The Role of Oxidative Stress on Necrotizing Enterocolitis in Very Low Birth Weight Infants

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Abstract: Necrotizing enterocolitis (NEC) is a devastating and common disease of very low birth weight (VLBW) infants with a mortality rate of 10% to 50% and a significant cause of morbidity in survivors. The incidence of NEC has increased from 5% to 7% in the last decades and this rate is likely to rise because of the increased survival of infants born at 24 weeks gestation, which are at high risk of developing NEC. NEC etiology is multifactorial: ischemia, infections, cytokines, enteral feeding and reactive oxygen species or free radicals (FRs) may contribute to the disruption of the immature gut barrier. In particular, ischemia, hypoxia-reperfusion, infection and inflammation are mechanisms capable of producing high levels of FRs, perturbing the normal redox balance and shifting cells to a state of oxidative stress (OS).

Despite advances in neonatal medicine, the early diagnosis of NEC remains a major challenge. Early clinical signs are non specific and the laboratory findings are not fully reliable. Therefore, its delayed occurrence after birth, its rapid onset, the highly fulminant nature, and its severe morbidity, as well as the possibility of progression to death, strongly require the identification of new prospective biomarkers specific for high NEC risk. There is evidences that OS biomarkers in cord blood allow the early identification of infants at risk for NEC and thereby can be used to develop novel therapies for this devastating disease which predominantly occurs in premature infants.

Keywords: Antioxidant, biomarkers, necrotizing enterocolitis, oxidative stress, preterm infants.

1. NECROTIZING ENTEROCOLITIS

a) Introduction

Necrotizing enterocolitis (NEC), a syndrome of acute intestinal ischaemic necrosis, is the most common gastrointestinal emergency in preterm infants. The incidence of NEC has increased from 5% to 7% in the last decades [1] and this rate is still rising due to the increased survival of extremely and very preterm infants resulting in a higher risk of developing NEC. Indeed the disease occurs in about 5% of all very preterm (<32 weeks’ gestation) or very low birth weight (VLBW: <1500 g) infants and in about 10% of all extremely preterm (<28 weeks’ gestation) or extremely low birth weight (ELBW: <1000 g) infants [2]. The pathogenesis of NEC is multifactorial and, according to epidemiologic studies, the principal risk factors include low gestational age, low birth weight, low Apgar scores, hyaline membrane disease, formula feeding, umbilical vessel catheterization, and intestinal ischemia [3].

In preterm infants the immaturity of the gastrointestinal tract contributes to NEC development [4]. The decreased intestinal peristalsis exposes the intestinal epithelium to noxious substances, specifically, intestinal peristalsis, macrophage, phagocytosis, gastric acidification and even epithelial barrier integrity have been shown to be impaired in premature infants as compared to full-term counterparts [5].

Prolonged antibiotic exposure is associated with an increased risk of NEC [1] and this association persists also in the multivariate analyses after the exclusion of the confounding factors (gestational age, birth weight, and sepsis) [6].

Other risk factors are sensitization to cow milk proteins [7] and genetic polymorphisms, such as mutations in carbamoyl phosphate synthetase [8], and vascular endothelial growth factor [9], and interleukin-10 and 12 [10].

Although many advances in the research field have been achieved, much remains to be elucidated on the pathophysiology of NEC. Some authors recently proposed that the underlying initial condition is the reduced ability of the neonatal gut epithelial cells to reduce oxidative stress (OS), and when epithelial gut cells are exposed to enteral feeding, the increased metabolic OS tips the population toward apoptosis, inflammation, bacterial activation, and eventual necrosis [11].

b) Last Updates in Pathophysiology

Preterm infants frequently suffer from ischemic events, such as hypotension, hypothermia, anemia, and patent ductus arteriosus during the intensive care stay. Severe anemia results in insufficient oxygen for the increased requirements of the mesenteric vessels after enteral feeding. Ischemia leads to endothelial cell disfunction and alters the endothelin-1/nitric oxide balance in favor of vasoconstriction, causing the expansion of ischemic intestinal lesions [12].

The ischemic conditions lead to the leakage of electrons from mitochondrial electron transport chain [13] and facilitate the availability of redox-active transition metals (copper
and iron), resulting in increased superoxide anion (O$_2^-$) production and hydrogen peroxide (H$_2$O$_2$) by the action of superoxide dismutase (SOD). H$_2$O$_2$ in conjunction with O$_2^-$ generates hydroxyl radicals that oxidizes cellular constituents such as enzymatic and structural proteins, membrane lipids and deplete glutathione. Lipid peroxidation results in the decrease of membrane fluidity, impaired iron transport and membrane integrity with loss of cellular functions [14]. OS appears to upregulate and enhance the activity of inducible nitric oxide synthase (iNOS) system accompanied by the overproduction of large amounts of nitric oxide (NO) that together with O$_2^-$ may produce, through iron catalyzed Haber-Weiss reaction, the peroxyxinitrites, greatly contributing to the cell damage [14]. OS also causes partial inactivation of cyclooxygenase-1 (COX- 1) and reduces the generation of gastroprotective prostaglandins (PG) that are known to inhibit gastric acid secretion, increase mucosal blood flow and stimulate mucus-HCO$_3^-$ secretion [15,16]. The decrease in mucosal PG, especially PGI2 and PGE2, might result in higher level of H$_2$O$_2$ in stressed gut that is accompanied by increased SOD activity. The changes in SOD activity may be considered as an adaptive mitochondrial response to excessive production of O$_2^-$: OS-induced ischemia and low oxygen (O$_2$) tension reduces the electron transport chain with subsequent leakage of electrons to increase the flux of O$_2$, which may release iron or copper, leading to excessive generation of hydroxyl (·OH), causing peroxidation of lipid cellular membranes and oxidative damage to proteins and other macromolecules. It is of interest that glutathione peroxidase (GPx), a major antioxidant enzyme in the gastric mucosa, was found to be inactivated during stress probably by excessively generated ·OH causing oxidative damage of GPx and this seems to play a significant role in stress-induced gastric ulceration [16].

Regardless of the triggering mechanisms, the resultant outcome is a significant inflammation of the intestinal tissues, release of inflammatory mediators, and down-regulation of cellular growth factors. In this context, the overproduction of free radicals (FRs) during inflammation significantly contributes to variable degrees of endothelial dysfunction and intestinal damage.

Several investigators have now examined the mechanisms that mediate the signaling response of the newborn intestine to bacteria, and have detailed the consequences of this signaling response to the pathogenesis of NEC. Hackam et al. have recently demonstrated that the expression of Toll-like Receptor-4 (TLR4) within the intestine rises during gestation in the mouse, then falls shortly before birth [17]. The finding that TLR4 expression rises in the intestine of the fetus during development suggests the distinct possibility that TLR4 may signal in response to agonists within the micro-environment of the developing intestine other than lipopolysaccharide (LPS) [18]. Following activation of TLRs a complex cascade of signals occurs, culminating in the translocation of the transcription factor NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) to the nucleus. Through the activation of NF-kB the most pro-inflammatory mediators, including TNF-α, IL-6, IL-1β and platelet activator factor (PAF) are released.

PAF is a potent lipid mediator that is released by a variety of cells including peritoneal cells [19] and macrophages [20], and is capable of eliciting pro-inflammatory responses in several cell types [21]. Caplan and colleagues as well as others [22–25] have elegantly demonstrated an important role for PAF in NEC pathogenesis. The neonatal intestine was found to have high PAF biosynthetic activity [22], and PAF levels were significantly increased in the stools of infants with NEC compared to healthy controls [23].

In animal models it has been demonstrated that in mother fed animals, there is a decrease in TLR4 expression in intestinal epithelium, while in formula-fed and asphyxia-stressed animals, TLR4 expression increases [26]. There is a suggestion that NEC occurs mainly in infants who have been fed, although there is no evidence that delaying introduction of enteral feeds decreases the incidence of the disease. Rather it has been demonstrated that NEC occurs more often when high volumes of enteral feeds are achieved [27] and that a prolonged use of trophic feeds is protective compared with quick increments in the first 10 days of life [28]. Promotion of breastfeeding reduces the incidence of NEC by three to ten-fold [29]. Moreover, it has recently been shown that TLR4 wild-type mice exposed to hypoxia and fed with formula have increased incidence of NEC when compared with TLR4 mutant mice. In this context, experimental data from NEC-induced animal models show that exogenous administration of Bifidobacterium infantis decreases the incidence of NEC and lowers plasma levels of toxins in treated rats [30]. A recently published meta-analysis systematically reviewed several randomized clinical trials searching to demonstrate the benefit of probiotic administration in populations of premature infants. The conclusion was that probiotics might reduce the risk of necrotizing enterocolitis in pre-term neonates with gestational age less than 33 weeks. Several questions related to dose, duration, and type of probiotic agents still remain open [31].

c Clinical Implications

NEC is a devastating disease with a mortality rate of 10% up to 50% [32-34]. The risk of mortality and serious morbidity rises with lower birth weight and gestational age. For infants with severe NEC which requires surgical intervention, reported mortality rates are 30–40%. Infants who develop NEC, if compared with gestation-matched controls, experience: 1) more nosocomial infections; 2) lower levels of nutrient intake; 3) slower growth; 4) higher incidence of bronchopulmonary dysplasia and retinopathy of prematurity; 5) longer durations of intensive care and hospital stay [35]. In those who survive to the acute phase of the disease, long-term consequences may follow such as higher morbidity, including growth restriction and neurodisability as a consequences of undernutrition and associated infections during a very vulnerable period of growth and development. The risk of adverse neurodevelopmental outcomes is higher in those infants with NEC who received surgical intervention [35, 36]. There is also evidence of a synergistic effect of NEC and invasive infections on the risk of severe neurodisability including microcephaly and cerebral palsy [37]. Short bowel syndrome is the most common long term gastrointestinal complication, affecting about one-fourth of NEC survivors (up to 42% in those who had surgical NEC). Not surprisingly, variables associated with severe NEC (low birth weight, antibiotic use, ventilator use, and greater extent of...
bowel resection) are also associated with the development of short bowel syndrome [38]. NEC is the most common cause of short bowel syndrome in childhood. Even patients who do not undergo surgery for NEC can develop short bowel syndrome because of poor absorptive capacity of the intestine that had been involved in the disease process [39].

In conclusion the prognosis of patients with NEC is strongly related to the maturation state of the bowel, the severity of injury and the need of a surgical intervention. Hence the importance of finding new preventive mechanisms and of an early diagnosis.

2. BIOMARKERS OF OXIDATIVE STRESS

a) Reliable Biomarkers of Oxidative Stress In vivo

FRs are defined as species containing one or more unpaired electrons, and it is this incomplete electron shell that confers their high reactivity. OS occurs when the production of FRs exceeds the capacity of antioxidant defenses [40]. Each cell is characterized by a particular concentration of electrons stored in many cellular constituents and the redox state of a cell with its oscillation determines cellular functioning [41]. Disturbances in the normal redox state of tissues can cause toxic effects through the production of peroxides and FRs that damage all components of the cell, including proteins, lipids and DNA. The identification of reliable biomarkers is essential for the characterization of OS and probably for the early discovery of OS related diseases. They can be used as “intermediate endpoints or early-outcome predictors” of disease development and for preventive purposes [42, 43]. They are also useful for monitoring pharmacologic response to antioxidant interventions. A complete and reliable biomarker should have biological validity, high sensibility and specificity, a standardized methodology of measurement and should be reproducible. Nevertheless, OS is difficult to be measured in vivo, because FRs have a very short half-life, thus, biomarkers evaluate host susceptibility to OS by measuring proteins, lipids and DNA damage (Fig. 1).

The most extensively studied DNA lesion is the formation of 8-Hydroxyguanosine (8-OH-G), which is a good biomarker of oxidative stress [44], but the measurement of lipid peroxidation has reached the highest in vivo specificity and sensitivity with the discovery of isoprostanes (IsoPs), a family of prostaglandin isomers derived from polyunsaturated fatty acids through a FR-catalyzed peroxidation of arachidonic acid [45]. These prostanoids are less reactive than other peroxidation products such as aldehydes or peroxy radicals so they can be easily found in plasma and urine. In particular, 8-iso-PGF2, a major isoprostane that is relatively chemically stable and measurable in biofluids, is a reliable biomarker of OS [46]. Oxygen tension can affect prostanoids profile. An oxygen insertion step diverts intermediates from the IsoPs pathway to form compounds, termed Isofurans (IsoFs) that contain a substituted tetrahydrofuran ring. Like the IsoPs, the IsoFs are chemically and metabolically stable and are well suited to act as in vivo biomarkers of oxidative damage. The ratio of IsoFs to IsoPs also provides informations about the relative oxygen tension where the lipid peroxidation is occurring. Determination of IsoPs and IsoFs requires sophisticated and expensive methods such as gas chromatography/mass spectrometry (GC–MS).

As plasma proteins are critical targets for oxidants, the detection of advanced oxidation proteins products (AOPP) in biologic fluid can be an optimal strategy to detect and to estimate the degree of oxidant-mediated proteins damage. Indeed AOPP are terminal products of proteins exposure to FRs without oxidant properties. AOPP can be measured using spectrophotometry on a microplate reader as described by Witko-Sarsat and colleagues [47]. Even if they don’t have oxidant properties, they may act as inflammatory mediators triggering the oxidative burst of neutrophiles, monocytes and...

Fig. (1). Biomarkers of oxidative stress.
T-lymphocytes thus leading to a phagocyte-derived OS. The importance of AOPP determination, as oxidative biomarker and inflammatory mediator, has been pointed out in several studies [48-51].

Lipids and proteins damage from FRs exposure leads to lipid hydroperoxide generation from lipids and to carbonyl formation and protein hydroperoxidation from proteins. Total hydroperoxides (TH) represent a measure of overall oxidative stress, because they are intermediate oxidative products of lipid, peptides and amino acids which can be measured collectively as organic hydroperoxide by a spectrometric assay [50, 51].

Without efficient protection by antioxidant factors, OS is responsible for a release of reactive form of iron (Fe), predisposing neonates to the risk of severe oxidative damage, due to the production and propagation of FRs reactions. Fe 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 [52]. In 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 [52]. These FRs may release even more Fe by mobilizing it from ferritin [53, 54]. This may lead to a cascade of iron release and FRs production, causing extensive cell damage. Fe ions cannot exist in plasma so the term non protein bound iron (NPBI) was introduced to indicate a low molecular mass iron form, free of high affinity binding to transferrin. NPBI plasma levels can be detected by high performance liquid chromatography (HPLC) [55]. Plasma NPBI has been found to be the best early predictive marker of neurodevelopmental outcome in newborns (gestational age 24-42 wks) with clinical signs of perinatal hypoxia at birth. This marker showed 100% sensitivity and 100% specificity for good outcome until the second year of age at 0-1.16 μmol/l and for poor outcome at > 15.2 μmol/l [56].

b) Necrotizing Enterocolitis and Biomarkers of Oxidative Stress

Despite advances in neonatal medicine, early diagnosis of NEC remains a major challenge. Early clinical signs are non specific and the laboratory findings are not fully reliable. Therefore, its delayed occurrence after birth, its rapid onset, highly fulminant nature, and progression to death, as well as its severe morbidity, require identification of prospective new biomarkers specific for high NEC risk [57].

The damaging effects of FRs in the perinatal period, by evaluating total TH, AOPP and non NPBI, as markers of biochemical markers of OS have been recently reported. An increased concentration of TH and AOPP in hypoxic preterm newborns whist a direct relation between the degree of hypoxia and the severity of oxidative damage in plasma at birth were observed [50]. Interestingly, it was demonstrated a predictive role of OS biomarkers for pathologies resulting from oxidative damage, grouped together and categorized as "free radicals related disease of the neonate" (FRD) [58, 59]. Such pathologies include retinopathy of prematurity (which in severe cases may lead to blindness), bronchopulmonary dysplasia (a particularly debilitating pulmonary lesion in the preterm infant), periventricular leukomalacia (an important cause of severe neurodisability in premature infants) and necrotizing enterocolitis.

Recently we found a strong association between NEC and cord blood concentration of AOPP, NPBI and TH, showing a clear correlation between intrauterine OS events and the risk of developing NEC [48]. Ozdemir et al. reported a significant increase of intestinal malondialdehyde (MDA) in preterm infants with NEC [60]. All-trans-retinoic acid (ATRA) treatment reduced the intestinal MDA elevation, suggesting an active lipid peroxidation in NEC disease. Consistent with this results, the administration of agents that either inhibit reactive oxygen species (ROS) production or act as antioxidant has been showed to reduce intestinal mucosa damaged by ischemia or inflammation [61, 62].

All these data establish the importance of perinatal oxidative insults in injured intestinal epithelial cells, proving a rational basis for formulation of intervention to interrupt those mechanisms. OS biomarkers in cord blood allow the early identification of infant at risk for NEC and thereby can be used to develop novel therapeutic strategies for this devastating disease which predominantly occurs in premature infants.

3. FUTURE PERSPECTIVES

Recent advances in neonatology have led to improved survival of extremely preterm infants, and to a resultant increase in the diseases of prematurity. NEC represents one of the most relevant source of mortality and morbidity. The enormous burden of human suffering and financial cost caused by FRD make the early diagnosis and prevention a major health care priority. Defining the causes, patterns and mechanisms of injury is crucial to develop rational protective strategies to reduce short and long term effect of FRD. Many potential antioxidant drugs have been investigated targeting different pathways leading to FRD in response to hypoxic-ischemic insult: agents that inhibit glutamate release, uptake, or blockade of glutamate receptors, blockade of removal FRs, blockade of downstream effects and inhibition of inflammatory effects. Currently there are no established therapies to prevent or ameliorate FRs mediated injury and altered organ development.

Antioxidants are critical in protecting against ROS-induced injury and several preclinical studies support antioxidant supplementation. Non-enzymatic proteins (transferrin, ferritin, lactoferrin), enzymes (superoxide dismutases, catalase, glutathione peroxidase), oxidizable molecules (glutathione, vitamins E, A, C, carotenoids, flavonoids), and trace elements (copper, zinc, selenium) all play a key role in maintaining a delicate balance between ROS production and oxidative damage to tissues and organs [9]. Among them, Lactoferrin (LF) is a very promising drug, this is due to its capacity to interfere with many of the pathological triggers of NEC.

LF is an 80 kDa, natural defense iron-binding glycoprotein, which is present in several exocrine secretions, including tears, nasal and bronchial mucus, saliva, intestinal and genital secretions [63]. In vivo, in normal subjects, LF is
only partially saturated with iron [64, 65]. Such iron-binding capacity gives LF the ability to primarily act as a chelator of free iron in the newborn’s gut. This may lead to the prevention of cell damage such as lipid peroxidation from any FRs generated by an iron-catalyzed Haber-Weiss reaction. Secondarily, the iron acceptor property may control the microbial pathogens and cellular inflammatory-induced oxidative damage [65-67]. It is important to emphasize that LF has a marked bacteriostatic and bactericidal effect on a wide range of microorganisms, not only because of the iron deprivation of the pathogens, as believed in the past, but also because of its active metabolite, the lactoferrin, a potent bactericidal peptide [68, 69]. A recent Italian multicentre trial examined the effect of enteral supplementation with bovine lactoferrin for up to 6 weeks, either alone or in combination with a probiotic lactobacillus, in very preterm infants. This good-quality trial found evidence that lactoferrin supplementation reduces substantially the incidence of invasive nosocomial infection and decreases the occurrence of NEC when combined with Lactobacillus rhamnosus GG [70]. Anyway till now there is no evidence of the efficacy of oral lactoferrin (given alone) in the prevention of NEC in preterm neonates. Well designed, randomized trials should address dosing, duration, type of lactoferrin (bovine or human) prophylaxis in prevention of sepsis and NEC [71]. It is possible that a smaller effect of lactoferrin on the risk of NEC may exist and further large trials are proposed to investigate this possibility.

4. CONCLUSION

Despite improvements in neonatal intensive care, NEC remains a critical disease in preterm infants with a high incidence of mortality and many severe complications. The development of NEC may be associated with a variety of factors, including colonization by pathogenic bacteria, secondary ischemia, insufficient maturation of the gastrointestinal tract and oxidative stress. At now due to the established importance of perinatal oxidative insults in injured intestinal epithelial cells, more studies are needed, providing a rational basis for formulation of interventions to interrupt those mechanisms. The use of supplemental antioxidants represents a logical strategy to prevent or ameliorate injury from excess production of ROS, but studies in animal models and in preterm infants have yielded mixed results.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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