

## Reactive Oxygen Species, Biomarkers of Microvascular Maturation and Alveolarization, and Antioxidants in Oxidative Lung Injury

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**ABSTRACT** | The lungs of extremely low gestational age neonates (ELGANs) are deficient in pulmonary surfactant and are incapable of efficient gas exchange necessary for successful transition from a hypoxic intrauterine environment to ambient air. To improve gas exchange and survival, ELGANs often receive supplemental oxygen with mechanical ventilation which disrupts normal lung developmental processes, including microvascular maturation and alveolarization. Factors that regulate these developmental processes include vascular endothelial growth factor and matrix metalloproteinases, both of which are influenced by generation of oxygen byproducts, or reactive oxygen species (ROS). ELGANs are also deficient in antioxidants necessary to scavenge excessive ROS. Thus, the accumulation of ROS in the preterm lungs exposed to prolonged hyperoxia, results in inflammation and development of bronchopulmonary dysplasia (BPD), a form of chronic lung disease (CLD). Despite advances in neonatal care, BPD/CLD remains a major cause of neonatal morbidity and mortality. The underlying mechanisms are not completely understood, and the benefits of current therapeutic interventions are limited. The association between ROS and biomarkers of microvascular maturation and alveolarization, as well as antioxidant therapies in the setting of hyperoxia-induced neonatal lung injury are reviewed in this article.

**KEYWORDS** | Antioxidants; Bronchopulmonary dysplasia; Chronic lung disease; Matrix metalloproteinases; Reactive oxygen species

**ABBREVIATIONS** | BPD, bronchopulmonary dysplasia; CLD, chronic lung disease; ECM, extracellular matrix; ELGAN, extremely low gestational age neonate; GPx, glutathione peroxidase; GSH, reduced form of glutathione; IH, intermittent hypoxia; MMP, matrix metalloproteinase; NAC, *N*-acetylcysteine; NOX, NADPH oxidase; PUFA, polyunsaturated fatty acid; RDS, respiratory distress syndrome; ROS, reactive oxygen species; SOD, superoxide dismutase; TIMP, inhibitor of metalloproteinase; VEGF, vascular endothelial growth factor

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## 1. INTRODUCTION

Extremely low gestational age neonates (ELGANs, < 28 weeks gestation and/or birth weight < 1250 grams) are usually intubated at birth and mechanically ventilated for respiratory support and treatment of respiratory distress syndrome (RDS) [1]. Although advances in respiratory care and management have led to the increased survival of ELGANs, approximately one-third of them will develop bronchopulmonary dysplasia (BPD), the most common form of chronic lung disease (CLD) that occurs in 45–68% of infants < 29 weeks gestation [2]. BPD/CLD is characterized by prolonged need for oxygen therapy, increased partial pressure of carbon dioxide (PCO<sub>2</sub>), and abnormal lung compliance and the known risk factors include preterm birth, respiratory failure, oxygen toxicity, and barotrauma [3], with devastating complications such as pulmonary hypertension occurring in as many as 17–24% [4] and rising to 59% in periviable infants, < 25 weeks [5]. Pathological characteristics include disrupted microvascular maturation and poor alveolarization secondary to lung inflammation, mechanical injury, and oxygen toxicity, leading to alveolar oversimplification and inadequate blood-air interface [6–8]. Many factors regulate normal and pathological angiogenesis and alveolarization, including, but not limited to, vascular endothelial growth factor (VEGF) involved in microvascular maturation, matrix metalloproteinases responsible for alveolarization, extracellular matrix (ECM) and lung basement membrane proteolysis and remodeling, and antioxidants responsible for scavenging of reactive oxygen species (ROS), all of which are deficient in ELGANs. While several reviews on BPD have been published, this review focuses on, and highlights, the important role of ROS

in the development of BPD, and examines the association between VEGF, ECM degrading enzymes or matrix metalloproteinases (MMPs), and ROS in its pathogenesis. It also discusses alternative treatments focusing mainly on antioxidant defenses for ROS scavenging and their likely therapeutic or preventive role in alleviating lung toxicities.

## 2. PERINATAL LUNG DEVELOPMENT

At about 22–25 days post conception, lung bud arises from the embryonic foregut. This is followed by subsequent branching of the bronchial tree [9]. Five major stages are identified that may overlap since lung structures develop simultaneously. These are embryonic, pseudo-glandular, canalicular, saccular, and alveolar stages [10, 11]. Between 16 and 28 weeks, lung growth is dominated by bronchial development, particularly, formation of alveolar ducts and terminal sacs. By around 24 weeks, these terminal sacs become abundant. The epithelium thins and Type 1 and II pneumocytes form. During this canalicular period, the network of blood vessels come much closer to the terminal saccules or primitive alveoli allowing for efficient respiration and gas exchange, despite bulk alveolarization occurring in the postnatal period [12]. Lung septation and alveolarization begin around 32–36 weeks gestation and are tightly coupled with vascular growth and branching [11], a complex process of endothelial cell differentiation and proliferation (vasculogenesis), formation and branching of new blood vessels (angiogenesis), and smooth muscle cell migration (arteriogenesis). The final stages of vascular development are characterized by increasing surface areas with thinning of alveolar walls surrounded by pulmonary capillaries

allowing postnatal gas exchange. Alveolar and vascular growth occur side by side involving delicately choreographed processes of cell proliferation, differentiation, migration, and apoptosis. Complex signaling pathways regulate these processes which are disrupted by preterm birth and exposure to supplemental oxygen.

The lungs of ELGANs born at < 28 weeks gestation are in the canalicular and saccular or early alveolar stages of development and are deficient of pulmonary surfactant, which normally matures in utero at much lower oxygen levels of 20–30 mm Hg necessary to promote branching morphogenesis, angiogenesis, and ECM deposition. They are incapable of efficient gas exchange, as the rate of transfer of gas across the lung is directly proportional to the surface area and inversely proportional to the diffusion distance between blood and air, according to Fick's Law of diffusion [4]. To improve gas exchange, ELGANs often receive supplemental oxygen with mechanical ventilation. Hyperoxia results in the production of harmful oxygen byproducts or ROS, and disrupts normal lung developmental processes including microvascular maturation and alveolarization [13]. Due to immature antioxidant systems to scavenge ROS, their accumulation in the lungs causes inflammation, lung tissue damage, and CLD/BPD [14]. While the etiology of CLD/BPD is multifactorial, overwhelming evidence suggests that oxidative stress and excessive ROS with inadequate antioxidant systems play a key role in its development and severity [15–17].

### 3. OVERVIEW OF ROS

ROS are abundant in nature because they may be formed endogenously or exogenously between cells. The unique molecular configuration of oxygen allows it to accept free electrons from normal oxidative metabolism [18] leading to the production of superoxide anion ( $O_2^{\cdot-}$ ), hydroxyl radical ( $OH^{\cdot}$ ), and hydrogen peroxide ( $H_2O_2$ ), of which the mitochondria and peroxisomes are major sources. About 90% of cellular oxygen uptake is due to mitochondrial respiration and 1–2% of the oxygen consumed is transformed into ROS [19, 20]. Uncoupling of the electron transport chain found in the mitochondria generates ROS and is the major producer [21, 22]. A good amount of ROS may also come from cytoplas-

mic and endoplasmic reticulum-bound enzyme systems and the plasma membrane [23, 24]. Generation of ROS is affected by the availability of oxygen, the redox state of the mitochondrial complexes, and mitochondrial membrane potential [25]. Multi-enzyme systems can also account for the production of ROS. These systems include flavoproteins that produce  $H_2O_2$ , cytochrome P450 monooxygenase system that produces superoxide, xanthine oxidoreductase that produces both superoxide and  $H_2O_2$ , and nitric oxide synthase that produces superoxide and nitric oxide (NO) [26–29]. NADPH oxidases (NOX 1–3), dual oxidases 1 and 2, and NOX 4 produce  $O_2^{\cdot-}$  endogenously [30, 31]. Other systems mainly, involved in inflammatory process, cyclooxygenase and lipoxygenase pathways in arachidonic acid metabolism can also generate ROS that contribute to the evolution of lung disease [32]. Superoxide anion reacts rapidly with nitric oxide producing peroxynitrite ( $ONOO^{\cdot}$ ) [33, 34]. Lipid peroxidation is initiated once peroxynitrite or hydroxyl radical reacts with membrane lipids forming more complex radicals [35]. ROS are also important regulators of nitric oxide bioavailability, affecting airway and vascular reactivity [30]. ROS directly damage cellular proteins, lipids, and nucleic acids via protein oxidation and nitrosylation, lipid peroxidation, and oxidation of nucleic acids [36, 37]. These injuries can lead to impairment in enzymatic functions adversely affecting growth factors [38]. Lipid peroxidation results in the activation of sphingomyelinase releasing ceramide from cellular phospholipids, triggering apoptosis [39]. Breakage in DNA strands after oxidation of nucleic acids leads to necrosis and maladaptive apoptosis [40].  $H_2O_2$ , a ubiquitous ROS [41, 42] permeates cell membranes through water channels [43–45], may act as a secondary messenger modulating cellular signaling [46].  $H_2O_2$  activates multiple signal transduction pathways, facilitating the actions of growth factors, cytokines, chemokines, and calcium signaling [47, 48]. During blood transfusion for anemia of prematurity, iron will react with  $H_2O_2$  forming hydroxyl radical that can injure cellular components [49, 50] leading to defective signaling pathways [51].

### 4. ROS AND PRETERM LUNGS

The premature lungs are highly susceptible to oxidative lung injury. Although necessary and essential for

their survival, exposure of the immature lungs to oxygen leads to accumulation of ROS, including superoxide anion, the first ROS responder. Superoxide is very unstable and is rapidly converted to the more stable  $\text{H}_2\text{O}_2$ , and  $\text{O}_2$  by mitochondrial superoxide dismutase (MnSOD) in the mitochondrial matrix and copper, zinc SOD (CuZnSOD) in the cytosol. SOD is the primary ROS-detoxifying enzyme in the cell [52]. Complete disposal of  $\text{H}_2\text{O}_2$  requires the action of catalase, predominantly located in peroxisomes, and glutathione peroxidase in the mitochondria and cytosol [53]. Accumulation of  $\text{H}_2\text{O}_2$  can lead to the formation of the highly reactive hydroxyl radical in the presence of ferrous iron via the Fenton reaction [54]. The hydroxyl radical oxidizes mitochondrial components such as lipids, proteins, and DNA via the self-propagating lipid peroxidation. Since mitochondrion lacks catalase, it relies on the reduced form of glutathione (GSH, about 10–20% of which is present in the mitochondria, the remainder is in the cytosol) to effectively scavenge  $\text{H}_2\text{O}_2$ . We and others have shown deficient antioxidant status in preterm infants [55, 56] and neonatal animal exposed to hyperoxia [57], suggesting an ROS/antioxidant imbalance that may lead to oxidative distress. Furthermore, ELGANs are often supplemented with iron for anemia. However, their low levels of plasma transferrin, the major iron-binding protein, predispose them to a higher risk for lipid peroxidation [58–62]. In the immature lungs, supraphysiological levels of oxygen and ROS result in a complex inflammatory reaction that is associated with accumulation of various cells (neutrophils and alveolar macrophages), and inflammatory mediators which result in increased microvascular permeability and lung damage [57]. Furthermore, exposure to high concentrations of oxygen during lung development results in alterations in capillary density [63], endothelial cell destruction [64], pulmonary inflammation [65], and inhibition of the process of alveolarization [66]. The immaturity of the lungs and inability of respiratory control result in apnea of prematurity and intermittent hypoxia (IH) events triggered by cessation of respiratory neural output [9, 67]. An IH event is usually defined as a decline in  $\text{SaO}_2$  by 5% lasting < 3 minutes in duration [67]. Re-oxygenation which occurs in hyperoxia or normoxia (IHR) reestablishes blood flow through damaged vessels, resulting in reperfusion injury. ROS are induced in both IH and IHR and may contribute to inflammation and cell death. Fluctuations

in  $\text{SaO}_2$  together with the underdeveloped antioxidant-scavenging mechanisms add to the complexity of CLD/BPD.

## 5. ROS AND VEGF

At any stage in pulmonary development, ROS may interfere with the well-coordinated processes influencing signaling pathways that regulate pulmonary vascular development which involves angiogenesis (sprouting of new vessels from existing ones required for central vessels) and vasculogenesis (formation of capillaries from endothelial cells). Using the baboon BPD model, Coalson et al. [68–70] demonstrated that the premature lungs exposed to hyperoxia and mechanical ventilation developed bronchial and bronchiolar epithelial lesions, decreased alveolarization, and interstitial and peribronchiolar fibrosis. Poor alveolarization and disrupted microvascular maturation involve altered angiogenic factors such as VEGF, ECM and lung basement membrane proteolysis by MMPs, and mitochondrial dysfunction [71–76]. VEGF is a potent mitogen and inducer of endothelial cell growth and proliferation [77]. Its expression is high in distal airway epithelial cells during differentiation of human fetal lungs [78]. VEGF is highly induced by hypoxia via the highly conserved transcription factor, hypoxia-inducible factor (HIF)-1 $\alpha$ , which regulates over 100 genes involved in proliferation and angiogenesis. Studies have shown that hyperoxia decreases neonatal lung VEGF protein and mRNAs [79]. Reduced lung VEGF mRNA and protein expression as well as decreased receptor Flt-1 were associated with characteristic patterns of alveolar simplification and dysmorphic microvasculature in the lungs of infants dying with BPD [80]. Premature infants who died with severe RDS had lower lung VEGF than survivors, and infants with BPD had lower tracheal VEGF [81–83]. We have shown similar findings in lungs from premature baboons delivered at 125 or 140 days gestation who received 100% oxygen with mechanical ventilation [84], and in preterm infants with BPD [85]. During recovery from hyperoxia, VEGF expression is increased in alveolar epithelial cells suggesting a role for VEGF in microvascular repair process [86]. Elevations in VEGF during hyperoxia reperfusion injury may involve mitochondrial ROS activation of HIF [50, 87].

## 6. ROS AND MATRIX METALLOPROTEINASES

Lung alveolarization, or the increase in gas-exchange surface area is characterized by the formation of alveoli and subdivision of sacculi [88]. During this stage, secondary septa are formed in the sacculi to create alveoli [12, 89]. Secondary septation occurs in parallel with microvascular maturation, the transition from a double-layered to single-layered capillary network [90]. This process involves MMPs, which regulate the structural changes associated with alveolarization and microvascular maturation [91].

MMPs are enzymes that degrade type IV collagen, the major constituent of ECM and lung basement membranes necessary for mechanical and functional properties of lung tissue. MMPs belong to a family of zinc endopeptidases that are collectively capable of degrading essentially all ECM components, and as such, play a major role in inflammation, tissue remodeling, angiogenesis, migration, and invasion [92–94]. MMPs are regulated by cytokines, chemokines, growth factors, and tissue inhibitors of metalloproteinases (TIMPs 1–4) which form a high affinity complex in a 1:1 ratio [95]. A balance between MMPs and TIMPs is responsible for maintenance of normal ECM architecture, whereas an imbalance is believed to result in parenchymal destruction in lung diseases such as CLD complicating pulmonary fibrosis and asthma [96, 97]. MMPs are classified into: (1) collagenases (MMP-1, MMP-8, MMP-13); (2) gelatinases A (MMP-2) and B (MMP-9); (3) stromelysins (MMP-3, MMP-10, MMP-11, MMP-12); and (4) membrane-type MMPs [98, 99]. MMPs and TIMPs are produced ubiquitously in the body. However, in the lungs MMPs are produced by structural cells such as fibroblasts, endothelial, and epithelial cells, as well as alveolar macrophages [97, 100]. Overproduction of MMPs during alveolar remodeling can contribute to inflammatory lung disease. There is evidence that MMPs are involved in neonatal lung injury secondary to hyperoxia and mechanical ventilation in infants [101–107] and animal models [108–110], including studies in our laboratory [111]. From those studies, MMPs are highly involved in arrested alveolarization and abnormal arterial remodeling, which are the hallmarks of BPD. These previous reports also demonstrate a strong association between MMPs, ROS, and oxidative lung injury.

## 7. ROS AND ANTIOXIDANT DEFENSES

A balanced redox state determines the vulnerability of an organism to oxidative stress. Exposure to oxidative stress has potential deleterious side effects, and in some cases, can be lethal. The lungs are particularly susceptible because, of all the organs in the body, it is the most exposed to a higher partial oxygen pressure [112]. However, homeostasis is maintained by efficient antioxidant defense systems that keep oxygen toxicity in check. Mitochondrion is not only the main generator of ROS, but also the main organelle for antioxidant ROS scavenging. Similar systems are found in the cytosol and peroxisomes. Antioxidants can be intracellular and extracellular or enzymatic and non-enzymatic [113]. They may also be categorized as primary (preventing ROS formation), secondary (scavenging ROS), and tertiary (removing or repairing oxidatively modified molecules) [114]. The major detoxifying antioxidant is SOD enzymes [115]. Copper and zinc-containing SOD (CuZnSOD or SOD-1) is found in the cytoplasm [116]. Manganese-containing SOD (MnSOD or SOD-2) is found in the mitochondria [117, 118], and extracellular SOD (ECSOD or SOD-3), as the name indicates, is present extracellularly [119]. These three forms allow the dismutation of superoxide into oxygen ( $O_2$ ) and  $H_2O_2$  [120]. Cytoplasmic and peroxisomal catalase breaks  $H_2O_2$  into water and  $O_2$ , and peroxiredoxins reduce it [121]. Catalase works best with high concentrations of  $H_2O_2$  [122, 123]. Reductases and peroxidases that detoxify ROS and lipid peroxides belong to the thioredoxin and glutathione systems [124]. Two forms of glutathione peroxidase (GPx) have been identified in mitochondria [125, 126]. Peroxidases clear up to 90% of  $H_2O_2$  produced in the mitochondria [127, 128]. Together these enzymes have been shown to have protective roles in lung diseases [129]. Multiple cell culture models suggested that overexpression of antioxidants prevents ROS-induced injury [130]. Other forms of free radical scavengers are small molecular weight compounds of the non-enzymatic type present endogenously or obtained through diet. GSH, cysteinyl tripeptide, uric acid, ascorbic acid, and tocopherols/tocotrienols are some of these compounds involved in protection from pulmonary diseases in neonatal and adult populations [131–135]. Recent literature highlights the importance of maintaining a delicate balance between ROS production in oxida-



tive stress and scavenging. Too much or faulty scavenging may lead to overexpression of SOD that could lead to excessive  $H_2O_2$  production and may have adverse rather than beneficial effects [136]. Studies in our laboratory have shown that the use of MnTBAP, an SOD mimetic that scavenges superoxide and peroxynitrite, can suppress oxygen-induced inflammatory MMP-9 levels in lungs of neonatal rats exposed to IH [137], while the use of anti-VEGF drugs causes pulmonary hemorrhage concurrent with elevations in MMP-2 and MMP-9 [138].

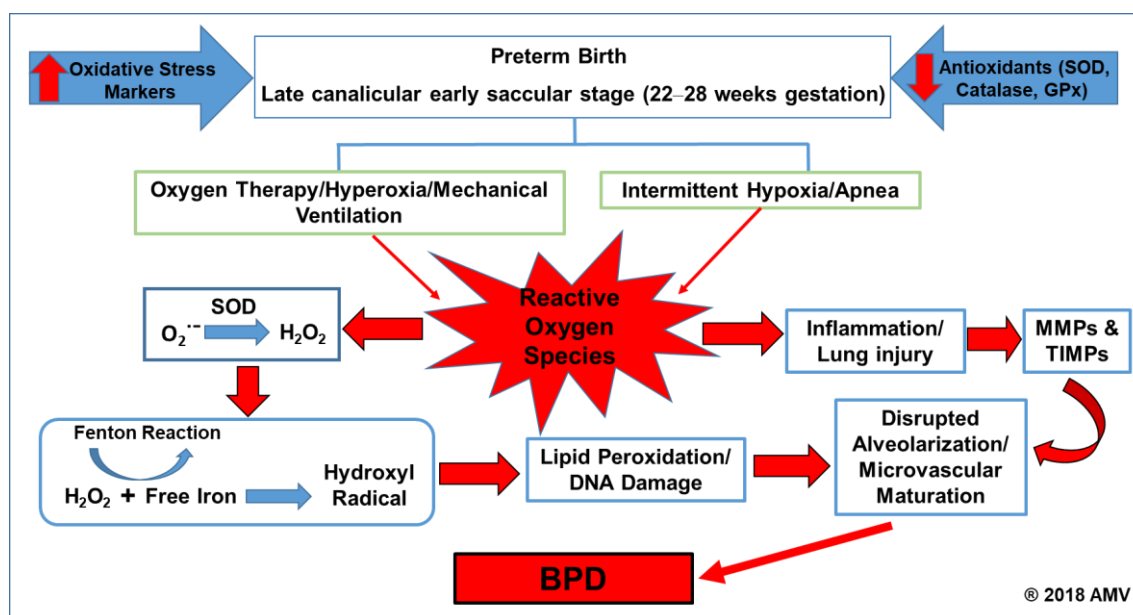
## 8. THERAPEUTIC CONSIDERATIONS

Despite the promising role of antioxidant therapy in CLD in ELGANs, clinical trials have only yielded limited success in treatment [139]. It is postulated that once the disease process has progressed to a critical point, antioxidant therapy may already be ineffective. In early stages, however, it may still have potential roles in prevention. Low doses of dexamethasone, which has anti-inflammatory and antioxidant properties, were beneficial in infants who remain ventilator dependent after 1–2 weeks [140]. However, studies done in our laboratory showed that a low-dose dexamethasone alters the imbalance in lung MMPs and TIMPs which may lead to further lung injury [141]. In lamb models of persistent pulmonary hypertension (PPHN), intratracheal antioxidant administration combined with NO inhalation is shown to be more effective than NO inhaled alone [142, 143]. Recombinant human SOD also reduces peroxynitrite-mediated protein nitration mitigating cell injuries [144]. Nosocomial infection is a predictor of BPD [145]. Exposure of lung epithelial cells to hyperoxia impairs phagocytosis and bacterial clearance with increased IL-8 production, and overexpression of SOD has beneficial effects [146, 147]. Data analysis has pointed to the critical role of SOD in preventing hyperoxia-induced lung injury and the preservation of the alveolar architecture which suggests a potential role for antioxidant therapy in CLD. Scavenging of ROS may also interfere with normal signaling pathways in early lung development and oxidative stress may be localized only in some areas, limiting the efficacy of some antioxidants [148]. Administration of early high doses of antioxidant vitamins had no beneficial effects to curtail pulmonary responses to hypoxia in the baboon BPD model

[149]. Multicenter trials using high-dose vitamin A in premature infants showed a 7% significant reduction of BPD, but long-term follow-up of treated subjects did not show long-term benefits [150, 151]. Vitamin C is thought to contribute to the regeneration of membrane bound alpha-tocopherol in preterm infants [152]. Vitamin E is relatively deficient in preterm infants, but clinical trials failed to demonstrate significant benefits [153, 154], and instead it may increase the risk of necrotizing enterocolitis (NEC) and sepsis [155]. Because iron is important in the production of ROS [54], studies showed that iron chelators effectively prevented ROS-induced lung injury [156]. *N*-Acetylcysteine (NAC) is a precursor of GSH. However, clinical trials comparing NAC against placebo in ventilated extremely low birth weight infants did not improve survival or decrease BPD at 36 weeks corrected age or improved pulmonary function at term [157, 158]. More recently, the use of omega-3 polyunsaturated fatty acids ( $\omega$ -3 PUFAs) has been proposed for the prevention of ROS-induced lung injury. Maternal  $\omega$ -3 PUFA supplementation appears to protect newborn rats from hyperoxia-induced lung injury [159, 160]. However, clinical trials show conflicting results. Preterm infants who received parenteral  $\omega$ -3 PUFAs had a lower incidence of BPD [161, 162], but a large, randomized multicenter trial showed no benefit for reducing BPD [163]. Further longitudinal outcome studies need to be undertaken to determine the efficacy of ROS scavenging and its use in treating BPD. For example, studies implementing synergistic pharmacological strategies to prevent ROS production, scavenge, and decrease ROS that have already been produced due to oxygen therapy, and at the same time, excrete and metabolize other toxic oxidative products such as  $H_2O_2$ , should be conducted in appropriate animal models.

## 9. CONCLUSION

Injuries to cells by oxidative stress is brought about by the arrest in the normal process of proliferation and differentiation. Damage to DNA, protein, and lipids in cell membranes, and disruption of regulatory pathways further amplify the initial injury. The balance between ROS generation and scavenging is crucial for maintaining homeostasis and to the survival of the organism (**Figure 1**). The inadequate an-



**FIGURE 1.** Graphic representation showing only the relationship between reactive oxygen species, matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), and bronchopulmonary dysplasia (BPD). Preterm infants who are at the highest risk for development of BPD are born in the late canalicular/early saccular stage of lung development. At this stage, the incidence of BPD is as high as 68%, and is characterized by alveolar oversimplification, disrupted alveolarization, reduced secondary septation, and aberrant microvascular maturation, all of which are associated with oxidative distress, inflammation, and MMPs and TIMPs. Preterm infants also have immature antioxidant systems. Superoxide anion ( $O_2^{\cdot -}$ ) produced from exposure to high levels of oxygen, undergoes dismutation to hydrogen peroxide ( $H_2O_2$ ) and  $O_2$ . Deficiency of antioxidants such as catalase and glutathione peroxidase (GPx), scavengers of  $H_2O_2$ , will lead to accumulation of  $H_2O_2$ . Preterm infants are often supplemented with iron, and excess free iron due to deficient iron-binding capacity leads to its reaction with  $H_2O_2$  via the Fenton reaction resulting in the formation of the hydroxyl radical, the highly reactive, self-propagating, and extremely powerful oxidant, that reacts with most organic molecules causing lipid peroxidation and DNA damage.

tioxidant system, the early stage of lung development in ELGANs, and their exposure to a hyperoxic environment after birth leads to the ontogeny of their lung disease. BPD/CLD is characterized by alveolar hypoplasia, and in some cases, with accompanying increased in pulmonary vascular resistance making it more challenging to maintain adequate ventilation and oxygenation. Prolonged exposure to oxygen and mechanical ventilation exacerbates oxidative stress and inflammatory injury to the premature lungs, leading to later fibrosis. Studies are underway to exploit the potential of antioxidant therapy to enhance the natural ROS-scavenging mechanisms in this population, in order to decrease morbidities and im-

prove their outcomes. Preliminary data, however, have yielded inconclusive reports. This may suggest that the timing for therapeutic intervention and the specificity of the antioxidant to its target may be key to the success of this novel approach.

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