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Biomarkers of oxidative stress in the fetus and newborn

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A B S T R A C T

Oxidative stress presents numerous opportunities for tissue injury through formation of reactive oxygen/nitrogen species. It is becoming more evident that oxidative stress is the final common endpoint for a complex convergence of events, some genetically determined and some triggered by an in utero stressor. Oxidative stress affects a complex array of genes involved in inflammation, coagulation, fibrinolysis, the cell cycle, signal trasduction and programmed cell death. It quickly becomes clear that a single pathway may be insufficient to provide clarification of oxidative stress action in the patogenesis of the so-called free radical diseases of the newborn.

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Oxidative stress in the fetus and newborn

Oxidative stress (OS) results from an imbalance between reducing agents and enzymes involved in the removal of free radicals (FR) and/or reactive oxygen species (ROS). The consequence of OS on fetal structure involves the activation of a complex array of genes involved in inflammation, coagulation, fibrinolysis, cell cycle and signal transduction.[1] It is now recognized that ROS are important for fertilization and developing embryos.[2] In moderate quantities and in presence of a good antioxidant capacity, FRs are continuously generated in the organism and are essential for cell aerobic metabolism and fetal growth, but they are toxic when overproduced, resulting in an attack of all classes of biological macromolecules, polysaccharides, nucleic acid, lipids and proteins.[3]

Hypoxia, hyperoxia, inflammation, Fenton chemistry, endothelial damage, arachidonic acid cascade are other mechanisms that lead to the formation of highly reactive products. FR reactions lead to DNA damage (fragmentation, apoptosis, base modifications and strand breaks), to lipid, protein and polysaccharides oxidation and as a consequence FR reactions may induce a wide range of biological toxic effects.[4]

The newborn-infant is very susceptible to FR-induced oxidative damage. First, because of its immaturity, the infant is frequently exposed to oxygen therapy and hyperoxia. At birth the newborn encounters an environment much richer in oxygen (PO2 100 torr) than the intrauterine environment (20-25 torr). This 4-5 fold increase exposes the newborn to a flood of FR.[5] Second, the antioxidant defense and its ability to be induced during an hyperoxic challenge are impaired.[6] Third, the preterm infant has an increased susceptibility to infection and inflammation, which increases OS.[8] Finally, free iron is found in the plasma and tissue of premature infants to a greater extent than in the term infants.[9]

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Increased OS in hypoxic fetuses and neonates has been demonstrated by assaying products of lipid peroxidation in expired air, serum malondialdehyde reaction, serum isoprostanes, serum total hydroperoxides, advanced oxidative protein products and increased non protein bound iron (NPBI) in serum. Low serum antioxidant power has also been observed in red cells.[9-12]

Initial data indicating early lipid peroxidation in hypoxic infants comes from our studies specifically focused on early identification of perinatal hypoxic cell damage in the fetus and newborns. The following have been examined: 1) FR generation; 2) lipid peroxidation; 3) protein oxidation; 4) antioxidant defences. We detected an increased concentration of total hydroperoxides and advanced oxidative protein products in hypoxic preterm newborns. We observed a direct relation between the degree of hypoxia and the severity of oxidative damage in plasma at birth.[13] We also found that

total hydroperoxides and advanced oxidative protein products increased from birth to seven days of life in both preterm and term babies, indicating that an OS occur early in life and newborns are particularly susceptible to oxidative damage.[14]

During the oxidation of proteins, carbonyl groups (–CO=O) are introduced into the side-chains of the proteins. Measuring carbonyl concentration may therefore allow an assessment of protein oxidation.

We demonstrated that albumin was the main plasma protein modified by OS in patients with high levels of NPBI. Since albumin is a major extracellular antioxidant, its susceptibility to oxidation suggests that albumin, as carrier of NPBI in plasma, is the major target of NPBI-induced OS. Oxidation of albumin can therefore be expected to decrease plasma antioxidant defences and increase the likelihood of tissue damage due to OS in the newborn.[15]

Recent data suggests that a delicate redox balance must exist to allow for proper growth and development.[2] We demonstrated OS in pregnancies with fetal growth restriction. Fetal growth restriction is often complicated by intrauterine hypoxia and impaired blood flow to the fetus.[16] Chronic restrictions in uterine blood flow elicit placental and fetal responses in the form of growth adaptation to hypoxia. Intrauterine hypoxia may induce FR generation and fetal OS. We found that isoprostane concentrations in amniotic fluid are a reliable marker of fetal growth restriction due to OS.[17]

Isoprostanes are a family of prostaglandin isomers derived from polyunsaturated fatty acids through a FR-catalyzed peroxidation of arachidonic acid.[18] They can be measured in biofluids such as circulating plasma, and later in urinary excretions. In particular, 8-iso-PGF2, a major isoprostane that is relatively chemically stable and measurable in biofluids, is a reliable biomarker of OS.[19] We measured the levels of F(2)-isoprostanes in plasma of newborns by gas chromatography/mass spectrometry and we found that F(2)-isoprostanes are significantly higher in preterm and term newborns compared to healthy adults.[20] A significant inverse correlation was found between the plasma levels of isoprostanes and the gestational age. Because no increase in F2-IP levels was found in plasma of mothers at delivery or during pregnancy, and no correlation was found between plasma F2-IP in mothers and newborns, the results suggested that some form of lipid peroxidation is active in the fetus.

Another aspect of the importance of ROS-induced damage to amniotic epithelium and chorioamniotic collagen was clarified by our recent data demonstrating that F2-IP concentrations were significantly higher in pregnancies with premature rupture of membranes than in normal ones.[21] ROS may disrupt amino acid binding in proteins and polynsatured fatty acids of the membrane lipid bilayers, causing cell dysfunction, modification of chorioamniotic biology and predisposing pregnancies to premature rupture of membranes.[22,23]

Considering the close relationships between FR release and phagocyte function and the relationship between phagocyte activation and infection, additional markers of infection could be exploited to determine the occurrence of OS.

Activated phagocytes release a large amount of oxygen radicals and proteases.[24] The superoxide anion, the most abundant radical species, is also the first stage of the bacterial killing reaction, which is followed by production of other FR, such as hydrogen peroxide (H_2O_2) by superoxide dismutase, hydroxyl radicals catalysed by transition metals and HOCl- by myeloperoxidase. These substances contribute to bacterial killing but also favour tissue damage.[25] Moreover, these agents produce increased capillary permeability that facilitates the passage of cytokines. The precise mechanisms of the interaction between inflammation and OS are not known. Preceding infection with cytokine production amplifies the effect of hypoxic-ischemic insults. It has also been suggested that the effect of infection may be mediated through ischemia-hypoxia. Finally, it should be noted that while pro-inflammatory cytokines are liberated in response to infection, several other conditions including ischemia and aspecific inflammation are also known to cause cytokine production. These cytokines could therefore enter in a *final common pathway* in the cascade of molecular interaction leading to tissue damage whether triggered by infection or ischemia.[26] It is known that FiO2 activates lung phagocytes and enhance ROS release in the lung and brain tissues.[27] Several investigations carried out in infants who develop cronic lung diseases demonstrated raised concentrations of pro-inflammatory cytokines (Il-6, TNF-α, IL-1) in the amniotic fluid and bronchoalveolar lavage at birth as well as in the first 2-3 weeks of life.[28-30]

The many observations of poor outcome in babies whose gestations were complicated by chorioamnionitis, and of high cytokines such as IL-6, IL-1β, TNF-α in serum of premature and full term newborns with placental lesions typical of chorioamnionitis suggest that it may at least be worthwhile assaying inflammatory mediators as markers of brain damage in utero.[31-35]

In an interesting multicenter study on the problem of inflammatory mediators and cerebral palsy,[36] found that B-lymphocyte chemoattractants, ciliary neutrophil factor, epidermal growth factor, IL-5, IL-12, IL-13, IL-15, macrophage migration inhibitory factor, monocyte chemoattractant protein-3, monokine induced by interferon-γ and tumor necrosis factor-related apoptosis-inducing ligands were significantly higher in those with cerebral palsy than those without. Preterm infants

with cerebral palsy had a higher epidermal growth factor and lower levels of granulocyte macrophage colony stimulating factor, IL-23, macrophage derived chemokine as well as pulmonary and activation regulated chemokine than matched controls.

The roles of inflammatory responses by the fetal system and multi-organ failure seems to be critically important for understanding the genesis of OS related diseases of the newborn. It is not known whether the inflammatory response is causal or modulatory in the cascade of events that occurs during an intrauterine or perinatal insult to the newborn.

Free iron

In the absence of efficient protection by antioxidant factors, OS is responsible for a release of reactive form of iron, predisposing neonates to the risk of severe oxidative damage, due to the production and propagation of FR reactions. Iron is normally sequestered in transport proteins such as transferrin and lactoferrin and stored in proteins such as ferritin and haemosiderin that maintain iron non-toxic, unable to engage in Fenton reaction.[37] During situations of iron-overload and low plasma pH, as occurs during ischaemia, transferrin releases its iron and chelatable forms of Fe (iron ions or redox active complexes of iron) escape sequestration in biological systems, producing FR.[38,39] These FR may release even more iron by mobilizing it from ferritin.[10,11] This may lead to a cascade of iron release and free radical production, causing extensive cell damage.

Erythrocytes were the first cells of newborns to reveal the susceptibility of the neonate to OS.[40] OS leads to oxidation of haemoglobin and damage to the erythrocyte membrane. Extensive investigations by our research group demonstrated the key role of OS and iron release in a reactive form causing membrane protein damage via the Fencton reaction and hydroxyl radical production.[10,11] The role of iron and the Fencton reaction in hydroxyl radical formation and red cell damage was demonstrated in experiments in which cells were incubated in a medium containing a number of oxiding agents.[41,42] Membrane structure alterations and modification of erythrocyte functions following OS have been studied not only by incubating red cells with oxidizing agents such as phenylhydrazine but also incubating them under aerobic and anaerobic conditions.[43,44] Erythrocytes depleted of glutathione demonstrated that membrane protein abnormalities observed after incubation with oxidizing agents also occurred after anaerobic incubation. It is interesting that membrane protein damage was related to the appearance of the senescence antigen.[45,46] Information on how oxidative injury of the red cell is triggered and the mechanism of OS may provide a partial answer to some questions about the causes of the anemia of prematurity and also about red cell involvment in neonatal hypoxia. Indeed, some peculiar characteristics of the neonatal red cell predispose it to oxidative damage.[47,48]

The risk of oxidative damage to red cells and other cells depends on the balance of production and elimination of ROS. There seems to be not only a general predisposition to oxidative hemolysis, but also relationships between oxidative injury, gestational age and clinical condition. Intraerythrocyte non protein bound iron (NPBI) has been found to be particularly elevated in cord blood of hypoxic newborns. Release of NPBI was associated with increased lipoperoxide products in plasma.[10,11,49] When experiments were carried out by incubating newborn red cells under hypoxic conditions, we found a much greater release of iron than in an equal period of normoxia. Hypoxia also induced faster formation of senescent cell antigen than did normoxia in erythrocytes of newborns and to a lesser extent, those of adults. In newborns the release of NPBI in erythrocytes is correlated with plasma NPBI: the released iron has a tendency to diffuse from erythrocyte into the surrounding medium, suggesting the appearance of plasma NPBI.[49] Iron chelators able to enter cells (ferrozine, quercetin, fluor-benzoil-pyridoxal hydrazone) prevent both membrane protein oxidation and senescent cell antigen formation, one of the major pathways for erythrocyte removal.[41]

After asphyxia in newborn infants there is an increase in intraerythrocyte and plasma NPBI, significantly correlated with neurodevelopmental outcome.[50] Leakage of plasma NPBI into the brain through a damaged barrier may occur and is particularly damaging, as it is taken up directly by cells in a manner that is independent of transferrin. OS may also result from iron delocalization induced by the superoxide anion, acidosis and anoxia.[51] Enhanced proteolytic activity occurring in injured tissue also releases iron from storage proteins.[52] When non protein bound iron gains access to the extracellular space, its uptake by cells is enhanced by intracellular calcium and paradoxically also by increased levels of intracellular iron.[53] The toxicity of iron is inversely proportional to the availability of ferritin to sequester and detoxify ferrous ion, and directly proportional to the quantity of hydrogen peroxide to produce hydroxyl radicals by the Fenton reaction. After hypoxia, the expression of transferrin receptors on brain macrophages increases.[54] This is a protective mechanism to facilitate the active uptake of excess iron that may be released by iron-rich oligodendrocytes, or may accumulate due to the disruption of its normal transport after hypoxic insult.

Nitric oxide

Nitric oxide (NO) is a FR synthesized by NO synthase (NOS) in endothelial cells and neurones in response to

biochemical cellular alteration induced by hypoxia.[55] NOS produces NO, citrulline and water from arginine, NADPH and oxygen. NO and superoxide radicals combine to produce peroxynitrite that spontaneously decomposes to form hydroxyl radicals, nitrogen dioxide and NO^{2+}.[56] Other potentially damaging metabolites of nitric oxide include the nitrogen dioxide radical NO2 and nitryl chloride (NO_2Cl), formed by reaction of nitrite, an end-product of nitric oxide metabolism, with hypochlorous acid (HOCl), itself produced by the action of myeloperoxidase in neutrophils.[57] Three types of NOS are known: neuronal NOS, inducible NOS and endothelial NOS.[52-54] Hypoxic stress up-regulates NO production in a wide variety of cell types (macrophages, endothelial cells, neurons and astrocytes) and ischemic-hypoxic areas are characterised by a prominent inflammatory response with synthesis of the inducible form of NOS.[58] Experimental studies have demonstrated that the initial nitric oxide-mediated vasodilation and enhanced perfusion that result from the activation of NOS 3 are neuroprotective, at least during the first 2 h of ischemic insult.[59] However, the overall effects of enhanced NOS 1 and NOS 2 activity after ischemia are detrimental.[60]

In additon to the participation in oxidative injury and exocitotoxic cascade, NO, intimately related with intracellular concentration of Ca^{2+}, $[Ca^{2+}]_i$, plays a fundamental role in the signal transduction controlling several cellular processes such as proliferation.[55] It is well known that in the central nervous system it regulates the expression of genes responsible for the activation of extracellular regulated MAP kinases ERK 1 and ERK 2 mediated by Ras/Raf/ERK cascade, which represent one of the most important signal transduction pathways regulating the cell cycle. All this indicates that both $[Ca^{2+}]_i$ and/or NO changes may alternatively lead to either block or activation of cell cycle[61,62] and that the decision whether the cell will live or die must be clearly a highly regulated phenomenon in which the duration and intensity of the Ca^{2+} and/or NO signals might play a fundamental role. In line with the hypothesis that under different circumstances the same NO/Ca^{2+} signal may have opposite effects, it was recently demonstrated that either the pyrogenic proinflammatory effect of interleukin 1β[63] or the neurodegeneration induced by hypoxia[55] are mediated by the interplay between the two messengers.

Conclusions

Oxidative stress presents numerous opportunities for tissue injury through formation of reactive oxygen/nitrogen species. Oxidative stress can occur early in pregnancy and continue until the first days of life. We have showed the occurrence of lipid and protein oxidative damage associated with oxidative stress in pregnancy. In clinical practice, early markers of oxidative stress indicate that prenatal prophylactic use of antioxidants could help to prevent or at least reduce oxidative stress related diseases in fetuses and newborns.

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