

Forum Review

Free Radicals and Diseases in Premature Infants

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ABSTRACT

Free radicals have been implicated in the pathogenesis of a wide spectrum of human diseases. Premature infants are probably developmentally unprepared for extrauterine life in an oxygen-rich environment and exhibit a unique sensitivity to oxidant injury. Diseases associated with premature infants, including bronchopulmonary dysplasia, periventricular leukomalacia, intraventricular hemorrhage, retinopathy of prematurity, and necrotizing enterocolitis, have been linked to free radical-mediated cell and tissue injury. With the advent of therapies designed to combat the injurious effects of free radicals, the role of these highly reactive chemical molecules in the pathogenesis of neonatal diseases needs to be fully determined. *Antioxid. Redox Signal.* 6, 169–176.

INTRODUCTION

FREE RADICALS are atoms or molecules that contain one or more unpaired electrons (29). Many radicals are highly reactive and can function as reducing or oxidizing agents by donating electrons to or removing electrons from other molecules. Small amounts of free radicals are constantly being generated in all living organisms. Although free radicals are potentially harmful to cellular components, a substantial body of evidence supports a role for these highly reactive chemical molecules in fundamental cellular reactions and cell-cycle regulation (2, 7, 8, 80). Innate cellular antioxidants provide protection against the noxious effects of chemical reactions involving free radicals (43, 44). However, occasionally biological processes can result in an increased generation of free radicals, which can exceed the capacity of the cell's antioxidant defense systems and result in oxidative damage to proteins, lipids, and DNA, with possible cell dysfunction or death (5, 20, 28, 46, 91). Free radicals and particularly oxygen-derived free radicals have been implicated as agents of cellular damage in many diseases associated with premature infants (Fig. 1), including bronchopulmonary dysplasia (BPD), periventricular leukomalacia (PVL), intraventricular hemorrhage (IVH), retinopathy of prematurity (ROP), and necrotizing enterocolitis (NEC) (58, 75, 95, 96). Although investigators occasionally use the term reactive oxygen species (ROS) to imply oxy-

gen-derived free radicals, it should be noted that not all ROS (for example, hydrogen peroxide) have an unpaired electron, and thus are not free radicals. This review will not differentiate specifically between free radicals and ROS, and will evaluate collectively the evidence supporting the link between free radicals (including ROS) and common diseases of prematurity (Fig. 2).

PREMATURE INFANTS AND SUSCEPTIBILITY TO FREE RADICAL-MEDIATED DISEASES

The concept that premature infants are developmentally unprepared to combat oxidant stresses was mostly derived from animal studies. Studies in newborn rabbits have demonstrated that marked increases in antioxidant enzyme activities occur during the last 10–15% of gestation (17). These developmental changes have also been identified in other newborn animals and appear similar to the maturation of the surfactant system, and probably represent a preparation for life in an oxygen-rich environment (17, 18). In addition, prematurely born animals have an impaired ability to adequately up-regulate antioxidant enzymes in response to oxidant stresses, making these immature animals highly susceptible to oxidant injury

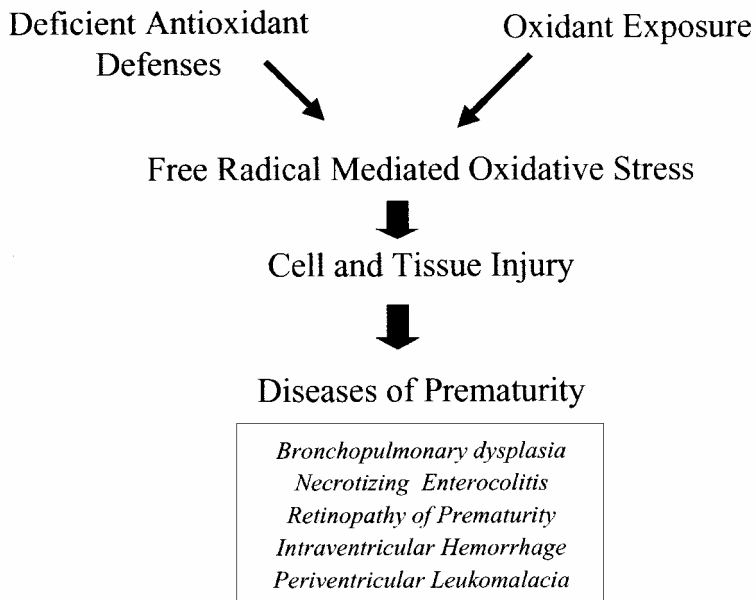
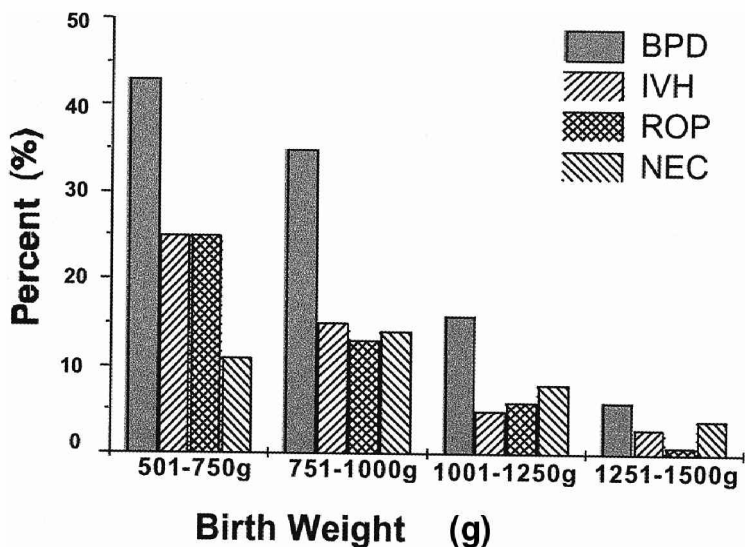


FIG. 1. Diagrammatic representation of factors contributing to free radical-mediated diseases in premature infants.

(19). In premature human infants, however, very few studies have directly examined the developmental expression of antioxidant enzymes in immature human tissues, and, with the exception of limited data supporting a developmental increase in the expression of lung catalase activity, the data have failed to demonstrate any late fetal surges in antioxidant enzyme activities in human tissues (21, 47, 81). In contrast, several clinical studies in premature infants have observed deficiencies in the antioxidant defenses, including, lower cord blood copper-zinc superoxide dismutase (SOD) activities (70), lower concentrations of glutathione in the bronchoalveolar lavage fluid (27), lower plasma glutathione concentrations (37, 78), and possibly decreased hepatic capacities for glutathione synthesis (92); these data suggest a susceptibility of the premature infant to free radical-mediated disorders.

Inadequate iron homeostasis may also contribute to the increased risk of free radical-mediated disorders in premature infants (82). The production of ROS is greatly enhanced by the presence of non-protein-bound iron. Ferrous ions can stimulate the formation of the highly reactive hydroxyl radical via Fenton-type reactions. Under normal circumstances, the plasma proteins transferrin, by retaining a considerable iron-binding capacity, and ceruloplasmin, whose ferroxidase activity enhances iron binding by catalyzing the oxidation of ferrous to ferric iron, act as powerful antioxidants toward iron-driven reactions. However, in premature infants, plasma levels of ceruloplasmin and transferrin are low and transferrin saturation is high, suggesting an increased availability of redox-active iron and an increased susceptibility to oxygen radical formation (39, 42, 54, 82, 83).

FIG. 2. Rates of serious neonatal morbidity by birth weight among infants hospitalized at Baylor College of Medicine affiliated nurseries from 1998 to 2002. BPD, bronchopulmonary dysplasia*; IVH, intraventricular hemorrhage grade 3/4; ROP, retinopathy of prematurity; and NEC, necrotizing enterocolitis. *Infants requiring oxygen beyond 36 weeks' gestational age. Data were obtained from the Section of Neonatology at Baylor College of Medicine, Houston, TX.



BPD AND FREE RADICAL-MEDIATED CELLULAR INJURY

Oxidant-mediated cellular injury has been implicated in the development of BPD, a severe lung disorder peculiar to premature infants. Although the etiology of BPD is unknown and probably multifactorial, increased generation of ROS during exposure of developmentally unprepared lungs to high concentrations of inspired oxygen probably contributes to the initial stages of lung injury via oxidation of biologically important cellular macromolecules. Several studies have addressed this hypothesis by evaluating the presence of redox-active iron in pulmonary secretions and products of lipid oxidation and protein oxidation in premature infants who developed BPD. Increased availability of redox-active iron, which can promote ROS formation, lipid peroxidation, and protein oxidation, has been identified in the bronchoalveolar secretions of infants who developed BPD (23, 24). Products of lipid peroxidation have been measured in plasma, urine, expired breath, and tracheal aspirates of premature infants. For example, investigators found that raised plasma levels of malondialdehyde (MDA) in premature infants were positively correlated with the duration of oxygen treatment and ventilator support (34), and elevated levels of urinary MDA were independently associated with oxygen therapy and BPD (76). Recently, increased pentane exhalation in sick premature infants was associated with adverse outcomes, including an increased risk for developing BPD (57, 88, 99). Further, Ogihara *et al.* observed increased plasma concentrations of heptanal, 2-nonenal, and 4-hydroxynonenal on the day of birth in premature infants that subsequently developed BPD (61). These data suggest that early increases in markers of lipid peroxidation, within the first few days after birth, correlate with adverse pulmonary outcomes, and concur with other data implicating an oxidative process initiated shortly before or after birth in the development of BPD.

Oxidation of proteins is typically accompanied by the introduction of carbonyl groups into the side chain of the protein, and quantification of the carbonyl content of proteins has been used to measure protein damage caused by oxidative stress (10). Elevated concentrations of protein carbonyls in the lung lining fluid of premature infants have correlated with adverse outcomes and the development of BPD. In a recent study, Schock *et al.* found that premature infants who subsequently developed BPD had a trend toward higher concentrations of protein carbonyls in bronchoalveolar lavage fluid within the first few days after birth than did infants that did not develop BPD (77). In a separate study, Varsila *et al.* found higher protein carbonyl contents in the tracheal aspirates of premature infants that subsequently developed BPD (89). Again these studies suggest that early increases in products of protein oxidation are associated with pulmonary disease in premature infants. Although some studies have failed to identify any correlation between products of oxidation and BPD in premature infants (72, 97), this may more likely reflect an aberration of the site of sampling (*i.e.*, plasma) than proof of an absence of oxidant-mediated pathology in the lung.

Further support for the concept of free radical-mediated cellular injury in the pathogenesis of BPD is provided by animal and, to a limited extent, human studies investigating the protective benefit of antioxidant therapies (14, 87, 94). For

example, survival of rats exposed to 100% oxygen was significantly increased when liposomes containing catalase and SOD were injected intravenously before and during hyperoxic exposure. The increased survival time in 100% oxygen was also associated with significantly less evidence of lung injury (87). In other studies, direct intratracheal administration of liposome-encapsulated SOD or catalase was associated with significantly greater survival and less lung injury in rats after 72 h of hyperoxic exposure than was noted in the control group (65). Similar studies in premature human infants with respiratory distress syndrome have demonstrated that intratracheal doses of recombinant human copper-zinc SOD (rhSOD) are well tolerated and significantly increase SOD activities in serum, tracheal aspirates, and urine (73, 84). In addition, tracheal aspirate markers of inflammation are reduced in rhSOD-treated infants compared with placebo controls, suggesting that less lung injury has occurred (73, 84). Although these clinical data are encouraging, they do not provide sufficient evidence to support the hypothesis that treatment with SOD will diminish the risk of developing BPD in premature infants, and further studies will be necessary to fully determine the clinical benefit of such therapeutic approaches (84).

PVL, IVH, AND FREE RADICAL-MEDIATED CELLULAR INJURY

PVL refers to necrosis of the cerebral white matter adjacent to the external angles of the lateral ventricles. In premature infants, PVL represents a major precursor for adverse neurological sequelae (100). Although the pathogenesis of PVL is mostly unknown and likely multifactorial, free radical-mediated cellular injury has been proposed as a key pathogenic factor in cerebral white matter injury in the premature infant. Support for this concept is derived mostly from indirect experimental data, particularly from studies on immature oligodendrocytes that have demonstrated not only the susceptibility of these immature neural cells to free radical-mediated injury, but also the benefit of free radical scavengers in protecting these cells from experimentally generated free radical-mediated cellular injury (3, 62, 93, 101). Direct evidence of a role for free radicals in the pathogenesis of PVL in premature human infants is mostly lacking. However, in a recent study, premature infants with subsequent evidence of PVL on magnetic resonance imaging at term had higher levels of cerebrospinal fluid protein carbonyls in comparison with healthy premature infants, term infants, and adult controls (36). In addition, significant differences in the levels of the lipid peroxidation products, 8-isoprostane and MDA, were apparent between premature infants with PVL and adult controls. There was also a trend toward higher levels of 8-isoprostane in the premature infants with white matter injury when compared with those without white matter injury. Although this was a relatively small single center study, these limited data provide direct support for an association between products of oxidation and PVL in premature infants, and further studies are warranted to determine the true nature of these associations.

Impaired autoregulation of the cerebral circulation is considered an important factor in the pathogenesis of IVH in premature infants. Experimentally induced hypovolemic hypo-

tension followed by reperfusion has been shown to produce IVH in the newborn beagle puppy (49). Considerable evidence supports an association between reperfusion injury and increased free radical production (45). SOD protects against the development of IVH in beagle pups exposed to hypovolemic hypotension followed by volume reexpansion (52). Other agents such as indomethacin and ethamsylate, whose beneficial action potentially involves a reduction in free radical production, have been shown to protect against IVH in experimental studies involving the beagle pup model (50, 51). Clinical studies support these findings; both ethamsylate and indomethacin have been reported to reduce the overall incidence of IVH in premature infants (4, 16). The role of vitamin E, a group of eight biologically active tocopherols including the potent antioxidant *d*- α -tocopherol, has been investigated in premature infants. Although there is no animal evidence linking vitamin E deficiency and IVH, the data from several clinical trials suggest that vitamin E administration resulted in a decreased incidence of IVH in premature infants (40). However, concerns regarding potential toxicity in premature infants preclude its routine use in the clinical setting.

ROP AND FREE RADICAL-MEDIATED CELLULAR INJURY

ROP is a vasoproliferative disorder of the immature retina seen in premature infants. Severe disease leads to visual impairment or blindness in a small but significant percentage of premature infants (85). The disease is brought about by the susceptibility of the developing retina to hyperoxia. Sustained hyperoxia produces vasoconstriction and retards the process of retinal vasculogenesis leaving the retina ischemic in some areas, which predisposes to the development of a vasoproliferative retinopathy (30). Several indirect lines of evidence suggest that free radicals play a role in the pathogenesis of ROP. First, premature human infants probably have an increased susceptibility to free radical-mediated retinal injury, as all major antioxidant systems are deficient in the immature retina (6, 9, 55, 64). In addition, increased levels of serum iron have been associated with the development of ROP, and in neonatal neural tissue, which shares its embryonic origin with ocular tissue, increased concentrations of iron are reported to be present, suggesting an increased potential for free radical generation via iron-catalyzed Fenton-type reactions (11, 35). The large body of evidence linking hyperoxia and ROP provides further support for the concept of free radical-mediated retinal injury in the pathogenesis of ROP (22, 79, 86, 96). Although the exact mechanisms of tissue injury in oxygen toxicity are not fully understood, exposure of cells to high concentrations of oxygen leads to increased production of ROS, which can react with cellular components causing cell and tissue injury (99). Oxygen exposure also reduces retinal antioxidant levels, creating an additional burden for retinal tissues responding to oxidant stresses (68). Animal models of oxygen-induced retinopathy confirm the predisposition of the immature retinal vessels to the toxic effects of oxygen, and, in some of these studies, products of lipid oxidation have been identified, strongly supporting a link between oxygen exposure, ROP, and free radical-mediated cellular injury (13, 48, 67). Finally, supplementation

of antioxidants has been reported to protect against oxygen-induced retinopathy in an animal model. For example, newborn rats supplemented with liposome-encapsulated SOD had significantly greater retinal SOD activities and reduced oxygen-induced vasoattenuation, as evidenced by increased retinal vessel density and decreased avascular area, when compared with littermates exposed to hyperoxia that received control liposomes (56). In other studies, Trolox C, a water-soluble analogue of vitamin E with potent antioxidant activity, facilitated the process of retinal vasculogenesis under conditions of hyperoxia in newborn rats (69). In the 1980s, several large clinical studies were conducted to investigate the benefit of prophylactic vitamin E in premature infants at risk for developing ROP (40). Results from these controlled trials have been mixed, and differences in ROP classification, vitamin E treatment regimens, and serum drug levels have limited the ability to compare the data from these studies. In addition to inconsistent findings from the clinical studies, concerns about the toxicity of vitamin E at concentrations close to that considered therapeutic diminished the enthusiasm for this therapy. However, a recent meta-analysis comparing data from six randomized clinical trials of vitamin E prophylaxis, which included a total of 704 very-low-birth-weight infants in the vitamin E prophylaxis groups and 714 in control groups, concluded that vitamin E prophylaxis is safe and results in a 52% reduction in the incidence of Stage 3+ ROP, and suggested that the role of the antioxidant vitamin E in reducing severe ROP be reevaluated (71).

NEC AND FREE RADICAL-MEDIATED CELLULAR INJURY

NEC is a common and often serious gastrointestinal disorder that predominately affects premature human infants. Although the pathogenesis of NEC remains uncertain, ischemia/reperfusion injury has been considered a major contributing factor (33). Free radical production during ischemia/reperfusion injury is likely to occur via the following mechanism: during the ischemic period, ATP is catabolized to hypoxanthine, which accumulates in the tissues; when the intestine is reperfused, oxygen is reintroduced into the tissues, and reacts with hypoxanthine and xanthine oxidase to produce the superoxide anion, hydrogen peroxide, and hydroxyl radical (26, 66). Several indirect lines of evidence support a role for this mechanism of free radical production in the pathogenesis in NEC. First, the intestine is a particularly rich source of xanthine oxidase, and, compared with other tissues, intestinal xanthine dehydrogenase is rapidly converted to xanthine oxidase during hypoxia, enabling brisk production of ROS during the reperfusion period (66). This hypothesis is further endorsed by the observation that pretreatment with allopurinol, a xanthine oxidase inhibitor, has been shown to ameliorate tissue injury from ischemia/reperfusion in many organs (26, 31, 32, 90). Finally, in a model of acute inflammatory intestinal injury induced by platelet activating factor, an endogenous phospholipid mediator, gastrointestinal damage is significantly attenuated by administration of allopurinol (12). However, a single clinical trial of prophylactic allopurinol in premature infants failed to identify any protective benefit against diseases of prematurity (74). Further evidence that free radicals

play a role in the pathogenesis of gastrointestinal injury in NEC is provided by animal studies investigating strategies to attenuate intestinal damage in experimentally induced NEC. For example, pretreatment with SOD prevents intestinal damage in experimental models of NEC (25, 53). In addition, deferoxamine, an iron chelator, protected rats from ischemic neonatal bowel necrosis, probably by limiting free radical generation via iron-catalyzed Fenton-type reactions (41). Further, in an experimental ischemia/reperfusion rat model of NEC, animals pretreated with vitamin E had milder histological evidence of the intestinal damage than matched controls (63). Evidence of lipid peroxidation has also been identified in intestinal tissues after experimentally induced NEC, and pretreatment with antioxidants resulted in decreases in these measured products of lipid peroxidation (1, 63). In all, this body of data provides suggestive evidence of a role for free radicals in the pathogenesis of NEC, but further studies are warranted to determine the exact nature of these associations.

In summary, free radicals have been implicated as pathogenic agents in a wide spectrum of human diseases, including diseases associated with premature infants. Although the majority of evidence supporting these associations is derived from indirect experimental data, the limited amount of clinical data available strengthens the hypothesis. The notion that antioxidant therapies could be beneficial to disorders mediated by free radicals offers a strong incentive for investigators to fully determine the nature of the association between these highly reactive chemical molecules and disease states. Studies in cell cultures *in vitro* (15, 59, 60) and animal models *in vivo* (65, 87) demonstrating attenuation of oxidant-mediated cellular injury by augmentation of a variety of antioxidants hold the promise that such therapies may someday be routinely used in the clinical arena. It is highly probable that more than one antioxidant defense system will need to be augmented for such therapeutic strategies to be effective, as different types of free radicals may work in concert to effect cellular injury. In addition, determining which high-risk populations will need to be treated, and with what antioxidant cocktail/mixture, will need much further study. However, premature infants are particularly susceptible to free radical-mediated disorders, and therapies designed to combat this mechanism of cell and tissue injury could contribute greatly to improved morbidity and mortality of these vulnerable patients.

ABBREVIATIONS

BPD, bronchopulmonary dysplasia; IVH, intraventricular hemorrhage; MDA, malondialdehyde; NEC, necrotizing enterocolitis; PVL, periventricular leukomalacia; ROP, retinopathy of prematurity; ROS, reactive oxygen species; SOD, superoxide dismutase.

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