affymetrix™ One Step Amplification Kit. There was no globin reduction performed on any whole blood sample. Amplified cDNA was again assessed with the Bioanalyzer 2100 prior to biotinylation and fragmentation to normalize hybridization quantities. Approximately 15 µg of labeled and fragmented cRNA was hybridized onto the Affymetrix™ HG U133a microarray, comprised of approximately 14,500 genes. Following

hybridization, arrays were washed and stained in the Gene Chip Fluidics Station 200 (Affymetrix™) and scanned with the GeneArray Scanner (Affymetrix™).

Of note, the umbilical cord blood microarray data used in this study was part of a previously published report examining feto-maternal trafficking at term that considered both mother and newborn gene expression profiles [17]. These data are publicly available online with the article at <http://dx.doi.org/10.1172/JCI29959DS1>. In the prior paper, the neonatal cord blood expression data were used to compare genes in the mother that were up-regulated during pregnancy and down-regulated following delivery. Statistical analysis focused solely on genes that were up-regulated in the maternal antepartum and the neonatal samples. The current analysis represents solely the umbilical cord blood gene expression data, independent of the mother. Thus, while the neonatal array data are common to both studies, they have been mined completely differently with different emphasis and different techniques.

Bioinformatic Analysis

All arrays were initially assessed using the Bioconductor suite of packages for the R statistical programming environment (<http://www.bioconductor.org>). Arrays were subsequently summarized and underwent a normalization using the Affymetrix mas5 algorithm with default parameters in the “affy” package in Bioconductor [18]. Probes were filtered to remove those equal to background signal. K-means clustering divided the genes into quintiles based upon their probe intensities. Only those genes identified in the highest quintile ($\geq 80\%$) were considered in the analysis. The expression level of hemoglobin genes in the highest quintile served as positive controls, while quintile and intensity distribution analysis indicated that all arrays hybridized in a similar fashion. Genes expressed at the top quintile were entered into the commercially available Ingenuity® Pathway Analysis (IPA) software system to assess gene–gene relationships, biological functions and pathways. IPA generates networks in which the differentially-regulated genes can be related according to previously known associations between genes or proteins [19]. All genes identified within the four statistically significantly over-represented biological systems were further analyzed with *Entrez Gene* and PubMed to better understand their role in each respective functional pathway.

Results

After background filtering, 14,000 probes remained. Of those, 440 probe sets representing 282 genes (2.0%) were identified in the highest quintile of expression across all arrays (Supplemental Table 1). Signal intensities were consistent across arrays and thus, no single array skewed the data. The hemoglobin genes alpha 1 (*HBA1*), beta (*HBB*), epsilon (*HBE1*), delta (*HBD*) and gamma (*HBG2*) were all present and expressed in the upper quintile. The top four statistically significantly over-represented functional pathways identified by IPA were: ‘oxidative stress’ ($p < 8.9 \times 10^{-4}$), ‘oxidative stress response mediated by nuclear factor-erythroid 2-related factor 2’ (Nrf2) ($p < 3.7 \times 10^{-3}$), ‘hypoxia-inducible factor signaling’ ($p < 1.72 \times 10^{-2}$), and ‘mitochondrial dysfunction’ ($p < 2.5 \times 10^{-2}$). A full list of the gene transcripts contained within each of these systems can be found in Table 2. The commonly known antioxidant enzymes superoxide dismutase 1 (*SOD1*), catalase (*CAT*), and glutathione peroxidase 1 (*GPX1*) were all present. Additionally, lesser known antioxidant genes associated with the thioredoxin (*PRDX1*, *PRDX6*), ferritin (*FTH1*, *FTL*), and mitochondrial uncoupling family of proteins (*UCP2*) were represented in the highest quintile of expressed genes. For the remainder of the paper, all gene transcripts will be italicized, while their corresponding proteins will remain in normal font, per convention.

Discussion

Normal neonatal physiological responses to the birth process are complex. In particular, a newborn must adapt to abrupt changes in oxygen concentrations and the increased generation of ROS following entry into a normoxic environment shortly after birth. Premature infants, in particular, are at high risk for morbidities due to this increased oxidative stress. While several studies have examined the role of antioxidant enzymes (e.g. SOD, CAT), vitamins (C, E), and other proteins (e.g. ceruloplasmin, bilirubin, transferrin) in the neonatal population, few studies have examined gene expression levels of these key modulators of oxidative stress [20], and only a small number of investigators have performed gene expression analyses on umbilical cord or neonatal blood [21-22]. By performing a discovery-driven genomic analysis on umbilical cord blood from healthy term infants, it is clear that multiple oxidative stress pathways are involved in the successful transition of term newborns to ambient oxygen concentrations (Figure 1). Each of the four significantly over-represented pathways in our analysis involves oxidative stress and its downstream effects. These pathways not only include commonly known gene transcripts for antioxidant enzymes that scavenge ROS, but also antioxidant reactions at the mitochondrial level, oxidative stress responses mediated by the Nrf2 signaling pathway, and antioxidant pathways involving thioredoxins and ubiquinones.

Interestingly, the umbilical cord whole blood samples obtained for this study are representative of the *in utero* environment of a term neonate shortly before birth. While we acknowledge that in our cohort of subjects, there is a three week range of gestational ages, all of these infants transitioned to room air without difficulty, had Apgars at 1 and 5 minutes of ≥ 8 , and did well clinically. Thus, these infants are representative of normal neonatal transitioning. Importantly, at time of blood collection, these infants had yet to be exposed to ambient air and its accompanying oxidative stress burden. Therefore, this genomic analysis suggests that the fetuses prepared for their impending transition with highly expressed levels of several antioxidant enzymes and associated pathways. Based upon the range of gestational ages in our cohort, this up-regulation of key genes involved in oxidative stress likely occurs during the last few weeks of gestation. Future studies comparing the genomic profiles of preterm infants, as well as term infants who have failed to successfully transition to an extrauterine environment (*i.e.* persistent pulmonary hypertension of the newborn), are needed to determine if inducing expression levels of the many genes involved in the specific redox pathways highlighted here will reduce neonatal morbidity.

Not surprisingly, the more well-known antioxidant gene transcripts *SOD1*, *CAT*, and *GPX1*, are strongly expressed in umbilical cord blood at term. This finding coincides with other studies that have demonstrated increased antioxidant concentrations in the plasma of term newborns [2]. Each of these genes is expressed within the top 2% of all genes detected in our expression microarray analysis, which suggests that healthy term neonates require extremely high levels of these enzymes to ensure a normal transition to ambient oxygen concentrations. Thus, a genomic approach to oxidative stress in the newborn not only confirms previous findings, but also identifies novel pathways regarding the physiologic activity of intricate antioxidant transcription pathways in the term newborn at birth.

Analysis of our data also suggests that oxidative stress response mediated by Nrf2 plays an important role in term neonatal redox balance. Nrf2 is a redox-sensitive transcription factor that regulates an antioxidant response element (ARE), and is involved in the expression of many different antioxidant enzyme systems [23]. While Nrf2 commonly exists in the bound and inactive form in the cytoplasm, physiological circumstances, such as increased levels of ROS, can lead to activation of Nrf2, resulting in increased gene transcription of antioxidant enzymes. Imhoff and associates recently demonstrated that activation of Nrf2 “causes the re-

regulation of the extracellular redox environment and may provide a mechanism by which extracellular oxidative stress may be alleviated” [23]. Term neonatal cord blood has statistically significantly over-represented genes within this signaling pathway, including both the ferritin heavy (*FTH1*) and light (*FTL*) chain gene transcripts. Interestingly, over-expression of *FTH1* has been shown to result in cytoprotection against oxidative stress [24]. Tsuji has shown that *JunD*, which was also present in our gene list, is a regulatory protein that activates *FTH1* transcription [24]. To our knowledge, we are the first to demonstrate the potential role of Nrf2 signaling in the term neonate at birth, and future studies are warranted to elucidate the complex regulation of this pathway in neonates.

The thioredoxin pathway is another example of how redox balance alters gene expression in the term neonate. Genes associated with this system, such as peroxiredoxin 2 (*PRDX2*) and peroxiredoxin 6 (*PRDX6*), are present and highly expressed in umbilical cord blood. A well studied cellular redox system, thioredoxins are known to alter gene expression. Their role in development, neonatal life, and premature disease has recently been described in a series of review articles [25-27]. In particular, the peroxiredoxins play an important adjunct role in the removal of hydrogen peroxide from the cellular milieu. Their presence in the top quintile of expressed genes suggests that this is yet another alternative pathway that the term neonate uses to maintain redox balance. Additionally, genes within ‘mitochondrial dysfunction’ functional pathway, such as uncoupling protein-2 (*UCP2*), were seen in this analysis. While the precise physiological role of *UCP2* remains under debate, it has been shown to diminish mitochondrial superoxide production, thus protecting the cell from ongoing oxidative stress damage [28,29], and may modulate cellular apoptosis [30]. Each of these pathways may provide important insights into the normal developmental regulation of redox balance in the term neonate.

A potential limitation of our study is that only infants delivered via cesarean section were included in this analysis, and as a result, we did not examine whether mode of delivery directly affects gene expression. In recent years, there have been conflicting reports about oxidative stress in the neonate based upon mode of delivery. Inanc and colleagues reported that infants undergoing elective cesarean section had significantly higher activity of SOD and CAT [31], while Vakillin and associates demonstrated that infants born via vaginal delivery had increased biomarkers of oxidative stress [32]. Conversely, Fogel *et al.*, showed that type of delivery had no impact on oxidative stress in the neonate [33]. Future comparative studies between infants delivered vaginally and those delivered via cesarean section will need to be performed to determine if mode of delivery alters the neonatal oxidative stress response at the genomic level.

In summary, genomic analyses of umbilical cord whole blood samples at term reveal that genes involved in multiple oxidative stress pathways are highly expressed at birth and are likely required to process the increased oxidative stress burden associated with transitioning to ambient air. This work demonstrates that normal neonatal redox balance is an elaborate interplay of oxidative stress, feedback loops and antioxidant transcription regulation. Understanding the delicate redox balance of the healthy term neonate at the genomic level lays the foundation for future comparative studies with vulnerable premature and sick term neonates, and may lead to new treatment strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

ARE	antioxidant response element
CAT	catalase
FTH1	ferritin, heavy polypeptide 1
FTL	ferritin, light polypeptide
GPX1	glutathione peroxidase 1
HBA1	hemoglobin, alpha 1
HBB	hemoglobin, beta
HBD	hemoglobin, delta
HBE1	hemoglobin, epsilon 1
HBG2	hemoglobin, gamma G
HIF	hypoxia inducible factor
JunD	jun D proto-oncogene
NQO1	NADPH:quinine oxidoreductase-1
Nrf2	nuclear factor-erythroid 2-related factor 2
PRDX2	peroxiredoxin 2--
PRDX6	peroxiredoxin 6
REDOX	oxidation-reduction
ROS	reactive oxygen species
SOD1	superoxide dismutase 1
TR1	thioredoxin 1
TRX	thioredoxin
UCP2	uncoupling protein 2

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Figure 1. Neonatal Defensive Cellular Response Mechanisms to Oxidative Stress at Birth
Schematic figure highlighting the complex cellular redox pathways involved in successful neonatal transitioning at birth.

1. **Free Radical Scavenging:** Superoxide dismutase 1 (SOD1) scavenges oxygen free radicals while catalase (CAT) converts hydrogen peroxide to water and oxygen.
2. **Thioredoxin System:** Peroxiredoxins reduce hydrogen peroxide by transferring electrons from thioredoxin (TRX) via PRDX2 or another donor such as glutathione via PRDX6 [25]
3. **Nrf2 Signaling Pathway:** Increased levels of ROS within the cellular milieu lead to an uncoupling and subsequent activation of Nrf2. Nrf2 then translocates to the nucleus, binds to ARE, and causes the upregulation of antioxidant genes such as glutathione transferase (*GST*), thioredoxin 1 (*TR1*), and NADPH:quinone oxidoreductase-1 (*NQO1*) [23].
4. **Mitochondrial Dysfunction:** Activation of UCP2 in the inner mitochondrial membrane diminishes the protonmotive force, attenuates mitochondrial ROS production, and reduces cellular oxidative stress damage [28].

Table 1

Subject demographics

Subject	Gender	GA	Birth Weight (g)	Apgars (1, 5 min)	Indication for Cesarean Section	Oxygen in delivery room (mode)
1	Female	39 1/7	3625	8, 9	Repeat	Yes (blow by)
2	Female	39	4136	9, 9	Repeat	No
3	Male	39	3372	8, 9	Repeat	No
4	Male	36 5/7	1735	9, 9	Multiple Gestation *	Yes (blow by)
5	Male	36 5/7	2194	8, 9	Multiple Gestation *	No
6	Male	39 2/7	4708	8, 9	Repeat	Yes (blow by)
7	Male	37 3/7	3159	9, 9	Onset of labor w/prior uterine surgery	No
8	Male	39 5/7	2897	9, 9	Repeat	No
9	Female	39 2/7	2949	8, 9	Repeat	Yes (blow by)
10	Male	39 1/7	3444	8, 9	Repeat	No

GA: gestational age in weeks

* Dizygotic twins

Table 2

Gene transcripts found within each over-represented functional pathways

Pathway	Gene symbol	Gene name	Description
Oxidative stress ($p < 8.9 \times 10^{-4}$)	PRDX2	peroxiredoxin 2	antioxidant enzyme regulation of cell cycle
	S100A9	S100 calcium binding protein A9	progression, differentiation; inhibition of casein kinase
	SOD1	superoxide dismutase 1, soluble	binds copper, zinc; free radical destruction
	PRDX6	peroxiredoxin 6	antioxidant enzyme; redox regulation
	CAT	catalase	antioxidant enzyme
	GPX1	glutathione peroxidase 1	antioxidant enzyme
Oxidative stress response mediated by Nrf2 ($p < 3.7 \times 10^{-3}$)	CAT	catalase	antioxidant enzyme
	SOD1	superoxide dismutase 1, soluble	binds copper, zinc; free radical destruction
	ACTG1	actin globin	non-muscle cell protein
	JUND	jun D proto-oncogene	anti-p53-dependent senescence, apoptosis
	ACTB	actin, beta	non-muscle cell protein
	FTH1	ferritin, heavy polypeptide 1	intracellular iron storage
	FTL	ferritin, light polypeptide	intracellular iron storage
	GSTO1	glutathione S-transferase omega 1	stress response protein; cellular redox homeostasis
	HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	chaperone (signal transduction, protein folding/degradation)
Hypoxia inducible factor-signaling ($p < 1.7 \times 10^{-2}$)	RBX1	ring-box 1	ubiquitin polymerization
	EIF1	eukaryotic translation initiation factor 1	
	HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	chaperone (signal transduction, protein folding/degradation)
	LDHA	lactate dehydrogenase A	anaerobic glycolysis target proteins for degradation;
	UBE2B	ubiquitin-conjugating enzyme E2B	post-replicative DNA damage repair
Mitochondrial dysfunction ($p < 2.5 \times 10^{-2}$)	UBE2D3	ubiquitin-conjugating enzyme E2D 3	ubiquitination of p53
	CAT	catalase	antioxidant enzyme
	COX6A1	cytochrome c oxidase subunit VIa polypeptide 1	terminal electron transfer in mitochondrial respiratory chain
	COX6B1	cytochrome c oxidase subunit VIb polypeptide 1	terminal electron transfer in mitochondrial respiratory chain
	COX7A2	cytochrome c oxidase subunit VIIa polypeptide 2	terminal electron transfer in mitochondrial respiratory chain
	SNCA	synuclein, alpha (non A4 component of amyloid precursor)	component of amyloid plaques

Pathway	Gene symbol	Gene name	Description
	UCP2	uncoupling protein 2	mitochondrial anion carrier protein