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Neonatal Intermittent Hypoxia, Reactive Oxygen Species, and Oxygen-Induced Retinopathy

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Abstract

Most of the major morbidities in the preterm newborn are caused by or are associated with oxygen–induced injuries and are aptly called “oxygen radical diseases in neonatology or ORDIN”. These include bronchopulmonary dysplasia, retinopathy of prematurity, periventricular leukomalacia, intraventricular hemorrhage, necrotizing enterocolitis and others. Relative hyperoxia immediately after birth, immature antioxidant systems, biomolecular events favoring oxidative stress such as iron availability and the role of hydrogen peroxide as a key molecular mediator of these events are reviewed. Potential therapeutic strategies such as caffeine, antioxidants, non-steroidal anti-inflammatory drugs, and others targeted to these critical sites may help prevent oxidative radical diseases in the newborn resulting in improved neonatal outcomes.

Keywords

Intermittent hypoxia oxygen-induced retinopathy; Oxidative stress; Reactive oxygen species

1. INTRODUCTION

Reactive oxygen species (ROS) play a key role in the development of a wide range of neonatal diseases including intraventricular hemorrhage (IVH) [1], periventricular leukomalacia (PVL) [2], chronic lung disease/bronchopulmonary dysplasia (CLD/BPD) [3–6], necrotizing enterocolitis (NEC) [7], apnea of prematurity (AOP), and retinopathy of prematurity (ROP) [8–10], thus giving rise to the term “oxygen radical diseases in

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neonatology (ORDIN)" [11, 12]. Extremely low gestational age neonates (ELGANs) who are born at 23–27 weeks of gestation, and weighing <1250 grams are particularly vulnerable to oxidative stress and often require oxygen therapy with mechanical ventilation which accentuate their susceptibility to injury. At this gestational age, lung development and respiratory control are extremely immature [13] and predispose the ELGAN to continuous fluctuations in arterial oxygen saturation (SpO_2), or intermittent hypoxia (IH) episodes, many of which are not responsive to an increased inspired oxygen concentration [3, 13]. Unlike IH in adults and older children with sleep apnea, neonatal IH is a developmental disorder. The lungs of ELGANs are in the canalicular stage of development and the respiratory control mechanisms are underdeveloped [14]. Combined immature respiratory control, relatively supraphysiological oxygen, and immature antioxidant defense mechanisms to scavenge damaging oxygen byproducts, contribute to the pathophysiology of many neonatal diseases.

The goal of this review is to summarize the known mechanisms underlying oxygen induced retinopathy (OIR) focusing on IH and ROS, and to highlight recent data emerging from our laboratory that utilizes a unique OIR model that simulates AOP and IH experienced by ELGANs and produces characteristics consistent with severe ROP. Mechanisms learned from the OIR model may be potentially applicable to all neonatal diseases related to oxidative injuries. This review emphasizes the biomolecular vulnerability of the preterm newborn to oxidative injuries and organ damage caused by the interactive actions of relative hyperoxia after birth, immature oxidative stress defenses, biochemical events favorable to oxidative injuries, and the importance of hydrogen peroxide in the pathogenesis of these diseases (Figure 1). Our recent findings point to the importance of curtailing ROS accumulation and oxidative stress during the early postnatal period. Timely use of appropriate pharmacological agents to prevent rather than treat oxidative stress may mitigate severe OIR.

2. APNEA OF PREMATURITY AND NEONATAL INTERMITTENT HYPOXIA

AOP is defined as cessation of breathing lasting longer than 15–20 seconds and/or accompanied by arterial oxygen desaturation and bradycardia in ELGANs. The incidence of AOP varies with the degree of prematurity, from 7% at 34–35 weeks of gestational age to 15% at 32–33 weeks, 54% at 30–31 weeks, and nearly 100% at <28 weeks [14], and is the most common cause of IH in ELGANs [3, 4, 13, 15]. Apneas of less than 10 seconds in duration can result in a reduction in oxygen saturation of up to 40% [15] even though these episodes may not be recorded on regular cardiorespiratory monitors currently employed in the neonatal intensive care unit (NICU). IH is defined as brief, repetitive cycles of arterial oxygen desaturations followed by re-oxygenation or IHR (recovery from IH) in normoxia or hyperoxia with supplemental oxygen [16]. An IH event is usually defined as a decline in SaO_2 by 5% lasting <3 minutes in duration [13–18]. Episodes of IH, often called desaturations, frequently occur independent of apnea in preterm babies on mechanical ventilation. IHR that follows IH induces damaging mitochondrial ROS, which not only causes oxidative stress and injury, but also activates signaling mechanisms to counteract those induced by IH. After numerous episodes, occurring within minutes of each other, a

"critical" point is achieved where the mechanisms induced by IH become indistinguishable from those induced by IHR [19].

The merging of the above mechanisms has profound deleterious effects on mitochondrial homeostasis and redox state [20]. IH is inflammatory and can lead to impairment of multiple neonatal systems including the brain [21], liver [22], and kidneys [23–25]; the latter two are major sites of drug metabolism and excretion. This is especially important since ELGANs are exposed to numerous drugs during the first few weeks of life. Due to its relatively high lipid content and ability to produce ROS, the liver is uniquely susceptible to lipid peroxidation (a self-propagating chain reaction that involves hydrogen peroxide reacting with elemental iron to form the hydroxyl radical) and IH injury [23]. Studies have shown that IH impairs drug metabolizing ability of several commonly used drugs in the NICU, including gentamicin, phenobarbital, acetaminophen, and theophylline [26], which lead to substantial variation in pharmacokinetic profiles and drug responses in ELGANs [27, 28].

3. MITOCHONDRIA AND ROS

Mitochondria are present in each cell's cytoplasm. The total number per cell varies from less than a hundred up to several thousand, depending on the amount of energy required by the cell [29]. Mitochondria participate in intracellular signaling, apoptosis, and in the metabolism of amino acids, lipids, cholesterol, steroids, and nucleotides. However, the primary role of mitochondria is the generation of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) and oxygen consumption. Therefore, mitochondria are important oxygen sensors. During normoxia, energy from ATP is produced in the mitochondrial respiratory chain, a group of five enzyme complexes situated on the inner mitochondrial membrane [29]. Reduced cofactors (NADH and FADH2) generated from the metabolism of carbohydrates, proteins, and fats donate electrons to complex I and complex II. These electrons flow between the complexes down an electrochemical gradient shuttled by complexes III and IV and by two mobile electron carriers, ubiquinone (ubiquinol, coenzyme Q10) and cytochrome c. The liberated energy is used by complexes I, III, and IV to pump protons out of the mitochondrial matrix into the intermembrane space. This proton gradient is harnessed by complex V to synthesize ATP from adenosine diphosphate (ADP) and inorganic phosphate [30–33]. The process of OXPHOS depends critically on the integrity and impermeability of the inner mitochondrial membrane; defects in the complexes, cofactors, or the machinery that transcribes, assembles, and maintains them result in interruptions of mitochondrial ATP supply [33].

Mitochondrial respiration accounts for about 90% of cellular oxygen uptake, and 1–2% of the oxygen consumed is converted to ROS [34, 35]. The factors that control ROS production include the oxygen availability to the mitochondria, the redox state of the mitochondrial complexes, and mitochondrial membrane potential [36]. In addition to its role in energy production, mitochondrion is the major producer of ROS [37], and a prime target for the damaging effect of ROS [38]. The principal ROS is superoxide anion ($O_2^{\cdot -}$) which is rapidly dismutated to the more stable hydrogen peroxide (H_2O_2) and O_2, generated as byproducts of normal aerobic metabolism [39]. Although complexes I and III are main sites of mitochondrial $O_2^{\cdot -}/H_2O_2$ production, complex III is the major source during oxidative stress

[40–42]. Under physiological conditions $O_2^{\cdot-}/H_2O_2$ serves to communicate between mitochondria and the rest of the cell [43] whereas under hypoxic conditions, the ROS are released resulting in the stabilization of hypoxia inducible factors (HIFs) and the induction of genes responsible for metabolic adaptation to low oxygen [44]. It has become increasingly clear that ROS are produced not only during IHR, but also during ischemia [45–47] and hypoxia. During ischemia $O_2^{\cdot-}/H_2O_2$ levels increase, antioxidant defenses are overwhelmed, and other ROS are formed by ROS-induced ROS release [39, 48]. The source of $O_2^{\cdot-}/H_2O_2$ generation during cell hypoxia is likely complex III [49]. $O_2^{\cdot-}/H_2O_2$ generated at complex III would be released into the inner mitochondrial membrane rather than into the matrix as H_2O_2 is readily permeable to the inner mitochondrial membrane [50, 51]. This leads to opening of the mitochondrial permeability transition pore [52] and initiation of apoptosis [53]. Therefore, it is the stable and membrane-permeable H_2O_2 that is the most abundant reactant that, in excess, likely leads to damage to cell structure and function. Indeed, H_2O_2 is central to the tissue and organ damage in oxidative stress and injury.

4. ROS AND ANTIOXIDANTS

ROS are scavenged by mitochondrial, cytosolic, and peroxisomal antioxidant systems including superoxide dismutases (SODs). SODs are the primary ROS-detoxifying enzymes [54], and 3 types exist in the cell: copper- and zinc-containing SOD (Cu, ZnSOD, or SOD1), manganese-containing SOD (MnSOD, or SOD2), and extracellular SOD (ECSOD, or SOD3) [55]. SOD1 is found primarily in the cytoplasm [55]. SOD3 is localized to the extracellular region [56]. SOD2 is found exclusively in the mitochondrial matrix [57, 58]. All 3 forms of SOD catalyze the dismutation of $O_2^{\cdot-}$ into H_2O_2 and O_2. SOD does not prevent the formation of H_2O_2, and in some cases, can actually enhance H_2O_2 production. H_2O_2 is one of the most abundant ROS [59], and being membrane permeable, it easily and rapidly diffuses through biological membranes by using water channels, or aquaporins [51, 60]. H_2O_2 is tightly regulated and is the only ROS that requires several enzymes for deactivation and removal. Under physiological conditions it acts as a second messenger and modulator of cell signaling [61], allowing the mitochondria to act as oxygen sensors [62].

Although a therapeutic goal may be to reduce ROS, particularly during oxidative stress, too much scavenging or the wrong kind of scavenging may eradicate protective physiological mechanisms. We reported that administration the SOD mimetic, MnTBAP alone actually worsened OIR in rats [63], suggesting that overexpression of these enzyme systems could lead to excessive H_2O_2. Because ROS also play a significant physiological role, the effects may be deleterious. For example, SOD activity is dynamically regulated within optimal ranges—the lower limit is sufficient to remove mitochondrial $O_2^{\cdot-}$ production and the upper limit is kept low enough to avoid excess H_2O_2 production [64]. SOD expression is induced or suppressed to match ROS production, such that more ROS lead to more SOD, more SOD leads to more H_2O_2. The cell uses multiple enzyme systems to catalyze the decomposition of H_2O_2 into water and O_2. One involves glutathione peroxidase (GPx), two forms of which have been identified in mitochondria [65, 66]. Catalase is another important enzyme used by cells to decompose H_2O_2 [67, 68]. Catalase converts two molecules of H_2O_2 into two molecules of H_2O and one molecule of O_2, and therefore, catalase works best with high concentrations of H_2O_2 [54]. Catalase is found primarily in peroxisomes, but can also be

found in the cytoplasm. Peroxidases are responsible for the detoxification of up to 90% of mitochondrial H_2O_2 and even more than that of cytosolic H_2O_2 [69, 70].

Antioxidant systems are compromised in ELGANs who require oxygen therapy [71]. Immature antioxidant systems can lead to H_2O_2 accumulation in the mitochondria and subsequent accumulation in the cytosol. Many critically ill oxygen-exposed ELGANs require blood transfusion for anemia which gives them a large dose of iron; H_2O_2 accumulation will likely react with iron to form the highly reactive hydroxyl radical which damages cellular components such as proteins, lipids, and DNA [72, 73] resulting in defects in signaling pathways [74]. Diverting iron to the path of erythropoiesis using recombinant human erythropoietin (EPO) has been shown to decreases oxidant injury in premature rabbits [75]. H_2O_2 and hydroxyl radicals readily attack the polyunsaturated fatty acids of the fatty acid membrane in the retina, initiating a self-propagating chain reaction, a key mechanism underpinning the development of severe ROP [76].

5. ROS AND HIF1α

Mitochondria are natural O_2 sensors because complex IV is where O_2 binding and O_2 consumption occur [77], and O_2 sensing by the cell is actually carried out by ROS [78–80]. Therefore, ROS are not only deleterious, but are essential participants in cell signaling [81–83]. For example, H_2O_2 are principal regulators of the HIF family of transcription factors [78–80, 84–86]. H_2O_2 damages DNA, cellular membranes, and organelles resulting in autophagy and HIF activation [87–89]. H_2O_2 is a master regulator of oxidative stress-induced endothelial cell dysfunction. It has been referred to as the “fertilizer” of cancer metabolism [90]. If the accumulation of H_2O_2 is not limited, it can diffuse to the cytosol or participate in a chain of reactions that generate more ROS, and/or activate and stabilize HIF1α [78, 91, 92]. Both HIF1α and HIF2α can be modified by ROS in a direct and indirect manner [93]. Both HIF1α and HIF2α could be prevented from hydroxylation and degradation by increasing ROS generation. Therefore, ROS are important regulators of the HIF system, and the crosstalk between ROS and HIF is an important pathophysiological link [91, 93, 94].

HIF1α is a transcription factor that regulates the cellular response to O_2 homeostasis. HIF1α upregulates the expression of many genes including those responsible for angiogenesis, glycolysis, cell growth, cell survival, and metastasis [95, 96]. When O_2 is adequate, HIF1α is hydroxylated by prolyl hydroxylase domain-containing proteins (PHDs). Upon hydroxylation, HIF1α binds to the Von Hippel-Lindau tumor suppressor protein (pVHL) which leads to its ubiquitination and subsequent degradation [97–99]. During hypoxia, the energy for hydroxylation and ubiquitination is inadequate in the cell and HIF1α does not bind to pVHL. Thus, it stabilizes, accumulates, and translocates to the nucleus where it binds to the hypoxia responsive elements (HREs) within about 100–200 genes including vascular endothelial growth factor (VEGF) and EPO [100], initiating their expression and activation of glycolysis, angiogenesis, and erythropoiesis [95–103]. In order to activate gene transcription, HIF recruits a range of gene-specific co-factors that acetylate histones, change chromatin structure, and facilitate epigenetic modifications [104]. Multiple studies have demonstrated that ROS regulate the hypoxia signal transduction pathway that mediates

HIF-1α stabilization [105–107]. During reoxygenation following an IH episode, or IHR a cascade of events occurs leading to hemorrhage and cell death. Hemorrhage occurs as a result of restitution of flow through severely injured microvasculature allowing leakage of intravascular fluids and cells into interstitial spaces. We have shown this phenomenon in our oxygen-induced retinopathy model [19, 63, 108, 109], suggesting that IHR accelerates cell injury.

6. ROS AND RETINOPATHY OF PREMATURITY

ROP is a leading cause of childhood blindness worldwide. In the United States, approximately 16,000 preterm infants develop ROP annually, and with improving neonatal care and survival of ELGANs, the incidence is rising in developing countries [110, 111]. ROP is a developmental vascular disorder characterized by abnormal growth of retinal blood vessels in the incompletely vascularized retina of ELGANs [112–114]. It is especially severe in the sickest, most immature infant requiring long-term mechanical ventilation and oxygen therapy. The incidence of severe ROP varies from 33% to 50% at 23 weeks of gestation, 13% to 23% at 24 weeks of gestation, and 9% to 17% at 25 weeks of gestation [115]. The incidence of detached retinas and blindness has not appreciably declined, and only 20% of infants with threshold ROP achieve normal vision despite early treatment. Early studies by Ashton et al. [116–118] demonstrated that exposure to oxygen causes vaso-obliteration and vaso-proliferation when room air breathing is resumed. Those early studies led to a two-phase hypothesis of ROP where vaso-obliteration or phase 1 begins at preterm birth with the transition from an intrauterine to extrauterine environment causing a rise in PaO_2 of 30–35 mm Hg to 55–80 mmHg and loss of placental and maternal growth factors. During this phase, exposure to supplemental oxygen, suppresses retinal growth factors such as VEGF, insulin-like growth factor (IGF)-1 and EPO, which are already compromised due to preterm birth and poor nutrition [119] leading to arrest and retraction of the developing retinal vessels. This is followed by vaso-proliferation or phase 2 which begins at approximately 32–34 weeks [120]. As the infant matures, the avascular retina becomes metabolically active, inducing a second phase of retinal neovascularization [121]. This phase of ROP is driven by hypoxia and subsequent upregulation of VEGF and IGF-1 which leads to abnormal vascular overgrowth into the vitreous, retinal hemorrhages, retinal folds, dilated, and tortuous posterior retinal blood vessels, or “Plus” disease, and retinal detachment [122].

With advancement in neonatal care, not only has ROP been eradicated in those relatively more mature preterm infants (>31 weeks), but survival of ELGANs increased, leading to a “new” form of ROP that involves AOP with IH and IHR. The retina is one of the highest oxygen-consuming tissues of the body, exceeding even that of the brain [123–125]. This high energy demand requires abundant numbers of mitochondria per cell [126] and the highest amount of mitochondria are in the photoreceptors. Defects in energy metabolism lead to visual deficits and blindness. The immature retina of ELGANs is hypersensitive to any disturbances or changes in oxygen [8], and it is now widely accepted that ROP is a disease resulting from oxidative stress [127–129]. Studies have shown that the vaso-obliterative phase of ROP may not only be caused by the lack of angiogenic factors, but also by endothelial cell damage due to increased levels of ROS [130–132]. The high susceptibility of the immature retina to ROS is further accentuated by compromised

autoregulation of the retinal blood flow [133], high rate of oxidative metabolism, significant stores of free iron that react with H_2O_2 by the Fenton reaction to form the highly reactive hydroxyl radicals that lead to lipid peroxidation [134–137]. Developmental deficiencies in defenses against ROS during neonatal IH also account for neonatal oxidative injuries [71, 138]. Specifically, high levels of H_2O_2 stabilize HIF1α and activate nuclear factor kappa B (NFkB) which promotes aberrant angiogenesis, neovascularization, and inflammation [57, 68, 77, 79].

Using a new paradigm that encompasses the strengths of the previous paradigm, we established different patterns of IH episodes and the outcome of severe OIR. The data showed that clustered IH episodes produced a more severe form of OIR than regularly dispersed IH episodes [108]. This finding was later corroborated in ELGANs [10]. In this model, grouping desaturations with minimal time for recovery between episodes caused the retina to remain hypoxic for a longer period of time. Timely treatment is difficult and may add to the reduction in therapeutic potential of drugs when administered in IHR. This led us to the question of how many IH episodes (or desaturations) can the immature retina sustain before a “point of no return” is achieved such that the retina is not responsive to treatment, and therefore, not salvageable. We discovered that the maximum number of clustered IH episodes that the rat retina can sustain before irreparable damage is achieved is 6. This was associated with accumulation of H_2O_2, SOD, and HIF1α accumulation during IHR [19]. Our unique model consistently resulted in severe OIR retinal hemorrhage, enlarged vessels, vascular tufts, vascular tortuosity, and vascular overgrowth [19, 63, 108, 109, 139]. Retinal hemorrhage occurring during IHR may be the result of restitution of blood flow through severely injured microvasculature allowing leakage of intravascular fluids and cells into interstitial spaces [140]. Each preceding episode contributed to limited recovery from the following episode, thus setting the stage for the irreparable damage. In addition to these classic ROP characteristics, this model produced persistence of hyaloid vessels, chronic gliosis, and retinal folds or rosettes, in the photoreceptor layer, and possibly retinal detachment. Using this IH/IHR model, we administered a MnSOD mimetic (MnTBAP) to rats during early postnatal life to determine whether exogenous SOD in IH is protective. We found that high doses of MnTBAP caused severe OIR during IHR [63]. This was associated with modifications of many genes that regulate OXPHOS. These findings suggested that exogenous SOD alone is not protective, but instead may actually induce ROS. As a follow-up, we examined whether co-administration of MnTBAP with catalase would improve H_2O_2 scavenging and ameliorate oxidative stress in human retinal endothelial cells. Our findings indicate that catalase or MnTBAP alone provided better protection than their co-administration (EUK-134) for HIF1α and VEGF reduction [141]. Collectively, our studies provide evidence that oxidative stress and ROS, particularly H_2O_2, are important regulators of IH and IHR-induced OIR, and that the molecular links between IH (and IHR), ROS and HIF1α involve regulation of MnSOD.

7. CONCLUSIONS

ROP is a marker of a much more sinister neonatal long term outcome. Motor impairment, cognitive impairment, and severe hearing loss were 3 to 4 times more common in children with severe ROP than those without [142]. The complex and multifactorial etiology of ROP

precludes the use of a single therapeutic agent, as no one therapy has proven to be effective without adverse effects. These drugs act through different mechanisms and synergistic approaches should be considered to target oxidative stress and ROS accumulation, as well as the inflammatory mechanisms associated with ROP. Caffeine citrate, which is used worldwide and is standard of care for AOP [143, 144] has been shown to have antioxidant properties [145], to significantly reduce the incidence of severe ROP [146], and to normalize aberrant retinal proteomic profiles in OIR [147]. Non-steroidal anti-inflammatory drugs (NSAIDs) have also been shown to reduce the incidence of ROP [148]. Caffeine and NSAID synergism was protective against severe OIR using our IH/IHR model [139]. This novel therapeutic approach to prevent and/or reduce severe ROP should be precisely timed to coincide with the "window" of ROS production. From our data, it is clear that H_2O_2 overproduction must be curtailed, or be disposed efficiently to prevent the downstream effects that lead to retinal neovascularization. The use of safe and effective pharmacological strategies targeting various biochemical and molecular pathways must be implemented to ultimately prevent severe ROP and avert a lifetime of blindness and severe neurocognitive impairment.

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ABBREVIATIONS

AOP	apnea of prematurity
ATP	adenosine triphosphate
ELGAN	extremely low gestational age neonate
EPO	erythropoietin
HIF	hypoxia inducible factor
IGF-1	insulin-like growth factor-1
IH	intermittent hypoxia
IHR	recovery from intermittent hypoxia
NSAID	non-steroidal anti-inflammatory drug
OIR	oxygen-induced retinopathy
ORDIN	oxygen radical diseases in neonatology
OXPHOS	oxidative phosphorylation
pVHL	Von Hippel-Lindau tumor suppressor protein
ROP	retinopathy of prematurity

ROS	reactive oxygen species
SOD	superoxide dismutase
VEGF	vascular endothelial growth factor

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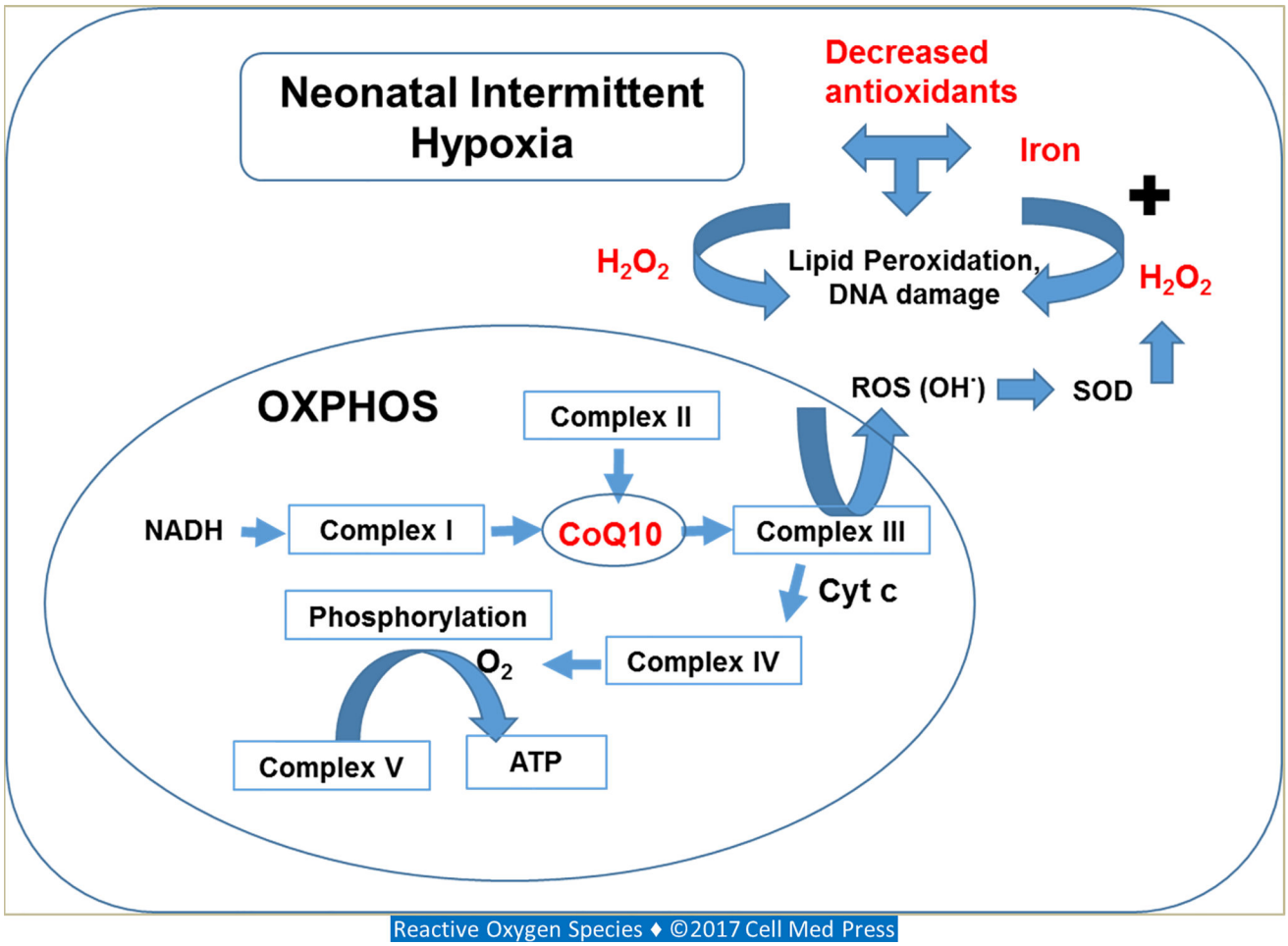

FIGURE 1. Mechanism of reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) interaction with iron to form lipid peroxidation in micro preemies

In OXPHOS, electrons are transferred down the redox enzyme complexes located within the mitochondrial inner membrane. The electrons enter at either complex I or II and are transferred through coenzyme Q to complex III, then to cytochrome c, on to complex IV, and finally to oxygen to generate H_2O. As a byproduct of OXPHOS, ROS are produced. When mitochondrial ROS production becomes excessive (as in the case of intermittent hypoxia and immature antioxidant systems) they can react with iron to cause lipid peroxidation.