0031-3998/01/5006-0681
PEDIATRIC RESEARCH
Vol. 50, No. 6, 2001
Printed in U.S.A.

REVIEW ARTICLE

Antioxidants as Therapy in the Newborn: Some Words of Caution

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ABSTRACT

Reactive oxygen and nitrogen species are considered to play a major role in the pathogenesis of a wide range of human disorders. This may be a particularly important pathogenetic mechanism in the newborn nursery. The phrase "oxygen radical disease of prematurity" has been coined to collectively describe a wide range of neonatal disorders based on the belief that premature newborns are deficient in antioxidant defenses at a time when they are subjected to acute and chronic oxidant stresses. This belief has led to a number of clinical trials of antioxidant therapies being undertaken in neonatal patients. The realization that reactive oxygen species play a critical role in neonatal illnesses has only recently been paralleled by an increased understanding of their physiologic roles. A major concern is that effective scavenging of reactive oxygen species, to attenuate their toxic effects, will also inhibit essential cellular functions such as growth in potential target organs such as lung, brain, intestine, and retina. **(*Pediatr Res* 50: 681–687, 2001)**

Abbreviations

AP-1, activator protein-1
GSH, reduced glutathione
H_2O_2, hydrogen peroxide
N-AC, *N*-acetyl-L-cysteine
$^{\cdot}NO$, nitric oxide
$O_2^{-\cdot}$, superoxide anion
$^{\cdot}OH$, hydroxyl radical
RNS, reactive nitrogen species
ROS, reactive oxygen species
SOD, superoxide dismutase

Implicit in the term "oxygen radical disease of prematurity" (1) is a belief in the central importance of free radical injury in a wide range of neonatal disorders. It is widely accepted, based largely on indirect evidence, that the premature newborn is uniquely susceptible to increased oxidant stress from a variety of sources, because of reduced antioxidant defenses (2–4). Inevitably, there has been considerable interest over the last two decades in the development and use of both natural and synthetic antioxidants as preventive and therapeutic agents for neonatal disease. Seemingly rational, and indeed laudable, as are these goals, our appreciation of how ROS cause cellular and tissue injury has recently been paralleled by the recognition that ROS also play a role in normal cell growth and, perhaps, differentiation. The purpose of this review is to reappraise the potential values and limitations of antioxidant approaches to diseases of the newborn in light of recent evidence.

REACTIVE OXYGEN AND REACTIVE NITROGEN SPECIES

For a detailed discussion of free radical biochemistry, the interested reader is referred to a number of excellent reviews on the subject (5–9). A free radical can be defined as any molecule capable of independent existence with one or more unpaired electrons. In 1924, it was established that molecular oxygen (O_2) has two unpaired electrons, although its reactivity is limited by quantum mechanical restrictions. The term ROS encompasses molecules that can be defined as free radicals as well as others that are oxidizing species, relative to molecular O_2, but do not possess an unpaired electron. The discovery of superoxide dismutase (SOD) by McCord and Fridovich in 1969 (10), and the finding that approximately 2% of the O_2

Received April 25, 2001; accepted June 28, 2001.
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Supported by a Group grant from the Canadian Institutes of Health Research. Dr. Tanswell holds the Women's Auxiliary Chair in Neonatal Medicine. Dr. Jankov is supported by a Clinician-Scientist Training Programme Fellowship from the Hospital for Sick Children Research Institute and a Postdoctoral Fellowship from the Canadian Lung Association and Canadian Institutes of Health Research.

reduced in mammalian mitochondria forms superoxide ($O_2^{\cdot-}$) or its dismutation product, hydrogen peroxide (H_2O_2), led to the realization that ROS are normally present in biologic systems. This discovery, in turn, led to an enhanced understanding of endogenous enzymatic and nonenzymatic antioxidants that have evolved to counter the adverse effects of endogenous ROS. In the presence of transition metals, such as iron and copper, $O_2^{\cdot-}$ and H_2O_2 can form highly reactive hydroxyl radicals ($^{\cdot}OH$) that are capable of reacting with any component of the cell at near diffusion-limited rates.

ROS are implicated in the pathogenesis of inflammatory, toxic, and metabolic insults, ischemia-reperfusion injury, carcinogenesis, and atherosclerosis (7). Conditions that are known to increase production of ROS include exposure to supraphysiologic concentrations of O_2 (11, 12), influx of inflammatory cells (13), increased free circulating transition metals (1), and ischemia-reperfusion (14). Increased formation of ROS is also caused by some drugs and chemicals through so-called "redox cycling." Examples include poisoning with paraquat or acetaminophen. Liver toxicity caused by acetaminophen is one of the few human diseases that is effectively treated with the timely use an antioxidant, *N*-acetyl-L-cysteine (N-AC) (15). A more recently appreciated group of molecules known as reactive nitrogen species (RNS) are derived from reactions involving another free radical, nitric oxide ($^{\cdot}NO$). Nitric oxide can act either as a pro-oxidant or as an antioxidant (16). The best-characterized pro-oxidant RNS is the peroxynitrite anion, formed by the reaction of $O_2^{\cdot-}$ with $^{\cdot}NO$, when present in equimolar concentrations (17). Its relative stability, high reactivity, and ability to act and release $^{\cdot}OH$, or an $^{\cdot}OH$-like ROS, at sites distant from its generation, make it a potentially very damaging molecule. Generation of peroxynitrite anion is less likely to occur when local concentrations of $^{\cdot}NO$ are greatly in excess of $O_2^{\cdot-}$, as is the case in the lung during therapeutic use of inhaled $^{\cdot}NO$. Data from animal experiments support an antioxidant role of $^{\cdot}NO$ at therapeutic concentrations (18). The potential roles of RNS in human disease are only just beginning to be properly appreciated (19–21).

SUSCEPTIBILITY OF PRETERM NEONATES TO OXIDANT INJURY

Neonatal morbidities in which oxidant stress has been hypothesized to play a causal role include bronchopulmonary dysplasia (22–24), retinopathy of prematurity (25), necrotizing enterocolitis (26), intraventricular hemorrhage, and periventricular leukomalacia (27, 28). In 1988, Saugstad coined the term "oxygen radical disease in neonatology" as a unifying hypothesis for the role of ROS in a wide range of neonatal morbidities, the implication being that they are, in fact, different manifestations of the same condition (29). This concept has been recently updated and reviewed in detail (2). Vulnerability to oxidant injury is predicated on the coexistence of two phenomena leading to "oxidant stress": endogenous antioxidant deficiency and increased generation of ROS and RNS.

Developmental aspects of antioxidant activity have been extensively reviewed by Frank (3, 30, 31). At the present time, the evidence for antioxidant deficiency in prematurely born humans is largely indirect. The concept of an immaturity-dependent enzymatic antioxidant (SOD, catalase, and glutathione peroxidase) deficiency is derived from data in animals where activity at birth appears to be lower and less inducible with greater immaturity (32–34). The only studies to examine enzymatic antioxidant activity in immature human tissues have not supported an immaturity-dependent deficiency (35, 36). However, a reduction in levels of another antioxidant, reduced glutathione (GSH), has been correlated with immaturity and male sex (37). Factors known to contribute to the production of ROS and RNS, as described above, are well recognized in preterm infants. Several studies in human neonates indicate that oxidation products increase with higher levels of inspired oxygen (38) and increasing immaturity (39).

SOURCES AND TARGETS OF OXIDANT INJURY IN NEONATES

ROS are capable of damaging all biologic macromolecules including proteins, DNA, and lipids *in vitro* (6). Unfortunately, a major difficulty in correlating oxidant stress with human disease has resulted from an inability to directly measure most ROS or RNS *in vivo* because of their high reactivity and relative instability. Thus, assessments of oxidative effects have generally been based on measurements of stable end products resulting from ROS or RNS interactions with cellular components. Measurement of these various compounds in the neonate have supported the notion that increased oxidant stress is associated with neonatal disease, particularly bronchopulmonary dysplasia, and that all cellular components are susceptible. The recent STOP-ROP trial has provided additional evidence for a contribution of oxidant stress, in the form of supplemental O_2, to pulmonary morbidity in neonates (40). Indices of oxidant stress that have been correlated with neonatal disease include measurement of the exhaled hydrocarbons ethane and pentane (41, 42), plasma malondialdehyde (43, 44), and plasma aldehydes (45) as markers of lipid peroxidation. Others include plasma allantoin (a ROS-derived product of uric acid) (46, 47) and plasma or airway-derived proteins modified by ROS (48–51) or RNS (52).

Peroxidation of biologic lipids have been the most often cited evidence for the involvement of ROS in human disease, though it has not definitively been shown to contribute to disease, with the exception of atherosclerosis (7, 53). Furthermore, the methodologies often applied to the measurement of thiobarbituric acid-reactive substances, as an index of lipid peroxidation *in vivo*, have frequently been flawed (54). Certain products of lipid peroxidation, including lipid hydroperoxides (55) and 8-isoprostane (56, 57), are biologically active and may contribute to disease. However, it remains unknown which radicals mediate injury, which oxidation products have roles in disease, and which cellular components are most susceptible, in specific disease entities of the newborn.

ANTIOXIDANT APPROACHES TO NEONATAL DISEASE

For a full appraisal of available antioxidants and their potential applications in clinical medicine, the reader is referred to several comprehensive reviews (5, 22, 58–61). Antioxidant

approaches to neonatal disease have also been recently reviewed by Rosenfeld and Davis (62). An "antioxidant" may be broadly classified as "any substance that can delay or prevent oxidation of a particular substrate" (63). Compounds that prevent cellular damage caused by ROS or RNS may act in numerous ways (6), ranging from prevention of their formation (such as chelators of metal ions and anti-inflammatory agents) to their interception once formed. Most antioxidants used clinically fall into the latter category. Antioxidants may be broadly classified into enzymatic (SOD, catalase, and glutathione or their precursors) or nonenzymatic (vitamins C, E, β-carotene, and allopurinol).

Enzymatic antioxidants have been well characterized for some time. As these enzymes are already present in cells, it appears logical to supplement them, especially in the premature neonate, who may be deficient. Studies in animals suggest that supplementation with a single antioxidant enzyme, such as SOD, is not protective; it must be used in combination with another enzyme, such as catalase (64, 65). Furthermore, these enzymes, when administered systemically, do not readily enter cells unless conjugated to polyethylene glycol or encapsulated in liposomes (65, 66). To date, the use of catalase has not been investigated in humans. An alternative approach is delivery of large doses directly to the target organ, for which the lung is ideally suited. Benefits from exogenously administered natural surfactant may at least partly relate to its antioxidant properties (67). Intratracheal delivery of recombinant human SOD has also shown promise in a piglet model of pulmonary O_2 toxicity (68).

The dietary antioxidant, vitamin E (α-tocopherol), was among the first to be used in the hope of preventing neonatal disease. The use of vitamin E was based on evidence that neonates are deficient and on the actions of this compound in preventing membrane lipid peroxidation, which is purported to contribute to disease. It has since been recognized that measurement of plasma vitamin E as a measure of sufficiency is seriously flawed (69). In addition, prolonged pharmacological dosage of vitamin E has been linked to an increased incidence of sepsis and necrotizing enterocolitis in neonates (70). This may have been caused by interference with oxidant-dependent mechanisms of bacterial killing by neutrophils and mononuclear cells, another theoretical hazard of antioxidant therapy. Recent clinical trials in adults have also emphasized a troubling reality that has already been described *in vitro*; exogenously administered dietary antioxidants in high doses (vitamin C and β-carotene in particular) may act as pro-oxidants (71) and actually increase mortality in some groups of patients (63, 72). Additives to pharmacological preparations of antioxidants may also cause harm, as was thought to be the case with E-Ferol, an i.v. preparation of vitamin E (α-tocopherol acetate) that led to a number of deaths in neonatal nurseries during the early 1980s (73, 74).

When proposing an antioxidant therapy for a human disease, the ROS or RNS involved, the site of injury, and the biologic molecule to be protected must be fully understood. As has already been described, this is not the case for the large number of neonatal diseases in which oxidant injury is hypothesized to play a role. Furthermore, an inherent requirement of an effective antioxidant is that such a therapy is effective against the specific ROS or RNS involved, and that sufficient quantities are present at the site of ROS and RNS formation. An effective single antioxidant must therefore be able to gain entry into all cell and subcellular compartments. Such diverse requirements mandate the use of antioxidant "cocktails" since few, if any, of the antioxidants that have been used clinically, are able to perform this function across a range of tissues and pathologic conditions when used individually. Although we have some idea of the biologic effects of dietary antioxidants and antioxidant enzymes, very little is known about the beneficial or adverse effects in humans of several newly available synthetic antioxidants, including Trolox (a water soluble analogue of vitamin E) (75), and the "lazaroids" (21-aminosteroids) (76).

The limitations described above may explain some of the disappointing results from trials of antioxidants for the prevention of neonatal disease, including vitamin E (77–79), allopurinol (80), and selenium supplementation (81). The outcomes of other antioxidant interventions in ongoing trials, such as intratracheal recombinant human SOD (82), are yet to be determined.

REDOX STATE AND CELL GROWTH

Evidence from a variety of sources supports the view that ROS play a role in cell growth, and perhaps in differentiation (83, 84), a process whereby cells undergo a transition from one state to another. The control of cellular proliferation, a recent area of intense discovery, involves qualitative and quantitative changes in gene expression and is characterized by the appearance of tissue-specific enzymes and other proteins. The mechanisms by which ROS affect these processes generally involve changes in "redox state," or the ratio of oxidizing to reducing equivalents. Redox state also varies in a predictable pattern during the cell cycle. The biologic impact of alterations in redox state may therefore be most profound during these cellular changes. More than 90% of the reducing equivalents in the cell are contained in GSH (85), though thioredoxins, other intracellular thiol buffers, also play an important role (86). The immediate effect of increased ROS is a shift in the redox state toward oxidizing equivalents. During cellular differentiation and proliferation, the redox state of cells shifts toward a more oxidized state, as indicated by a decline in GSH concentration (87), suggesting that redox state may be an important determinant of the ability of cells to grow and differentiate. This possibility is supported by evidence both *in vitro* and *in vivo*. We have shown that a "lazaroid" (21-aminosteroid) synthetic antioxidant inhibited somatic growth and lung cell division in newborn rats (88, 89). Furthermore, overexpression of antioxidant enzymes may also inhibit cell growth (90).

REACTIVE OXYGEN SPECIES AS SIGNALING MOLECULES

The combination of a surface receptor with an external ligand, such as a growth factor, cytokine, or hormone, transmits a signal into the cytoplasm that starts a cascade of signal transduction reactions. There is a growing body of evidence that $O_2^{-\cdot}$ and H_2O_2 are involved in cell-cycle-associated

changes in redox state that allow some types of cellular signaling. Changes in the activity of certain antioxidant enzymes, particularly SOD, are also closely associated with the cellular state of differentiation (91). Concentrations of H_2O_2 and $O_2^{\cdot-}$ in the nanomolar-to-low micromolar range stimulate the growth of various cell types *in vitro* (92, 93), whereas higher concentrations cause a reduction in cell growth and eventually cell death. The growth promoting properties of $O_2^{\cdot-}$ at low concentrations, and cytotoxic properties at high concentrations, are illustrated in Figure 1. Primary cultures of rat fetal lung epithelial cells were exposed to increasing concentrations of either paraquat, an $O_2^{\cdot-}$ generator, or TEMPO, an $O_2^{\cdot-}$ scavenger, and assayed for DNA synthesis or cytotoxicity as previously described (94, 95). Low concentrations of $O_2^{\cdot-}$ enhance cell DNA synthesis and protect against basal levels of cytotoxicity, whereas higher concentrations are cytotoxic and inhibit DNA synthesis. Scavenging $O_2^{\cdot-}$ inhibits DNA synthesis at noncytotoxic concentrations, suggesting a role for $O_2^{\cdot-}$ in DNA synthesis by control cells. This situation is analogous to the effects of $^{\cdot}NO$, which has regulatory functions or cytotoxic effects depending on the source and amount generated (96). In the case of exogenously added $O_2^{\cdot-}$, effects on cells are extremely rapid and distinct from those of H_2O_2, involving increased intracellular pH and Ca^{2+} ion concentration (97). Such effects are inhibited by an anion channel blocker, suggesting that $O_2^{\cdot-}$ exerts its effects directly after being transported into the cell, although the mechanisms of this response remain unclear. In contrast, H_2O_2, which can freely permeate cell membranes, has a much slower onset of response that is dependent on the presence of growth-stimulating proteins, such as insulin (98).

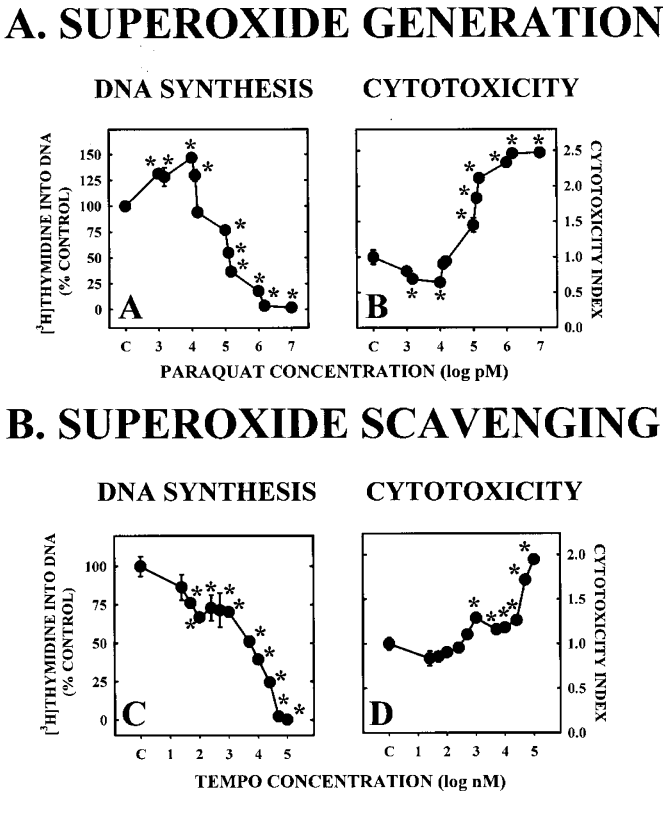

Figure 1. In primary cultures of rat fetal lung distal epithelial cells paraquat, a superoxide generator, (*A*) stimulated DNA synthesis at subcytotoxic concentrations of 1–12.5 nM, but DNA synthesis was inhibited by concentrations of paraquat ≥100 nM. (*B*) Concentrations of paraquat ≥100 nM, were cytotoxic, whereas at concentrations of 5–10 nM, paraquat reduced the basal levels of cytotoxicity evident in paraquat-free control cells. TEMPO, a superoxide scavenger, (*C*) inhibited DNA synthesis at subcytotoxic concentrations of 50–500 nM. (*D*) TEMPO was cytotoxic at concentrations ≥1 μM. At concentrations < 1 μM, the cytotoxicity index was no different to that observed with TEMPO-free control cells. ($n = 4$; M ± SEM; $^*p < 0.05$ *vs* additive-free control cells by ANOVA).

The slow onset of response to H_2O_2 may be explained by recent observations that H_2O_2 is crucial to signal transduction mechanisms downstream of the receptor-ligand interaction. One such pathway involves phosphorylation of proteins including tyrosine and serine/threonine. This process is reversible and dynamic and controlled by the opposing actions of protein kinases and phosphatases. Protein phosphorylation is of major importance to functions as diverse as mitogenesis, cell adhesion, cell differentiation, and apoptosis (99). H_2O_2 may act as an intracellular "second-messenger" by facilitating tyrosine phosphorylation (100) and inhibiting the actions of phosphatases (101). The actions of polypeptide growth factors such as platelet-derived growth factor (102), epidermal growth factor (103, 104), and insulin (101) involve protein phosphorylation that is accompanied by a rise in the intracellular level of H_2O_2. Blocking platelet-derived growth factor (102) and transforming growth factor-β1-associated (105) rises in H_2O_2 with catalase inhibited the cellular response to these mitogens. The signal transduction pathways of other mitogens may also require low endogenous levels of H_2O_2 or $O_2^{\cdot-}$ (106, 107). Those that are known include PTH and vitamin D_3 (108), Angiotensin II (102), transforming growth factor-α (109), IL-1β (108), and tumor necrosis factor-α (110).

Some ROS-derived products may also have a role in regulation of signal transduction through activation of phospholipases. Among these, phospholipase A_2, is a rate-limiting enzyme involved in the release of arachidonic acid from membrane glycerophospholipids. Arachidonic acid is the precursor of prostaglandins, thromboxanes, and leukotrienes, which are important regulators of inflammatory and immune responses. The action of oxidants in causing membrane lipid peroxidation, which causes the formation of lipid hydroperoxides, stimulates arachidonic acid production via increased phospholipase A_2 activity (111). Lipid hydroperoxide activation of phospholipases represents one plausible but yet unproven mechanism by which the actions of arachidonic acid products, growth factors, and hormones may be regulated according to redox state.

REACTIVE OXYGEN SPECIES AND REGULATION OF GENE EXPRESSION

Transcription factors are regulatory proteins that recognize and bind specific DNA sequences and regulate RNA synthesis. Transcription factors that are sensitive to cellular redox state include, among others, NF-κB (112) and activator protein-1 (AP-1) (113). The activation of the nuclear transcription factor NF-κB regulates the expression of genes related to acute phase proteins, cell surface receptors, and cytokines. H_2O_2, possibly

through redox regulation of tyrosine kinase activity (114), promotes the dissociation of I-κB from NF-κB, releasing the active transcription factor and permitting its translocation into the nucleus to bind DNA (110). Although the anti-inflammatory actions of some antioxidants (such as N-AC) are mediated partially through inhibition of NF-κB, overexpression of SOD may increase NF-κB activation through generation of H_2O_2 (115).

AP-1 is a complex of oncogene proteins of the Jun (c-Jun, JunB and JunD) and Fos (c-Fos, Fra-1, Fra-2, and FosB) families of nuclear transcription factors. AP-1 proteins, which include the Jun-Jun homodimer and the Jun-Fos heterodimer, are subject to regulation by many factors, including redox state, and function as intermediaries in processes leading to cell proliferation and differentiation (116). At least one redox-sensitive element of AP-1 has been identified in the Cys^{252} residue of the Jun protein. Oxidation of this amino acid has been shown to inhibit DNA binding of AP-1, and reduction has been shown to have the opposite effect (117, 118). AP-1 activities are strongly activated by the antioxidant N-AC (119) *in vitro*.

Thus, it is clear that oxidants and antioxidants may decrease or increase cellular differentiation depending on the tissues, cell types, and ROS or ROS-derived products involved. Redox-induced biochemical modifications, as described above, can have significant biologic consequences.

CONCLUSION

A widespread perception is that the use of antioxidant therapies is a "good" therapeutic objective. This idea is based on evidence in very few human disease processes (7). Antioxidant interventions have certainly been effective in various animal disease models. However, such studies have usually been of brief duration and have not generally been designed to look for adverse outcomes on cell growth and differentiation. A note of caution is in order. The concept that ROS also have important regulatory and signaling roles is now beyond doubt and carries significant implications for the use of antioxidants in the growing and developing newborn. While antioxidants may yet find a place in the growing list of neonatal therapies, we have attempted to highlight a major problem faced by clinicians and basic scientists, that "antioxidant" function is much more complex than simple scavenging of ROS and RNS, and supplementation of antioxidants, natural or otherwise, may perturb the natural balance of the cellular redox state and have unforeseen deleterious consequences (63). Added to these concerns is the current inability to easily determine which individuals might benefit from antioxidant therapy and which antioxidant, or combination of antioxidants, may be most beneficial in a given circumstance, a situation that is unlikely to change in the near future.

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