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Oxygen Radical Disease in the Newborn, Revisited: Oxidative Stress and Disease in the Newborn Period

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Abstract

Thirty years ago, there was an emerging appreciation for the significance of oxidative stress in newborn disease. This prompted a renewed interest in the impact of oxygen therapy for the newborn in the delivery room and beyond, especially in premature infants. Today, the complexity of oxidative stress both in normal regulation and pathology is better understood, especially as it relates to neonatal mitochondrial oxidative stress responses to hyperoxia. Mitochondria are recipients of oxidative damage and have a propensity for oxidative self-injury that has been implicated in the pathogenesis of neonatal lung diseases. Similarly, both intrauterine growth restriction (IUGR) and macrosomia are associated with mitochondrial dysfunction and oxidative stress. Additionally, reoxygenation with 100% O₂ in a hypoxic-ischemic newborn lamb model increased the production of pro-inflammatory cytokines in the brain. Moreover, the interplay between inflammation and oxidative stress in the newborn is better understood because of animal studies. Transcriptomic analyses have found a number of genes to be differentially expressed in murine models of bronchopulmonary dysplasia (BPD). Epigenetic changes have also been detected both in animal models of BPD and premature infants exposed to oxygen. Antioxidant therapy to prevent newborn disease has not been very successful; however, new therapeutic principles, like melatonin, are under investigation.

Keywords

Antioxidant therapy; Bronchopulmonary Dysplasia (BPD); Epigenetic changes; Growth; Gen-differentiation; Inflammation Mitochondria; Newborn; Oxidative stress; Persistent Pulmonary Hypertension of the Newborn (PPHN)

In the 1980s, there was an emerging understanding of the significance of oxidative stress in the newborn period, especially in premature infants who were shown to have reduced antioxidant defense systems compared to their term counterparts.[1] Furthermore, free iron,

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a transitional metal that promotes hydroxyl radical ($\text{OH}\cdot$) production through Fenton chemistry, was detected in preterm as well as in asphyxiated neonates, potentially resulting in increased generation of reactive oxygen species (ROS) in those infants.[2] A decade earlier Babior et al linked inflammation and oxidative stress through discovery of the leucocytes oxidative burst as an important part of antimicrobial defense.[3] The combination of these principles broadened the field's understanding of oxidative stress in the newborn as a combination of factors beyond just the level of oxygen exposure. In 1988, Saugstad introduced the term "oxygen radical disease in the newborn". The idea was that, during reoxygenation, oxygen radicals are formed in excess and different organs may be attacked. Thus, we may not be dealing with different diseases in bronchopulmonary dysplasia, necrotizing enterocolitis, or retinopathy of prematurity but different aspects of one disease; an "oxygen radical disease in neonatology," caused by a common pathogenic mechanism. The clinical manifestation of this disease may differ according to which organ is most severely affected.[4]

At the time, such considerations stimulated a new interest in newborn oxygenation, making it a major research priority. This led to resuscitation studies with different oxygen levels, especially air versus 100% oxygen, and large multicenter studies testing out the optimal oxygenation targets for immature newborn infants. Moreover, these considerations resulted in new therapeutic approaches designed to prevent oxidation injury. Unfortunately, most of these approaches have so far been disappointing. Today, 30 years after the first wave of interest in oxidative stress described above, similar questions are still in focus. ROS clearly are of importance for normal and abnormal development. Although ROS can be harmful, they primarily function as important signaling molecules. The effect of ROS at the mitochondrial, genetic, and epigenetic levels are just emerging. Moreover, new antioxidants are being tested or are in the pipeline as novel therapeutic approaches. Perhaps we still can talk about an "oxygen radical disease in the newborn" and should aim at interventions reducing oxidative stress? In this review, we summarize some of the recent evidence linking oxidative stress to newborn disease (Table 1). We put special emphasis on the relationship between inflammation, growth restriction, oxidative stress, and oxidant injury, and how oxidative stress may induce inflammation. The role of the mitochondria in oxidative stress is also summarized.

Oxygen and oxygen sensing

Shortly after the discovery of oxygen as an element in the 1790s, its dichotomy as a life-giving as well as a toxic substance was understood. Part of the mechanism behind this was clarified about 100 years later; that oxygen is magnetic. Oxygen receives its magnetic properties due to an inequality in the total electron spin and is therefore reactive. In energy metabolism, oxygen functions as an electron acceptor in the respiratory chain. By accepting 4 electrons, oxygen is reduced to water, but this process requires four steps. Each intermediate step occurs within the mitochondria and generates ROS including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and $\text{OH}\cdot$. O_2^- and $\text{OH}\cdot$ are free radicals, and therefore highly toxic, causing cell membrane damage by lipid peroxidation, protein and DNA oxidation. On the other hand, H_2O_2 is not a free radical, in spite of being a ROS, and

acts as a signaling molecule essential for cell cross talk (e.g. regulating blood flow within the ductus arteriosus and the pulmonary circulation).[5–8]

As indicated above, there are several reasons that newborn infants, especially those born preterm, are at risk of oxidative stress. First, relative oxidative stress is generally high within the first weeks of birth after transitioning from the low oxygen environment of the uterus to air.[9] Second, sick newborns may need supplemental oxygen if there is respiratory insufficiency. Third, the addition of asphyxia as well as free iron increases the risk of oxidative stress. Most importantly, preterm infants do not have sufficient anti-oxidant defense, missing either *de novo* or passively acquired from the mother in the 3rd trimester, which increases susceptibility to oxidative stress.

Mitochondrial ROS

Mitochondria are a major source of ROS production, easily surpassing cytosolic ROS sources.[10] Mitochondrial ROS include O_2^- produced by complexes I and III of the electron transport chain, and H_2O_2 generated through the dismutation of O_2^- by mitochondrial superoxide dismutase (MnSOD). Hypoxia leads to elevation of Krebs cycle intermediates, including α -ketoglutarate, succinate, and fumarate. Succinate creates the so-called reverse electron transport from Complex –II to Complex-I, which generates higher ROS compared to the conventional forward electron transport from Complex-I to Complex–II.[11]

Given the importance of mitochondria to ROS signaling, it is perhaps not surprising that exaggerated mitochondrial oxidative stress has been described in a number of animal models that mimic human neonatal disease, including models of persistent pulmonary hypertension of the newborn (PPHN), bronchopulmonary dysplasia (BPD), brain injury, and necrotizing enterocolitis (NEC).

While the etiology of neonatal lung disease is multifactorial, the relevance of mitochondrially-derived ROS to neonatal pulmonary injury has been previously demonstrated in animal models of PPHN and in models of hyperoxic lung injury. Studies utilizing prenatal ligation of the ductus arteriosus in fetal lambs have linked mitochondrial oxidative stress to maladaptive changes in nitric oxide (NO) signaling pathways and mitochondrial dysfunction with the pathogenesis of PPHN.[5, 12] These responses, including activation of phosphodiesterase-5 (PDE5), decreased cGMP-responsiveness to exogenous NO, and decreased expression of endothelial Nitric Oxide Synthase (eNOS), may promote additional injury through increased vasoconstriction, impaired responsiveness to vasodilators, and decreased availability of ATP.[13] Moreover, increased levels of H_2O_2 in pulmonary arteries have been associated with decreased expression of soluble guanylate cyclase and reductions in cGMP levels, likely contributing to pulmonary vasoconstriction. [14] H_2O_2 has also been linked to decreased activity of extracellular SOD (ecSOD), an antioxidant that's a major determinant of NO bioavailability.[6]

Similar to the PPHN model, exaggerated mitochondrial oxidative stress responses have been described in murine hyperoxic lung injury models utilizing chronic neonatal oxygen

exposure to mimic BPD.[15, 16] Mitochondrial oxidative injury in the alveolar epithelium is induced by O_2^- , H_2O_2 , $OH^\cdot$, or hypoxanthine/xanthine oxidase.[17, 18] Neonatal chronic hyperoxia increases NADPH oxidase, which induces alveolar epithelial cell apoptosis.[15, 16, 19] Oxygen-induced epithelial cell injury in rodent models are mitigated by MnSOD overproduction alone but not by intracellular SOD (CuZnSOD) or catalase (CAT) in isolation.[18, 19] Additionally, important developmental differences have been identified in murine models, resulting in increased mitochondrial ROS generation in neonatal rodents as compared to adults, suggesting that mitochondrial sources of oxidative stress are particularly important in neonatal responses to hyperoxia.[15] Finally, the possible role of mitochondrial ROS in preterm neonates has been suggested by a study examining genetic polymorphisms of superoxide dismutase (SOD) enzymes linking single nucleotide polymorphisms in MnSOD enzyme to increased risk of BPD development.[20] This retrospective study evaluating the allele frequency and genotype distribution of polymorphisms of SOD enzymes in a population of preterm infants, found that a specific single nucleotide polymorphism on exon 2 of MnSOD gene was associated with increased risk of BPD. Taken together, the available data suggests that ROS originating in the mitochondria are an important contributor to pathophysiology of neonatal lung injury.

In addition to being an important source of ROS, mitochondria are also a prime target of oxidative damage, resulting in defects in oxidative phosphorylation and mitochondrial dysfunction.[21] Mitochondrial dysfunction itself has been implicated in the pathogenesis of BPD and it is possible that the oxidative stress generated by the mitochondria may contribute to defects in mitochondrial function, further worsening lung injury.[22, 23] Mitochondria-derived free radicals have been shown to result in oxidative self-injury and mitochondrial dysfunction in a neonatal brain injury model induced by hypoxia-ischemia (HI).[24] In addition to their role in HI, mitochondrial ROS have also been implicated in brain injury induced by hypoglycemia, and implicated as a major source of intestinal apoptotic signaling in rodent models of NEC.[25, 26] Hypoxia, a common element used in animal models of NEC, is also an important source of oxidative stress and results in increased mitochondrial ROS generation.[27] In that context, it is interesting to note a recent meta-analysis of the five clinical trials investigating the safety and outcomes of different oxygen saturation goals in preterm infants, found that lower oxygen saturation targets were associated with increased rates of NEC, highlighting the importance of hypoxemia in pathophysiology of neonatal diseases associated with increased oxidative stress.[28]

Growth abnormalities and oxidative stress

Fetal growth abnormalities can induce oxidative stress that continues in the newborn period. Both extremes of fetal growth, small or large for gestational age (SGA or LGA), are associated with increased neonatal morbidity and mortality as well as long-term health complications.[29–31] Furthermore, extremely preterm infants are disproportionately affected by intrauterine growth restriction (IUGR) as evidenced by increased mortality, incidence of BPD and BPD-associated PH when using even a liberal definition of growth restriction as birth weight less than the 25th percentile at birth.[29, 32] The concept of “fetal origins of adult disease” was first described by Barker in the early 1990s, as the risk of growth restricted infants to develop cardiovascular disease, diabetes and other metabolic

disorders as an adult.[33, 34] In the ensuing three decades, epigenetic change due to oxidative stress has emerged as the mechanism for the life-long disease predilection.[35, 36] Moreover, recent evidence supports that effects of IUGR may be more imminent, especially for preterm infants, in childhood manifesting as global developmental delay as opposed to the previously described endpoints during adulthood.[37] Although fetal IUGR and macrosomia are very disparate growth states, both are associated with mitochondrial dysfunction and oxidative stress.[38]

Fetal growth extremes can be caused by either maternal or fetal factors; however, in high income areas of the world the most common cause is uteroplacental insufficiency (UPI) due to maternal factors, such as preeclampsia, chronic hypertension or obesity.[39] These maternal disease states appear to have overlapping pathogenesis with increased maternal cytokines and inflammatory markers that affect placental function and subsequently cause nutrient deficiencies and hypoxic effects on the fetus.[40, 41] Independent of the cause of the abnormal growth, human and animal data have demonstrated increased markers of oxidative stress along all parts of the maternal-placental-fetal axis.

Early embryonic and placental development occur naturally under hypoxic and oxidant conditions:[42–44] however, when this physiologic condition continues, as in IUGR, the result can be detrimental to the mother or pregnancy outcome.[45–47] Regarding IUGR pathogenesis, it is not known if maternal or placental tissues initiate the oxidative stress cascade. One longitudinal case-control study found elevated maternal urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine at 12 and 28 weeks of gestation in mothers who ultimately had a fetus affected by IUGR; interestingly, this major byproduct of DNA oxidation was present prior to the clinically evident IUGR.[48] Moreover, additional markers of oxidative stress that have been increased with IUGR include plasma protein carbonyls, H₂O₂, malondialdehyde (MDA), as well as corresponding decreases in antioxidants such as vitamins C and E, SOD and CAT.[49, 50] Many IUGR pregnancies are complicated by preeclampsia but when comparing IUGR with and without preeclampsia, Mert et al found similar measures of increased oxidative stress compared to control.[51] Despite evidence supporting an increased oxidative stress state in women with pregnancies complicated by IUGR, the inciting ROS cause remains unknown.

Although the antecedent for oxidative stress in pregnancies with abnormal growth trajectories is unknown, there is evolving evidence that UPI may result from abnormal placental implantation and development which initiates the pathogenic cascade.[51] The abnormal placental implantation, commonly affecting IUGR pregnancies, produces a state of a relative hypoxia within the intervillous space inducing oxidative stress for both the mother and fetus.[45] Protein carboxylation and lipid peroxidation within the placenta have been shown to impair neutral amino acid and glucose transport.[52, 53] Similarly, placental tissue from UPI affected pregnancies have evidence of insulin receptor and type-1-insulin-like-growth-factor receptor glycosylation; although this did not appear to affect ligand binding, receptor function could be impaired.[53, 54] Moreover, increased presence of CuZnSOD and MnSOD, glutathione peroxidase (GPx) and NADPH oxidase has been found in the vascular endothelium of chorionic and umbilical arteries from IUGR affected pregnancies.[55] In a swine model of natural growth restriction, placental concentrations of H₂O₂, MDA and 8-

hydroxy-2-deoxyguanosine were elevated in growth restriction without differences in NO concentration.[56] The findings in human placental tissues and the swine IUGR model placentas parallel the changes in response to oxidative stress commonly found in the premature neonate with increased free radical moieties, protein oxidation, and decreased antioxidant enzyme production.

Altered biochemical homeostasis is detected in LGA and SGA infants, likely secondary to ongoing oxidative stress and ROS production. In studies simultaneously comparing appropriately grown to both SGA and LGA cohorts, SGA and LGA infants have increased circulating levels of carbonyl proteins and H₂O₂ as well as decreased vitamin levels.[50] Independently, SGA infants have been found to have increased MDA and xanthine oxidase plasma levels, as well as decreased antioxidant capacity via reduced glutathione (GSH) and CAT levels, with similar signs of oxidative stress present in their mothers as well. The directionality of SOD changes is not consistent between SGA datasets; however, their SOD activity differs from controls by several fold. [40, 57] On the other hand, LGA infants appear to have increased SOD activity, likely a physiologic response to elevated oxidative stress, independent of a change in oxidative stress in their mothers.[50] Animal growth restriction models from several species have supported these finding with similar measures of decreased total antioxidant capacity and increased oxidative stress.[41, 56, 58–60] Similar to the maternal studies, the inciting ROS in LGA and SGA infants is generally unknown. These states likely lead to altered gene expression and epigenetic changes that indefinitely affect the aberrant grown infants through adulthood. With the rising incidence of obesity and common incidence of UPI, effects of abnormal fetal growth states are likely to have public health implications unless the oxidative stress state can be addressed.

Obesity affects more than a third of reproductive age women in the United States with a prevalence that has more than doubled over the last two generations. Although macrosomia is more common in offspring of obese women, affecting 20% of births, the incidence of IUGR in obese women is increased 2–3 fold compared to the general population as well.[61] Obese individuals are in a constant low, metabolic, inflammatory state induced by elevated levels of hypercholesterolemia, non-esterified fatty acid concentrations, hyperglycemia, insulin resistance and adiponectin.[61–63] In addition to this background, pregnant obese women have decreased SOD, GSH, Oxidized to Reduced Glutathione (GSSG/GSH) ratio, and antioxidant vitamin levels, as well as increased MDA, nitrite and carbonyl proteins.[64, 65] Similarly, there are signs of increased oxidative stress and decreased antioxidants in their placentas. Subsequently, the chronic physiologic stress alters the maternal environment, which has physiologic effects on the developing fetus and continues in the neonate following delivery (Table 2).

The antioxidant systems of term neonates from obese mothers are induced during gestation with increased relative activity of CAT and SOD at birth.[64] With the current obesity epidemic in the United States and other industrialized nations, the background oxidative damage caused of obesity needs to be recognized as a cofounder for fetal growth and a risk factor for perinatal disease origins.

Inflammation

For many critically ill neonates, exposure to inflammation begins prenatally. Prenatal inflammation has been associated with a variety of adverse neonatal outcomes, including preterm birth, neonatal sepsis, respiratory distress, NEC, intraventricular hemorrhage, and adverse neurodevelopmental outcomes, underscoring the close relationship between inflammation and oxidative stress in a pathogenesis of a wide variety of neonatal diseases. [66–69] Similarly, postnatal inflammation has long been recognized as an important contributor to a number of neonatal diseases. Studies showing increased inflammatory markers in infants with NEC,[70–72] as well as in neonates who go on to develop BPD,[73, 74] illustrate how ubiquitous inflammation is in neonatal diseases associated with increased oxidative stress. The close link between ROS and inflammation was first understood when the oxidative burst of PMNs were described almost 50 years ago.[3] Activated inflammatory cells release a large amount of oxygen radicals and proteases, resulting in direct tissue injury, while also leading to production of pro-inflammatory cytokines that also act as biomarkers of underlying injury. The inflammatory process can ultimately be a source of excessive ROS production with subsequent damage to cellular components and endothelial dysfunction when inflammatory responses are sustained. In addition, cytokines produced as a result of inflammation have been associated with changes in patterns of nitric oxide synthase (NOS) production, further contributing to oxidative stress through reactions of NO with O_2^- leading to even greater production of reactive oxidants such as peroxynitrite ($ONOO^-$).

The interplay of inflammation and oxidative stress has been studied in animal models of NEC where, through a variety of insults to the premature intestine, tissue damage and epithelial injury lead to an inflammatory response. Increased levels of pro-inflammatory cytokines have been described in neonates with NEC,[70, 72, 75] and in animal models of the disease.[76] One consequence of increased pro-inflammatory cytokine production is induction of intracellular nitric oxide synthase (iNOS) whose expression is regulated by cytokines.[77, 78] In addition to increased iNOS, NEC is also associated with decreased expression of eNOS and eNOS uncoupling. Collectively, these changes may promote excessive production of NO and result in increased levels of $ONOO^-$ when NO combines with superoxide anion, another byproduct of increased oxidative stress, at a rate that is even faster than the dismutation of O_2^- by superoxide dismutases .[79–81] $ONOO^-$ and other reactive nitrogen species can, in turn, increase gut permeability through induction of enterocyte apoptosis and changes in gap junctions that result in decreased intestinal integrity. [82, 83] Finally, mitochondrial ROS production can further exacerbate tissue injury in NEC by increasing production of pro-inflammatory cytokines and promoting apoptotic signaling. [25, 84]

Inflammation has also been implicated in pathogenesis of BPD with increased levels of pro-inflammatory cytokines documented in infants who go on to develop BPD.[74, 85] In addition, exposure to hyperoxia, a risk factor for development of BPD, has been associated with an inflammatory lung response in rodent models of hyperoxic lung injury.[74, 86, 87] Similarly to changes seen in NEC, hyperoxic lung exposure is associated with increased production of pro-inflammatory cytokines and increased expression of iNOS in the lung,[88,

89] resulting in production of ONOO^- and other reactive nitrogen species as evidenced by increased plasma 3-nitrotyrosine levels in premature infants who develop BPD.[90]

The complex interplay between inflammation and oxidative stress also appears to be important in pathogenesis of neonatal brain injury. Several studies have demonstrated increased oxidative stress in intraventricular hemorrhage, hypoxic-ischemic encephalopathy (HIE), and epilepsy.[91–93] Preterm neonates with severe perinatal hypoxia-ischemia have been shown to have increased concentrations of IL-6, a pro-inflammatory cytokine, and decreased activity of the antioxidant enzyme glutathione peroxidase in their cerebrospinal fluid, implicating both inflammation and oxidative stress in pathogenesis of HIE.[94] In addition, inflammatory activation of microglial cells in the brain has been associated with increased production of pro-inflammatory cytokines and ROS production.[95] In a newborn lamb model of asphyxia, expressions of IL-1 β , IL-12p40, TLR-2, and TLR-4 were increased in the cortex and subcortex after resuscitation with 100% O_2 compared with 21% O_2 indicating the pathways by which hyperoxia may induce inflammation in the newborn brain.[96] Finally, similar to the oxidative induction of iNOS in the lung and the intestine, increased iNOS expression has been reported in animal models of hypoxia-ischemia, likely contributing to oxidative stress burden,[97, 98] while selective inhibition of inducible and neuronal NOS has been associated with decreased formation of reactive nitrogen species and decreased apoptotic cell death in a newborn piglet model of HI.[99]

Although much work has been done to elucidate the interplay between inflammation and oxidative stress, the exact mechanisms linking inflammation, oxidative stress, and neonatal pathology remain poorly understood. The end result of excessive inflammation appears to be a vicious cycle whereby oxidative stress induces cytokine release, which then go on to increase ROS production further.[100] Additional studies are needed to investigate the cross talk between inflammatory injury and oxidative stress and their contribution to neonatal injury, and to delineate therapeutic strategies aimed at the inflammatory and oxidative response to injury.

Oxidative stress in neonatal pulmonary disease

While neonatal lung injury is often a result of a multitude of etiologic factors, oxidative stress has been recognized as a critical factor in pathophysiology of several neonatal lung diseases, including PPHN and BPD. Oxidative stress is also a common endpoint for multiple events, including inflammation, hyperoxia, and mechanical ventilation, that contribute to sustained lung injury and result in impaired lung function. The production of oxidative stress originates from a variety of sources and, when combined with immature antioxidant defenses, may ultimately result in oxidative lung injury in an at-risk neonate.

Evidence from animal models of PPHN linking increased oxidative stress to negative effects on NO signaling pathways and aberrant pulmonary vasodilatory responses supports the idea that ROS play an important role in the pathogenesis of PPHN. Lambs with PPHN demonstrate both antenatal and postnatal evidence of increased oxidative stress as compared to control animals, including increased levels of O_2^- and H_2O_2 in the pulmonary vasculature

of fetal animals,[14, 101, 102] and exaggerated ROS production in response to hyperoxia after birth.[103, 104]

When Dr. Northway first described BPD more than 50 years ago, he outlined the toxic effects of oxygen exposure on the preterm lung as it induced the disease phenotype.[105] Clinicians continue to struggle with the paradigm as the ideal oxygen saturation and exposure for premature infants remain unknown.[106] Recent work has shown that any amount of oxygen exposure in the first few hours of life, or even just in the delivery room, can induce oxidative stress and potentially put patients at higher risk for developing BPD. [107, 108] Murine models have demonstrated that various concentrations and intervals of oxygen exposure during critical developmental windows can induce alveolar simplification. [16, 109] High oxygen exposure is directly toxic to the alveolar epithelium, especially the alveolar type II epithelial cell of which a subpopulation functions as stem cells for growth and injury repair.[19, 110] This is particularly injurious to the lung because oxygen exposure stunts epithelial cell growth potential in culture models even after removal of the oxygen exposure.[18]

A variety of ROS sources have been demonstrated in the lamb model of PPHN as well as in neonatal hyperoxic lung injury models of BPD. In the lamb model of PPHN, mitochondrial ROS have been linked to aberrant changes in the NO signaling pathways while O_2^- production from uncoupled eNOS and from NADPH oxidase has been associated with impaired vasodilatory responses and decreased angiogenesis.[102, 111–113] Rodent models of hyperoxic lung injury, on the other hand, demonstrate hyperoxic injury to pulmonary endothelial cells via NADPH oxidase resulting in cell death.[114–116] Similarly, hyperoxia exposed human fetal lung endothelial cells demonstrate increases in the NADPH pathway and H_2O_2 production.

Finally, impaired antioxidant capacity in the premature pulmonary endothelium and epithelium can potentially result in an imbalance between ROS production and antioxidant abilities of the lung, leading to increased oxidative stress. Reductions in superoxide dismutase enzymes, including ecSOD and MnSOD, have been described in PPHN lambs, [111, 117] while treatment with antioxidants results in increased eNOS, decreased PDE5 activation, better vasodilatory responses, and improved oxygenation.[103, 117–119] Preterm human lung tissues are deficient in their ability to increase MnSOD expression when exposed to hyperoxia.[120, 121] Moreover, in premature infants, early high oxygen exposure increases GSSG/GSH ratio that continues for the first several days of life and correlates with later development of BPD.[108]

Due to the crosstalk between the pulmonary endothelium and epithelium during development,[122] the fact that PPHN and BPD have overlapping oxidative stress and antioxidant mechanisms is not surprising. While pulmonary epithelial and endothelial cells have distinct mechanisms of oxidative stress production in hyperoxic lung injury models, [17, 18, 114–116] there are similarities in mechanisms of ROS generation between the PPHN and BPD disease models.[16, 102, 111–113, 123] These cell-type specific responses highlight the difficulty of targeting antioxidant systems for PPHN and BPD therapy as the

lung is comprised of more than 40 cell types that may respond differently to oxidative stress stimuli.

Oxidative stress, gene regulation and epigenetic changes

Gene regulation in response to oxidative stress is complex and incompletely understood. As gene expression and regulation are organ specific, and in many cases cell-type specific, research on neonatal organ development and response to injury can be a challenge. Recent work with the LungMAP project has demonstrated that the neonatal period is a dynamic phase of shifting gene expression in order to direct organ maturation. Therefore, neonatal disease modeling is further complicated by this superimposed developmental trajectory.[124, 125] Current knowledge on gene expression or epigenetic changes in human neonates is mostly obtained from cells in blood samples and umbilical cord blood, however this does not allow for systematically revealing temporal pathogenesis at molecular level in lungs, brain, heart etc. Neonatal murine hyperoxic lung injury models are versatile and allow research at the molecular level in tissues. When rodents are born at term, their lungs are developmentally in the saccular stage. However, as opposed to humans, rodents are biologically prepared to meet the extrauterine (21% O₂) world in the saccular stage with oxidative defenses genetically programmed to handle this postnatal environment.

In a murine neonatal hyperoxic lung injury model inducing BPD-like alveolar simplification, we determined mRNA gene expression in lung tissue of young mice using microarray technique. 117 genes related to pulmonary vascular disease were differentially regulated, including down-regulation of genes related to contractile elements, as well as up- and down-regulation of factors involved in vascular tone and tissue specific genes. Another group found close to 665 differentially regulated genes in neonatal hyperoxic lung injury; when isolating the genes not affected by sex differences, the remaining ~200 genes were predominantly in the angiogenesis pathways.[126] Moreover, in our hyperoxic lung injury model, 69 genes relevant to the immune system had differential expression including B-cell specific genes. (Revhaug C, Saugstad OD, unpublished data) Additionally, significant enrichment was observed regarding cytokine-cytokine receptor interactions, the P13-AKT, and B cell receptor signaling pathways.[127] Oxidative damage could therefore be partly responsible for the increased susceptibility to respiratory viruses and infections seen in premature infants with BPD through dysregulated genetic pathways involved in immunity and inflammation.

Any stress or alteration in cellular environment results in some cell or organ response. Gene regulation will change to produce proteins that may protect and repair the cell after damage or exposure to potentially damaging forces. Oxidative stress changes gene expression preferably by up-regulating antioxidant genes and regulating genes responsible for repairing cell damage. ROS also act as messengers to induce expression of genes via signal transduction. ROS from outside the cell (hyperoxia) or intracellular ROS (mostly from the mitochondria but also from cytokines and growth factors) activate redox-sensitive signaling pathways like NF- κ B and AP-1, p38 mitogen-activated protein kinase, and JNK and result in expression of antioxidant and proinflammatory genes.[128]

In addition to the direct effects on gene expression, oxidative stress can also result in epigenetic changes. Epigenetic mechanisms regulate the availability of genes for transcription, resulting in altered gene expression without changes to the DNA sequence. Epigenetic research is not new, the term and concept already introduced in the 1940 when epigenetics were studied in regard to evolution and development. [129] Epigenetic changes are responsible for considerable variability in the phenotypes of complex organisms and it is likely true for pathophysiological and disease phenotypes. The main methods of epigenetic regulation are DNA methylation and histone modifications (chromatin structuring and remodeling) that can lead to short-term and long-term effect on gene regulation. There are some studies that have looked at oxygen treatment and ROS [130] and the effect of oxygen and redox systems in the cells on the enzymes methylating DNA and histones [131]. ROS can have both direct and indirect effects on epigenetic mechanisms. OH⁻ can directly attack DNA bases, leading to the formation of 5-hydroxymethylcytosine (5hmC) from 5-methylcytosine (5mC). 5hmC can then lead to indirect demethylation of CpG sites resulting in changes in gene transcription.[132, 133] An example of lasting epigenetic effect of oxygen in the newborn period was published in 2012, where adult rats exposed to hypoxic episodes in the neonatal period showed increased sleep disordered breathing and hypertension thought to be a result of DNA methylation in redox genes.[134]

Studies on human autopsy lung samples, from preterm infants and stillborn fetuses, have demonstrated a developmental shift in gene methylation affecting gene expression. In patients with established BPD, 32 genes were shown to have differential methylation compared to controls including differential expression of genes in pathways like GSH-mediated detoxification.[135] Moreover, decreased expression of the MnSOD gene was associated with DNA hypermethylation of a single CpG dinucleotide close to the transcription start site. Indirectly, ROS can affect gene regulation via epigenetic changes both locally and globally.[133, 134] Hyperoxic treatment of newborn mice in a BPD model resulted in lasting global hypermethylation of lung tissue homogenates. In this hyperoxic lung injury model, significant global DNA hypermethylation was present in oxygen-exposed animals, as well as enrichment of the TGF- β pathways.[136] Recently, epigenetic data from newborn infants show that the amount of oxygen provided during postnatal stabilization changes the DNA methylome in preterm infants. These authors found 1567 genes to be associated with oxygen load and, of these, 85% were hypomethylated acutely following oxygen exposure. An oxygen load starting at >500 mL O₂/kg changed the methylation pattern of pathways involved in cell cycle progression, DNA repair, and oxidative stress. [137]

In the future the role of posttranslational epigenetic changes, like N⁶-methyladenosine (m⁶A), may be of interest, as these mechanisms are largely not well understood. A recent study demonstrated that hypoxia induces an increase in methylation of mRNA and that increased m⁶A stabilizes mRNA, however, the impact of these changes remains to be further elucidated. [138] In conclusion, hypoxia and stress are believed to modify RNAs by complex and poorly understood mechanisms and more work is needed to improve our understanding of these processes. [139]

Antioxidant defense and antioxidant therapy

There is strong evidence that the antioxidant defense system in premature infants is profoundly underdeveloped; however, attempts to therapeutically target these systems have yielded inconsistent results.[121, 140–143] Developmentally, the antioxidant system rapidly matures during the third trimester.[15, 142] This is likely an evolutionary change to adapt from the relatively hypoxic fetal environment, with a partial pressure of oxygen at 3.3 kPa, to the high oxygen tension extrauterine environment after birth.[144] As preterm infants are prematurely exposed to this environmental shift, relevant antioxidants that have been clinically targeted to prevent complications of prematurity include enzymes like CuZnSOD, MnSOD and ecSOD, GSH; vitamins such as A or E; and trace elements which are enzyme cofactors, such as selenium or L-arginine; however, development of these as clinical therapeutics have been limited by biochemical and physiologic factors. Antioxidant therapy developments have been limited by biochemical impediments such as compound half-life, poor cell penetrance, and difficulty targeting intracellular organelles. Moreover, there is a lack of physiologic knowledge regarding ideal antioxidant levels, gestationally appropriate enzyme activity, the relationship between trace element cofactor levels and enzyme activity in the premature neonate, as well as the appropriate disease endpoints to assess therapeutic effect.[120, 145, 146]

The very act of labor may prime the antioxidant system to deal with the transition to extrauterine life. Labor and vaginal delivery change the oxidative state in the mother, as well as in the fetus and the neonate, due to fluctuations in tissue oxygenation tension. Increased neonatal SOD, maternal MDA and placental xanthine oxidase activity have been reported in women who labored versus deliveries accomplished by caesarean without labor.[147, 148] In preterm infants, especially, when this physiologic response occurs it could overload their available antioxidant systems.[148] However, Georgeson *et al* found that preterm infants were able to produce higher CAT and GPx activity when delivery occurred by spontaneous vaginal delivery as opposed to cesarean section at comparable gestational ages. This supports the theory that the oxidative stress burden produced during labor and delivery induces antioxidant enzyme expression and activity to deal with the transition to extrauterine life.[149]

Animal models have shown beneficial effects of antioxidant therapies regarding prevention and improvement of neonatal disease phenotypes. Mitochondria-targeted antioxidants improve adult mouse survival in hyperoxia, and attenuate alveolar simplification and right ventricular hypertrophy in neonatal hyperoxic lung injury models.[16, 19] Extensive work with MnSOD, CuZnSOD and ecSOD, in hyperoxic lung injury models, have shown improvement in lung epithelial cell function and alveolarization through oxygen scavenging in the pulmonary epithelium.[150–154] In PPHN models, exogenous MnSOD treatment increases eNOS and improves vasodilatory responses in isolated pulmonary arteries.[117] Furthermore, treatment of PPHN lambs with recombinant human-CuZnSOD (rhSOD) improves vasodilation and increases iNO responsiveness,[119] resulting in improvements in oxygenation and decreased oxidative stress,[103] as well as attenuation of hyperoxic PDE5 activation with improvements in cGMP levels.[118] In vitro and in vivo, hydrocortisone treatment is associated with reductions in ROS levels along with decreases in hyperoxic

PDE5 activation.[155, 156] Moreover, hypoxic-ischemic brain injury can also be attenuated in neonatal mice by targeting ROS production at the level of mitochondrial complex I, a major source of mitochondria-derived O_2^- . [157] In a rat NEC model, all-*trans*-retinoic acid injections were protective against NEC mediated by increased SOD and GPx activity. Supplementation of several different antioxidants, such as all-*trans*-retinoic acid, N-acetylcysteine, or astragaloside, all decreased the incidence of experimental NEC in the rat model likely through convergent mechanisms of lowering intestinal lipid oxidation as measured by MDA levels.[158] However, some antioxidant supplementation studies have exacerbated the disease of interest,[159, 160] highlighting our limited understanding to the optimal antioxidant balance in the developing neonatal animal.

Due to the direct oxygen toxicity on the lungs that occurs following premature birth, many groups have attempted to prevent BPD with antioxidant therapies ranging from vitamin and cofactor supplementation to replacement of antioxidant enzymes deficient in preterm infants. Supplementation with vitamin E and the enzyme co-factor selenium did not decrease the incidence of BPD in several studies.[161, 162] A non-enzymatic antioxidant intervention to show promise for BPD prevention is intramuscular vitamin A injections for the first month in extremely premature infant. The significant treatment effect was small, but the implementation of this therapy has been variable, likely due to the lack of prolonged clinical effect. Although underpowered for this outcome, Vitamin A therapy showed no difference in neurodevelopmental or pulmonary outcomes at 18–22 months.[163, 164] With regards to enzyme replacement, one of the most promising clinical trials regarding prevention of long-term pulmonary complications was intratracheal administration of rhSOD although this therapy has not become standard of care in the 15 years since the publication. Although the rhSOD intervention did not decrease early death or BPD, at one year, survivors at one year had decreased pulmonary disease burden quantified by less emergency room visits, medications and pulmonary readmissions.[165] On the other hand, attempts to target GSH deficiency in preterm infants with N-acetylcysteine did not affect BPD incidence or lung function in infancy.[166, 167] Similarly, despite promising animal studies, trials using inhaled NO in the early preterm period have not been beneficial in preventing either BPD or brain injury; at this time, routine use is not recommended.[168–172] By contrast, a recent study even indicated increased risk of childhood cancer after NO exposure to newborn infants (Adjusted OR 8.6, 95% CI 4.3– 17.4).[173]

Similar to BPD, animal models and patient studies have implicated oxidative stress and ROS generation in other neonatal disorders such as NEC, IVH and HI; but therapies have not become standard of care as the investigations have been limited by marginal effect or study size.[158, 174] For example, a systematic review regarding the use of L-arginine supplementation was a protective effect against NEC; however, the combined trials within the review only included 235 infants limiting confidence in adapting this therapy as a practice change.[175] However, one emerging therapy to show promise may be the use of melatonin (*N*-acetyl-5-methoxytryptamine) as an adjunct to hypothermia treatment to prevent HI injury. Melatonin has anti-oxidant, anti-apoptotic and anti-inflammatory properties, and has shown promise in published results from pre – and clinical intervention trials.[176, 177]

Physiologic reasoning supports using antioxidant therapy to prevent or address neonatal diseases where oxidative stress and ROS generation are central to the pathophysiology; despite the promising results in preclinical animal studies, human trials for antioxidant therapies have demonstrated a marginal benefit at best and in most cases no disease effect (Table 3). In addition to the biochemical and physiologic limitations listed above, successful transition of antioxidant therapies from pre-clinical to clinical studies have been limited by poor understanding of the correlations between histologic changes in animal models and physiologic changes in human disease. Many animal models demonstrate a histopathologic change in the target organ and the treatment effect is similarly assessed; however these findings are difficult to translate to the clinical disease spectrum present in humans.[109, 178, 179] Moreover, many of neonatal diseases discussed above are diagnosed on a constellation of clinical parameters, not on the histologic changes that are used to evaluate animal models.[180–182] A current search of clinicaltrials.gov yields a list of 59 studies regarding premature infants and antioxidants, with specifically 13 studies investigating antioxidants and BPD as the outcome.[183] In general, there is a lack of strong evidence in humans to encourage the use of antioxidant therapy as standard of care. Further work is needed to understand the antioxidant system in premature neonates, determine ideal enzyme activity and understand how histopathologic improvements in animals models correlate with physiologic outcomes in human neonates.

Discussion

Oxidative stress is involved in a wide spectrum of newborn disease. In this review, we have summarized evidence for oxidative stress involvement not only in pulmonary diseases, such as BPD and PPHN, but also in HIE, NEC and fetal growth, as well as vascular changes. Although not addressed in as much detail here, previous reviews have detailed the evidence regarding retinopathy of prematurity as an oxygen dependent condition.[184, 185] Moreover, we summarized the epigenetic changes and subsequent effects on the transcriptome caused by oxidative stress and the subsequent long-term health implications of these neonatal changes.

Since those early articles linking neonatal disease to oxidative stress more than 30 years ago, the complexity and interplay between different ROS mechanisms and cell types have progressed significantly. The specific role and significance of mitochondrial derived oxidative stress has enhanced our understanding how oxidative stress may contribute to disease and injury especially in the newborn. Taken together with a better understanding of risk factors for oxidative stress, such as growth restriction and neonatal oxygen exposure, there is a hope that development of a new generation of antioxidants, in combination with other modulators, may contribute to prevention of oxidative stress induced injury of the newborn.

Genetic and epigenetic regulation occurs through several mechanisms; moreover, evidence supports that oxygen exposure induces epigenetic changes. Whether these are transitory or more permanent remains to be proven. However, such genetic and epigenetic investigations are also of importance for augmentation of our understanding how to prevent newborn

disease; perhaps most importantly, how neonatal diseases and interventions have lasting effects into adult life, and thus subsequently contribute to adult diseases.

In figure 1 are summarized some of the concepts discussed in this review. Aberrant growth, maternal disease, such as obesity and UPI, infection, like chorioamnionitis, may all have more profound effects on the premature than the term infant. Oxidative stress with or without inflammation may be translated to injury of several organs such as the lung, brain, intestine and eye.

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Figure 1.
Mechanisms contributing to Oxygen Radical Disease in the Newborn

Table 1

Oxidative Stress related factors contributing to neonatal disease

Newborn Disease	BPD	PPHN	NEC	IVH	HIE	Growth Aberrancy
Mitochondrial OS	+	+	+			+
Inflammation	+	+	+	+	+	+
Genetic/Epigenetic	+					+

Table 2.

Summary of the oxidative stress state related to altered fetal and maternal growth status.

Aberrant Growth State	Antioxidant Reserve		Free Radicals Present
	Enzyme	Vitamin	
Maternal Obesity	↓SOD, ↓GSH ↓GSSH/GSH	↓Vitamins B ₆ , C and E	↑MDA
Small for Gestational Age	↑Xanthine oxidase, ↓GSH, ↓CAT, ↓ SOD	↓Vitamins C and E	↑MDA, ↑H ₂ O ₂
Large for Gestational Age	↑SOD	↓Vitamin A and E	↑H ₂ O ₂

Table 3.

Summary of trials evaluating antioxidant therapy for prevention of neonatal disorders.

Therapy	Mechanism/ROS Target	Trial Outcome	Reference
<i>BPD</i>			
Inhaled NO	NO	No disease effect	Hasan et al.[171]; Askie et al. [172]; Askie et al. [28]
N-acetylcystine	GSH	No disease effect; no long term effect on lung function	Ahola et al.[167]; Sandberg et al.[166]
rhSOD	CuZnSOD	No decrease in death or BPD; survivors with less pulmonary disease burden in infancy	Davis et al.[165]
Selenium	Enzyme co-factor supplementation	No disease effect	Darlow et al.[162]
Vitamin A	Supplementation	Decreased incidence of BPD No pulmonary effect at 18–22 months	Ambalavanan et al. [163]; Tyson et al. [164]
Vitamin E	Supplementation	No disease effect	Watts et al. [161]
<i>NEC</i>			
L-arginine	Enzyme co-factor Supplementation		Mitchell et al.[175]
<i>Neurodevelopment</i>			
Inhaled NO	NO	No effect for preventing IVH	Askie et al.[172]
Melatonin	GSH, MnSOD, ecSOD		Aly et al. [176]
Vitamin A	Supplementation	No developmental effects at 18–22 months	Tyson et al. [164]