Oxygen Toxicity in the Neonate
Thinking Beyond the Balance

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KEYWORDS
- Oxygen • Prematurity • Bronchopulmonary dysplasia • Retinopathy of prematurity
- Necrotizing enterocolitis • Glutathione • Antioxidants • Mitochondria

KEY POINTS
- Oxidative stress has traditionally been presented as an imbalance between oxidants and antioxidants but the situation is far more complex.
- Neonatal O2 toxicity has been primarily characterized by macromolecular indices of damage that are nonspecific and are inadequate to capture dynamic biochemical processes.
- In premature infants, the fetal to neonatal transition occurs during a period of marked susceptibility to oxidative stressors caused by deficits in antioxidant defenses and impaired endogenous antioxidant response activation.
- The molecular effects of O2 on subcellular compartments and developmental pathways are poorly understood.
- State-of-the-art oxidation-reduction biology techniques will enable more robust understanding of the global impact of O2 toxicity in preterm neonates.

INTRODUCTION
Fetal development occurs normally in a relatively hypoxic (~20–25 Torr) environment in utero, meaning that the transition into room air at birth represents significant oxidative stress for prematurely born neonates. However, the transition from the hypoxic environment of the womb to the relatively hyperoxic extrauterine environment occurs during a period of marked susceptibility to oxidative stressors. Preterm neonates are more susceptible to the effects of O2 toxicity because of developmental deficits in antioxidant defenses and developmental impairments in the ability to mount rapid antioxidant responses to hyperoxia. In general, the toxicities of O2 during the neonatal period have been characterized by macromolecular indices of oxidative...
protein, lipid, and/or DNA damage. An expanding body of evidence has defined the molecular effects of hyperoxia on developmental pathways that guide organogenesis. The sudden and dramatic increase in lung and systemic O2 tension on preterm delivery significantly influence transcription factor activation and related downstream pathways. However, the global impact of O2 toxicity in preterm neonates is incompletely characterized because of the lack of sensitive and specific oxidation-reduction (redox) biological techniques that adequately capture these complex biochemical reactions that undoubtedly contribute to the observed morbidity and mortality in this highly vulnerable patient population.

BASIC TENETS OF OXIDATIVE STRESS

Sources of Reactive O2 Species

A redox reaction refers to a transfer of electrons between molecules. It is essential to remember that matter is neither created nor destroyed in chemical transformations. In the simplified scheme (Fig. 1), molecule A loses an electron and becomes oxidized and molecule B accepts an electron and becomes reduced. Thus, the net reaction is simply the transfer of the electron from molecule A to molecule B. In Fig. 1, “n” and “m” refer to the oxidation state of molecules A and B, respectively. When electrons are lost, the oxidation number increases (A\(^{n+1}\)). In contrast, when electrons are gained, the oxidation number decreases (B\(^{m-1}\)).

In order to fully comprehend the effects of O2 tension on neonatal pathophysiology, the complexities of redox biology must be appreciated. Conceptually, this understanding must extend beyond the oxidant/antioxidant balance concept, which is that oxidative stress represents a deficiency of antioxidants in a setting of enhanced oxidant generation. This overly simplistic model suggests that oxidative stress can be overcome by exogenously administered antioxidants to restore balance. In reality, the complex biochemical reactions responsible for the reduction of O2 are dynamic, highly compartmentalized, sensitive to clinically relevant factors such as pH and temperature, and extremely difficult to characterize in vivo with currently available techniques.

Diatomic O2 is highly reactive because of an unpaired electron in its outer orbital, and it requires 4 electrons for complete reduction (Fig. 2). O2 is also the primary cellular metabolic fuel for aerobic metabolism. Under normal conditions, the reactive O2 species (ROS) generated in the process of the 4-electron reduction of O2 to H\(_2\)O are quickly reduced (Fig. 3). ROS generated during cellular metabolism include superoxide (O\(_2^-\)) and hydrogen peroxide (H\(_2\)O\(_2\)). Additional oxidants, including

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\begin{align*}
A^n & \rightarrow A^{n+1} + e^- & \text{oxidation of A} \\
B^m + e^- & \rightarrow B^{m-1} & \text{reduction of B} \\
A^n + B^m & \rightarrow A^{n+1} + B^{m-1} & \text{net reaction}
\end{align*}
\]

Fig. 1. Basic scheme of redox reactions. Molecule A loses an electron and becomes oxidized and molecule B accepts an electron and becomes reduced. Thus, the net reaction is simply the transfer of the electron from molecule A to molecule B. “n” and “m” refer to the oxidation state of molecules A and B, respectively. When electrons are lost, the oxidation number increases (A\(^{n+1}\)). In contrast, when electrons are gained, the oxidation number decreases (B\(^{m-1}\)).
peroxinitrite (ONOO$^-$), generated from the nonenzymatic reaction between $O_2^{**}$ and nitric oxide (NO$^*$), and hydroxyl radical (•OH), generated from the reaction between $H_2O_2$ and iron (Fe$^{++}$) or copper (Cu$^+$), are primarily formed in situations in which endogenous antioxidant systems are unable to sufficiently provide electrons for reductive processes. Although the primary focus of this article is $O_2$ toxicity, it is important to understand that excessive ROS generation in preterm infants comes from a variety of sources, including ischemia/reperfusion, infection, inflammation, mitochondrial respiratory chain, free iron and Fenton reaction, and hyperoxia.$^{12-14}$ The generation of ROS can lead to the disruption of normal physiologic events.$^{15}$ The extent of the effects of ROS on physiology depends on specific molecular interactions, cellular locations, and timing of exposure.$^{15}$

The effects of ROS contribute to quantifiable cellular, tissue, and organ damage that underlies many of the morbidities of prematurity.$^{12}$ These damaging processes occur in both the placenta and the developing fetus.$^{13}$ Although premature infants that develop prematurity-related morbidities are usually exposed to only the least required amount of supplemental $O_2$ postnatally, they show marked evidence of oxidant stress.$^{6,12,14}$ There is evidence that excessive ROS production contributes to retinopathy of prematurity, bronchopulmonary dysplasia, intraventricular hemorrhage,
periventricular leukomalacia, necrotizing enterocolitis, kidney damage, and hemoly-
sis. Pathophysiologically, many diseases of prematurity likely represent a
convergence between injury and ROS-induced alterations in development, probably
leading to increases in susceptibility to chronic diseases in adulthood, and perhaps
more rapid aging as well.

The appreciation of ROS as something other than a negative entity has grown in the
last 20 years. Several cellular processes are actively modulated via ROS production.
ROS serve as cell signaling molecules for normal biological processes. For example,
nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) produce O$_2^-$
and/or H$_2$O$_2$ in tightly regulated and highly specific intracellular events. As such,
these processes are governed by transcription factors that are influenced by the redox
environment of the tissue, cell, or subcellular compartment in which they are
expressed. Changes in electron flux through these pathways, whether it be through
reduction of O$_2$ or through NOX influence signaling. NOX-dependent ROS production
influences developmental programming by acting on redox-sensitive transcription
factors, including hypoxia-inducible factors (HIFs) and nuclear factor kappa-light-
chain-enhancer of activated B cells (NF-kB). Dysregulation of HIFs and NF-kB have
been linked to one another and to negative outcomes in prematurely born infants.
NOX isoforms contribute to signaling during lung development and injury and their
function influences pulmonary airway and vascular cell phenotypes, including prolifera-
tion, hypertrophy, and apoptosis. Oxidative stress is also associated with altered
nitric oxide (NO) signaling in which ROS and reactive nitrogen species production
are increased and bioavailable NO is decreased.

Antioxidant Systems
Antioxidants are substances that inhibit or prevent oxidation of a substrate. Highly
conserved antioxidant systems have developed to rapidly and robustly respond to al-
terations in cellular and subcellular redox perturbations. In the context of the previ-
ously mentioned 4-electron reduction of O$_2$, antioxidant systems serve as electron
donors, as illustrated in Fig. 3. Antioxidants that protect against and repair O$_2$-medi-
ated injury include flavin-containing enzymes, superoxide dismutases (SODs), the
glutathione (GSH) and thioredoxin (Trx) systems, heme oxygenases, and small-
molecular-weight antioxidants. Antioxidant capacity is lower in preterm new-
borns than in term infants.

Birth represents an oxidative challenge. In the days preceding full gestation, antiox-
idant systems are upregulated and nonenzymatic antioxidants cross the placenta in
increasing amounts. These developmental changes provide for the transition from
the relative hypoxia of intrauterine development to the O$_2$-rich extraterine environ-
ment. Furthermore, endogenous antioxidant production is upregulated immediately
before birth in term infants and is further upregulated on exposure to atmospheric
O$_2$. Remembering that development occurs in a hypoxic environment in utero
(~20–25 Torr), exposure to even room air constitutes hyperoxia for prematurely
born neonates. Premature infants are at a distinct disadvantage for many reasons
because they do not receive maternal antioxidants before delivery, have impaired abil-
ity to induce endogenous antioxidants before birth, and are unable to further induce
endogenous antioxidant responses following delivery. Although much has been
outlined regarding associations between oxidative damage and neonatal morbidities,
significant gaps in knowledge still exist regarding the role of oxidative injury in the
pathogenesis of neonatal diseases.

Therapeutic strategies to mitigate ROS-induced diseases in premature infants
have included both enzymatic and nonenzymatic antioxidant preparations.
Although logically based on the idea of antioxidant imbalance, studies in animal models and in preterm infants have yielded mixed results.\(^5,15\) Cysteine is a precursor of GSH, the most abundant intracellular antioxidant in the body. Cysteine chloride supplementation in parenteral nutrition improved nitrogen balance in preterm infants; however, increased metabolic acidosis was also reported. \(N\)-acetylcysteine has shown promising results in preclinical models by acting as a precursor for de novo GSH synthesis. However, routine \(N\)-acetylcysteine supplementation was not found to be effective in improving respiratory outcomes in extremely low birth weight infants.\(^23\)

One of the most promising catalytic antioxidants to undergo extensive clinical investigation in the prevention of bronchopulmonary dysplasia (BPD) was superoxide dismutase (SOD). Although the incidence of wheezing was lower in SOD-treated infants, a Cochrane meta-analysis indicated there is insufficient evidence to draw firm conclusions about the efficacy of SOD in preventing chronic lung disease of prematurity; however, it seems to be well tolerated and has no serious adverse effects.\(^24\) Post hoc analyses of the data from infants with retinopathy of prematurity (ROP) in this trial indicated that severity greater than stage 2 was present in 42% of placebo-treated infants versus 25% of SOD-treated infants, suggesting that SOD may reduce the risk of developing ROP.\(^25\)

**O\(_2\)** Toxicity–Related Sequelae of Birth

*Macromolecular Oxidation*

In general, similar pathophysiologic mechanisms contribute to \(O_2\) toxicity–related morbidities in infants. As described earlier, ROS generated from metabolism, ischemia/reperfusion, infection, hyperoxia, and inflammation, when present in excess amounts, result in detectable byproducts of oxidation. These byproducts are highlighted in Fig. 4. Although nonspecific, the detectability of these byproducts has enabled associations between \(O_2\) toxicity and neonatal disorders including BPD, intraventricular hemorrhage (IVH), ROP, necrotizing enterocolitis, and periventricular leukomalacia.\(^13,16\)

GSH is the most abundant intracellular antioxidant in the body and cycles between thiol (GSH) and disulfide (GSSG) species. The GSH redox ratio (GSH/GSSG) is often used as a noninvasive measure of in vivo redox status. A significant negative correlation was reported between the arterioalveolar \(O_2\) and blood glutathione redox ratio, with improved oxygenation inversely associated with decreased GSH/GSSG ratio.\(^26\) Further, associations between BPD, lipid hydroperoxide (LOOH), and GSH concentrations in bronchoalveolar lavage fluid levels have suggested that early LOOH level increases in preterm infants developing BPD suggest that lung biochemical monitoring of sick infants might be possible and that BPD could be predicted early by evaluating biomarkers.\(^27\) Extremely preterm infants have low GSH levels that impair their ability to detoxify ascorbylperoxide (AscOOH), an oxidant commonly found in parenteral nutrition. Higher first-week urinary AscOOH levels are associated with an increased incidence of BPD or death.\(^28\)

White matter in the brains of premature infants is vulnerable to oxidative damage because of delayed expression of SOD, catalase, and GSH peroxidase enzymes.\(^29\) Isoprostanones are a quantifiable marker of ROS-mediated tissue injury and concentrations of \(F_2\)-isoprostanate in preterm lesions are similar to those measured in moderately severe cerebral cortical hypoxic-ischemic lesions in term infants.\(^29\) Diffuse white matter injury involves maturation-dependent vulnerability of the oligodendrocyte lineage with selective degeneration of late oligodendrocyte progenitors triggered by oxidative
stress and other insults. Oxidative damage triggers cell death in preterm human white matter and the magnitude of oxidative damage is comparable with that sustained in the cerebral cortex after severe perinatal asphyxia.

Redox-Dependent Alterations in Cell Signaling

As presented earlier, there has been increasing recognition of O$_2$ toxicity as an alteration in redox-dependent cellular and subcellular function. When viewed from this perspective, even subtle changes in redox balance can have persistent effects on organogenesis, tissue repair, and cellular function. As an example, multiple growth factors and signaling cascades play important roles in normal lung vascular development. One of the most extensively studied endothelial growth factors is vascular endothelial growth factor (VEGF). VEGF, a potent endothelial cell mitogen produced by type 2 alveolar epithelial cells, is significantly involved in alveolar development and its expression is regulated by HIFs. Numerous studies in newborn animal models have shown the importance of normal VEGF signaling to lung alveolar...
Premature delivery has deleterious effects on the O2-dependent biological processes that mediate lung development; in particular, the HIF/VEGF pathways.8 NF-kB regulates angiogenesis by acting upstream of HIF/VEGF.20 Direct effects of ROS on signaling pathways include redox-sensitive transcription factors (eg, HIF; nuclear factor, erythroid derived 2, like 2 [Nrf2]; and NF-kB) as well as indirect effects through inactivation of NO-based signaling.15 For example, NF-kB is a direct regulator of VEGF receptor-2 (VEGFR2), in the neonatal pulmonary vasculature.42 Similar to BPD, altered HIF/VEGF signaling also mechanistically contributes to ROP. O2 toxicity can directly damage pulmonary parenchyma and vessels.43 Treatment with iNO can enhance additional ROS formation in the form of ONOO\(^-\) leading to NO depletion and enhanced arterial pulmonary vascular constriction.43

O2-mediated activation of NOX enzymes modulates angiogenesis or apoptotic pathways in the retina and contributes to the pathophysiology of ROP. The magnitude of NOX activation from O2 fluctuations is associated with the degree of ROP.44 VEGF-induced VEGFR2 alters the interaction between NOX and phosphorylated VEGFR2, suggesting that NOX4 may be a target to alter ROS generation to modulate VEGFR2 signaling and reduce ROP.45 Patients with BPD frequently show alterations in pulmonary vascular remodeling and tone that manifest as pulmonary hypertension (PH).46 ROS and NO signaling pathways are disrupted in PH, as shown by increased NOX expression, uncoupling of endothelial NO synthase, and reduced mitochondrial number and function.21

**Redox Effects in the Mitochondria**

More than 90% of ATP in mammalian cells is produced by oxidative phosphorylation through the action of mitochondrial ATP synthase.47 Mitochondrial bioenergetic dysfunction has been proposed as a cause of altered organ development in premature infants (see Fig. 4).48 Mitochondria are now thought of as among the cell’s most sophisticated and dynamic responsive sensing systems.49 Specific signatures of mitochondrial dysfunction that are associated with disease pathogenesis and/or progression are increasingly recognized as being important.49 Although the specific pathways that regulate alveolar and white matter development are different in premature infants, both postnatal pulmonary and white matter development depend on proper mitochondrial function.48,50 At birth, both the lungs and brains of premature infants are structurally and functionally immature, and growth also requires substantial energy.48 Mitochondrial dysfunction is increasingly appreciated as a key pathologic feature in the development of lung disease.49,50

Mitochondria govern the response to altered O2 tension and mitochondrial quality control.51 Premature neonates show lower mitochondrial functional capacity, likely because of maturational delays in critical mitochondrial complexes and increased degradation of mitochondrial proteins.47 Although the role of mitochondrial processes in diseases of prematurity is complex, recent evidence suggests that mitochondria offer the potential for novel diagnostics and therapeutics in lung diseases.49 Vascular endothelial mitochondrial function at birth was recently shown to be a potential biomarker for BPD susceptibility in preterm infants.50 Mitochondrial dysfunction in human-derived vascular endothelial cells isolated from umbilical cords at the time of birth strongly predicted the risk of poor pulmonary outcomes.50 In vitro, hyperoxia causes reduced O2 consumption, increased uncoupling, and altered insulin secretion in human beta cells. Using ultradepth sequencing, Kleeberger and colleagues52 identified mitochondrial DNA (mtDNA) sequence variation and differences in heteroplasmy between inbred mouse strains that associate with pulmonary phenotypes on
hyperoxic exposure in neonatal mice. The effects of these differences on mitochondrial function is an area of active investigation for the Kleeberger group. Ballinger and colleagues\textsuperscript{53} recently showed that differences in mitochondrial bioenergetics and mtDNA damage associated with maternal ancestry may contribute to endothelial dysfunction and vascular disease. Collectively, these data highlight the need for a greater understanding of the impacts of mitochondrial dynamics, mitochondrial metabolism, mtDNA sequence variability, and mitochondrial protein expression in the context of neonatal diseases.\textsuperscript{49}

**GAPS IN KNOWLEDGE**

**Effects of Genetics on Redox Biology in the Neonate**

O\textsubscript{2} toxicity alters developmental pathways through a variety of mechanisms.\textsuperscript{54} Similarly, differential responses to O\textsubscript{2} toxicity are also influenced by genetics in individual patients, including ROS production, antioxidant responses, and genetics of underlying developmental pathways. VEGF and endothelial nitric oxide synthase (eNOS) haplotypes are associated with differential effects of O\textsubscript{2} on the development of RDS, BPD, IVH, and ROP in a population of 342 neonates less than 29 weeks old.\textsuperscript{55} Collectively, the data indicated that haplotypes of VEGF and eNOS genes may also independently affect birth weight and gestational age, and act as protecting or risk markers for prematurity complications.\textsuperscript{55}

With respect to antioxidants, genetic polymorphisms of SOD and catalase were recently shown to influence the incidence of morbidities in premature infants.\textsuperscript{43} Genetic variations in antioxidant enzymes may contribute to the pathogenesis of redox-mediated prematurity complications. In an investigation of a cohort of 451 infants less than 30 weeks old, a single-nucleotide polymorphism related to the Nox family altered the susceptibility to oxidative stress–related complications of prematurity, including RDS, BPD, and ROP.\textsuperscript{56} Furthermore, it has been estimated that the effects of gestational age and the duration of supplemental O\textsubscript{2} administration may account for up to 70% of the variance in ROP susceptibility.\textsuperscript{57}

In general, SNPs of antioxidant enzymes have been poorly studied.\textsuperscript{43,58} With respect to GSH metabolism during the neonatal period, levels of oxidative stress markers in boys are greater compared with girls. This discrepancy is likely caused by alterations in estrogen metabolism, which promotes the activation of glutathione metabolism.\textsuperscript{59} Thus, it is possible that considerations regarding sex must be factored into nutritionally focused antioxidant therapies that target GSH metabolism.\textsuperscript{59} After adjustment for epidemiologic confounders, sequence variants of NAD(P)H quinone oxidoreductase-1 and Nrf2 SNPs were associated with BPD and severe BPD, respectively.\textsuperscript{60} Additional study of genetic polymorphisms could help identify high-risk populations that would benefit from targeted antioxidant strategies.\textsuperscript{43}

**Enhancing Endogenous Antioxidant Responses**

Nrf2 is a transcription factor that coordinates the basal expression and inducible activation of antioxidant and xenobiotic genes. For a comprehensive overview of Nrf2 and associated processes, the reader is directed to the excellent review by Tonelli and colleagues\textsuperscript{61} (Fig. 5). Briefly, Nrf2 regulates de novo GSH synthesis, NADPH production, as well as autophagy, stem cell activation, and the unfolded protein response.\textsuperscript{61} O\textsubscript{2} is a potent Nrf2 stimulus and, based on the availability of binding partners, competition or cooperation with other activators and repressors, and crosstalk with other signaling pathways, Nrf2 epigenetically alters target gene promoters.\textsuperscript{61} Nrf2 is currently being
investigated as a potential therapeutic target to enhance endogenous antioxidant responses to attenuate the impacts of O₂ toxicity on the premature infant.

Trace elements, including copper, zinc, iron, and selenium (Se), are essential for normal antioxidant enzyme function. Preterm infants have well-documented perinatal deficiencies in Se, as recently reviewed by our group.⁶² Data indicate that trace mineral supplementation could optimize total antioxidant capacity.⁶³ Although Se supplementation was associated with a reduction in sepsis in preterm infants, it did not improve survival, reduce BPD, or reduce ROP incidence.⁶⁴ Using BPD models, the Kleeberger group has used bioinformatics to identify novel Nrf2-dependently modulated genes that regulate downstream targets in order to screen for chemicals or drugs that modulate expression. These types of approaches could help lead to the identification of new Nrf2 modulating therapies to prevent morbidities of prematurity.⁶⁵ There is much interest in understanding the intersection between trace mineral status on the efficacy of Nrf2 modulating therapies in diseases of prematurity.⁶⁶

Methodologically, analyses of oxidative stress biomarkers have not translated into routine clinical practice because of lack of automation and cost.⁶⁷ In addition, the lack of specificity, especially as it relates to redox-regulated developmental processes, creates significant technical challenges, and economic difficulties constitute a challenge for the immediate future because accurate evaluation of oxidative stress would contribute to improve the quality of care of our neonatal patients.⁶⁷ New techniques such as surface-enhanced Raman spectroscopy may improve the ability to measure oxidative stress biomarkers using low sample volumes and in real time.⁶⁷,⁶⁸

**O₂ TOXICITY: BEYOND THE BALANCE**

It is clear that ROS have important regulatory and signaling roles in the newborn. Thus, antioxidant manipulation is likely to have implications for redox-sensitive developmental pathways that guide proper organogenesis.¹⁶ Given the evolving understanding of oxidative stress in the neonate, future research must include evaluations of the prognostic and therapeutic value of oxidative stress biomarkers and antioxidants in premature infants.¹² The lack of enhanced induction of antioxidants by O₂ in preterm infants highlights the need to better understand the mechanisms responsible for differential responses and burden of disease in this highly vulnerable population.¹⁹ Clinicians are also currently unable to determine which infants are likely to achieve maximal benefit from therapies that replace antioxidants or enhance endogenous antioxidant responses.¹⁶
NF-kB has a major role in lung and brain development, suggesting that therapeutic strategies to selectively block or enhance discrete components of this pathway may hold promise in preventing or treating diseases of prematurity.\textsuperscript{20,42} It is also possible that preservation of mitochondrial function or prevention of mitochondrial dysfunction may be a novel strategy to prevent morbidities in prematurely born infants.\textsuperscript{43} Enhancement of NO signaling and prevention of eNOS uncoupling by NOX inhibition could help prevent mitochondrial dysfunction and/or restore mitochondrial function.\textsuperscript{21} In addition, use of high-throughput evaluation of mitochondrial biology of human umbilical vein endothelial cells or peripheral blood mononuclear cells may help modify therapeutic strategies to decrease risk for adverse outcomes in susceptible infants.\textsuperscript{50}

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