*Send Orders for Reprints to reprints@benthamscience.net*

*Current Pediatric Reviews*, 2014, *10*, 00-00 

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

Serafina Perrone[1], Maria Luisa Tataranno[1], Antonino Santacroce[1,*], Simona Negro[1] and Giuseppe Buonocore[1]

[1]*Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy*

**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

*Address correspondence to this author at Department of Molecular and Developmental Medicine, University of Siena, Policlinico Santa Maria alle Scotte, Viale Bracci 36, 53100 Siena, Italy; Tel.: 0039 0577 586542 / 523; fax: 0039 0577 586182. E-mail address: santacroce.antonino@gmail.com

1573-3963/14 $58.00+.00 

and iron), resulting in increased superoxide anion ($O_2^{-\cdot}$) production and hydrogen peroxide ($H_2O_2$) by the action of superoxide dismutase (SOD). $H_2O_2$ in conjugation with $O_2^{-\cdot}$ 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 $O_2^{-\cdot}$ may produce, through iron catalyzed Haber-Weiss reaction, the peroxynitrites, 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-HCO3- secretion [15,16]. The decrease in mucosal PG, especially PGI2 and PGE2, might result in higher level of $H_2O_2$ 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 $O_2^{-\cdot}$. 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 preterm 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 peroxyl 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

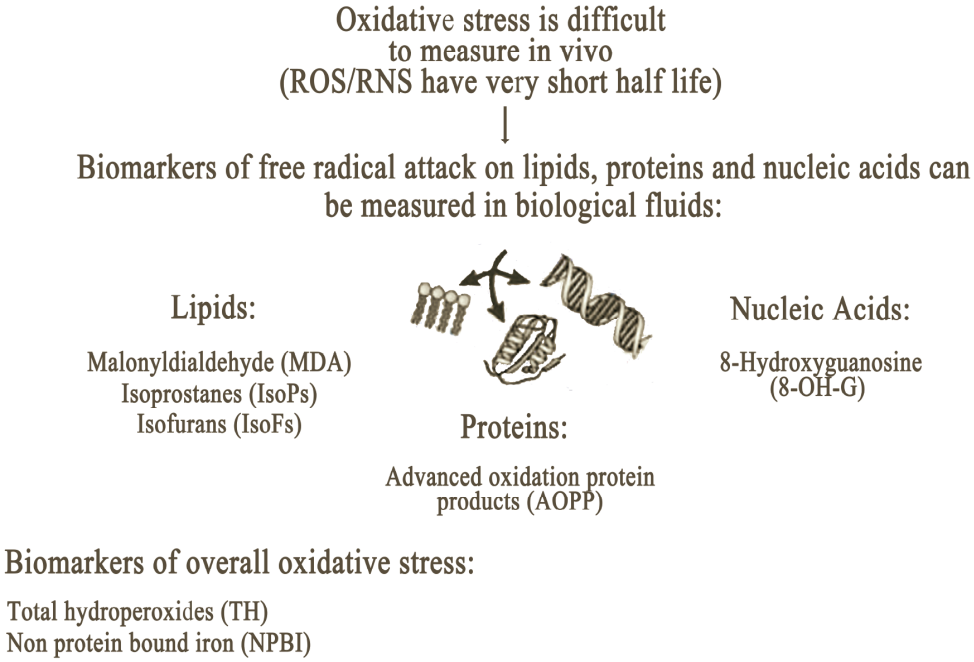


**Fig. (1).** Biomarkers of oxidative stress.

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 hydroperoxides generation 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].

Thedamaging effects of FRs in the perinatal period, by evaluating total TH, AOPP and non NPBI, as markers of biochemichal markers of OS have been recently reported. An increased concentration of TH and AOPP in hypoxic preterm newborns whit 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 malonyldialdehyde (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 devasting disease wich 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 glatamate release, uptake, or blockade of glutamate receptors, blockade or removal FRs, blockade of downstream effects and inhibiton 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]. 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 lactoferricin, 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 occurence 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.

## ACKNOWLEDGEMENTS

We are very grateful to EURAIBI (EURope Against Infant Brain Injury) foundation for its support.

Grants from: Italian Ministry of Health, Research Project RF-2009-1499651: Early identification of newborns at high risk of brain injury through a validated biomarkers profile and specific neuro-imaging patterns. A follow-up study of clinical and neurodevelopmental outcome in a large cohort study.

## REFERENCES

[1] Holman RC, Stoll BJ, Curns AT, Yorita KL, Steiner CA, Schonberger LB. Necrotising enterocolitis hospitalisations among neonates in the United States. Paediatr Perinat Epidemiol 2006; 20: 498-506.

[2] Morgan JA, Young L, McGuire W. Pathogenesis and prevention of necrotizing enterocolitis. Curr Opin Infect Dis 2011; 24(3): 183-9.

[3] Boccia D, Stofli I, Lana S, Moro ML. Nosocomial necrotizing enterocolitis outbreaks: Epidemiology and control measures. Eur J Pediatr 2001; 160: 385-91.

[4] Lee JH. An update on necrotizing enterocolitis: pathogenesis and preventive strategies. Korean J Pediatr 2011;54(9): 368-72.

[5] Milla PJ, Fenton TR. Small intestinal motility patterns in the perinatal period. J Pediatr Gastroenterol Nutr 1983; 2: S141-4.

[6] Alexander VN, Northrup V, Bizzarro MJ. Antibiotic exposure in the newborn intensive care unit and the risk of necrotizing enterocolitis. J Pediatr 2011; 159(3): 392-7.

[7] Abdelhamid AE, Chuang SL, Hayes P, Fell JM. In vitro cow's milk protein-specific inflammatory and regulatory cytokine responses in preterm infants with necrotizing enterocolitis and sepsis. Pediatr Res 2011; 69(2): 165-9.

[8] Moonen RM, Paulussen AD, Souren NY, Kessels AG, Rubio-Gozalbo ME, Villamor E. Carbamoyl phosphate synthetase polymorphisms as a risk factor for necrotizing enterocolitis. Pediatr Res 2007; 62(2): 188-90.

[9] Bányász I, Bokodi G, Vásárhelyi B, *et al*. Genetic polymorphisms for vascular endothelial growth factor in perinatal complications. Eur Cytokine Netw 2006; 17(4): 266-70.

[10] Treszl A, Kaposi A, Hajdú J, Szabó M, Tulassay T, Vásárhelyi B. The extent to which genotype information may add to the prediction of disturbed perinatal adaptation: none, minor, or major? Pediatr Res 2007; 62(5): 610-4.

[11] Kim M, Christley S, Alverdy JC, Liu D, An G. Immature oxidative stress management as a unifying principle in the pathogenesis of necrotizing enterocolitis: insights from an agent-based model. Surg Infect (Larchmt) 2012; 13(1): 18-32.

[12] Nankervis CA, Giannone PJ, Reber KM. The neonatal intestinal vasculature: contributing factors to necrotizing enterocolitis. Semin Perinatol 2008; 32(2): 83-91.

[13] Loschen G, Azzi A, Richter C, Flohé L. Superoxide radicals as precursors of mitochondrial hydrogen peroxide. FEBS Lett 1974; 42(1): 68-72.

[14] Maréchal A, Mattioli TA, Stuehr DJ, Santolini J. Activation of peroxynitrite by inducible nitric-oxide synthase: a direct source of nitrative stress. J Biol Chem 2007; 11; 282(19): 14101-12.

[15] Konturek SJ, Konturek PC, Brzozowski T. Prostaglandins and ulcer healing. J Physiol Pharmacol 2005; 56 Suppl 5: 5-31.

[16] Bandyopadhyay D, Chattopadhyay A. Reactive oxygen species-induced gastric ulceration: protection by melatonin. Curr Med Chem 2006; 13(10): 1187-202.

[17] Gribar SC, Sodhi CP, Richardson WM, *et al*. Reciprocal expression and signaling of TLR4 and TLR9 in the pathogenesis and treatment of necrotizing enterocolitis. J Immunol 2009; 182: 636-46.

[18] Dai S, Sodhi CP, Cetin S, *et al*. Extracellular high mobility group box1 (HMGB1) inhibits enterocyte migration via activation of toll like receptor 4 and increased cell-matrix adhesiveness. J Biol Chem 2010; 285: 4995-5002.

[19] Shindou H, Ishii N, Uozumi T, Shimizu T. Roles of cytosolic phospholipase A2 and platelet activating factor receptor in the Ca-induced biosynthesis of PAF. Biochem Biophys Res Commun 2000; 271: 812-7.

[20] Svetlov SI, Liu H, Chao W, Olson MS. Regulation of platelet-activating factor (PAF) biosynthesis via coenzyme A-independent transacylase in the macrophage cell line IC-21 stimulated with lipopolysaccharide. Biochim Biophys Acta 1997; 1346: 120-30.

[21] Izumi T, Shimizu T. Platelet-activating factor receptor: gene expression and signal transduction. Biochim Biophys Acta 1995; 1259: 317-33.

[22] Muguruma K, Gray PW, Tjoelker LW, Johnston JM. The central role of PAF in necrotizing enterocolitis development. Adv Exp Med Biol 1997; 407: 379-82.

[23] Amer MD, Hedlund E, Rochester J, Caplan MS. Platelet-activating factor concentration in the stool of human newborns: effects of enteral feeding and neonatal necrotizing enterocolitis. Biol Neonate 2004; 85:159-66.

[24] Caplan MS, Hedlund E, Adler L, Lickerman M, Hsueh W. The platelet activating factor receptor antagonist WEB 2170 prevents neonatal necrotizing enterocolitis in rats. J Pediatr Gastroenterol Nutr 1997; 24: 296-301.

[25] Caplan MS, Lickerman M, Adler L, Dietsch GN, Yu A. The role of recombinant platelet activating factor acetylhydrolase in a neonatal rat model of necrotizing enterocolitis. Pediatr Res 1997; 42: 779-83.

[26] Jilling T, Simon D, Lu J *et al*. The roles of bacteria and TLR4 in rat and murine models of necrotizing enterocolitis. J Immunol 2006; 177: 3273-82.

[27] Owens L, Berseth CL. Is there a volume threshold for enteral feeding and necrotizing enterocolitis? Pediatr Res 1995; 37: 315A.

[28] Berseth CL, Bisquera JA, Paje VU. Prolonging small feeding volumes early in life decreases the incidence of necrotizing enterocolitis in very low birth weight infants. Pediatrics 2003; 111: 529-34.

[29] Lucas A, Cole TJ. Breast milk and neonatal necrotizing enterocolitis. Lancet 1990; 336: 1519-23.

[30] Caplan MS, Miller-Catchpole R, Kaup S *et al*. Bifidobacterial supplementation reduces the incidence of necrotizing enterocolitis in a neonatal rat model. Gastroenterology 1999; 117: 577-83.

[31] Deshpande G, Rao S, Patole S. Probiotics for prevention of necrotizing enterocolitis in preterm neonates with very low birth weight: a systematic review of randomized controlled trials. Lancet 2007; 369: 1614-20.

[32] Kosloske AM. Epidemiology of necrotizing enterocolitis. Acta Paediatr Suppl 1994; 396: 2-7.

[33] Thompson AM, Bizzarro MJ. Necrotizing enterocolitis in newborns: pathogenesis, prevention and management. Drugs 2008; 68(9): 1227-38.

[34] Schnabl KL, Van Aerde JE, Thomson AB, Clandinin MT. Necrotizing enterocolitis: a multifactorial disease with no cure. World J Gastroenterol 2008; 14(14): 2142-61.

[35] Leviton A, Dammann O, Engelke S, *et al*. The clustering of disorders in infants born before the 28th week of gestation. Acta Paediatr 2010; 99: 1795-1800.

[36] Rees CM, Pierro A, Eaton S. Neurodevelopmental outcomes of neonates with medically and surgically treated necrotizing enterocolitis. Arch Dis Child Fetal Neonatal Ed 2007; 92: F193-F198.

[37] Martin CR, Dammann O, Allred EN, *et al*. Neurodevelopment of extremely preterm infants who had necrotizing enterocolitis with or without late bacteremia. J Pediatr 2010; 157: 751-6.

[38] Duro D, Kalish LA, Johnston P, *et al*. Risk factors for intestinal failure in infants with necrotizing enterocolitis: a Glaser Pediatric Research Network study. J Pediatr 2010; 157: 203-8 e201.

[39] Stanford A, Upperman JS, Boyle P, Schall L, Ojimba JI, Ford HR. Long-term follow-up of patients with necrotizing enterocolitis. J Pediatr Surg 2002; 37(7): 1048-50.

[40] Buonocore G, Groenendaal F. Anti-oxidant strategies. Semin Fetal Neonatal Med 2007; 12: 287-95.

[41] Buonocore G, Perrone S, Tataranno ML. Oxygen toxicity: chemistry and biology of reactive oxygen species. Semin Fetal Neonatal Med 2010; 15: 186-90.

[42] Bonassi S, Au WW. Biomarkers in molecular epidemiology studies for health risk prediction. Mutat Res 2002; 511: 73-86.

[43] Ogino K, Wang DH. Biomarkers of oxidative/nitrosative stress: an approach to disease prevention. Acta Med Okayama 2007; 61: 181-9.

[44] Halliwell B. The wanderings of a free radical. Free Radic Biol Med 2009; 46: 531e42.

[45] Morrow J, Roberts L. The isoprostanes. Current knowledge and directions for future research. Biochem. Pharmacol 1996; 12: 1-9.

[46] Roberts LJ, Morrow JD. Measurement of F(2)-isoprostanes as an index of oxidative stress *in vivo*. Free Radic Biol Med 2000; 28(4): 505-13.

[47] Witko-Sarsat V, Friedlander M, Capeillère-Blandin C, *et al*. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney International 1996; 49: 1304-13.

[48] Perrone S, Tataranno ML, Negro S, *et al*. May oxidative stress biomarkers in cord blood predict the occurrence of necrotizing entero colitis in preterm infants? J Matern Fetal Neonatal Med 2012; 25S1: 128-31.

[49] Alderman CJ, Shah S, Foreman JC, Chain BM, Katz DR. The role of advanced oxidation protein products in regulation of dendritic cell function. Free Radic Biol Med 2002; 32: 377-85.

[50] Buonocore G, Perrone S, Longini M, Terzuoli L, Bracci R. Total hydro peroxide and advanced oxidation protein products in preterm hypoxic babies. Pediatr Res 2000; 47: 221-4.

[51] Buonocore G, Perrone S, Longini M, *et al*. Oxidative stress in preterm neonates at birth and on the seventh day of life. Pediatr Res 2002; 52: 46-9.

[52] Papanikolau G, Pantopoulos K. Iron metabolism and toxicity. Toxicol Appl Pharmacol 2005; 202: 199-211.

[53] Buonocore G, Zani S, Perrone S, Caciotti B, Bracci R. Intra erythrocyte non protein-bound iron and plasma malondialdehyde in the hypoxic newborn. Free Radic Biol Med 1998; 25: 766-70.

[54] Buonocore G, Zani S, Sargentini I, Gioia D, Signorini C, Bracci R. Hypoxia induced free iron release in the red cells of newborn infants. Acta Paediatr 1998; 87: 77-81.

[55] Paffetti P, Perrone S, Longini M, *et al*. Non-protein-bound iron detection in small samples of biological fluids and tissue. Biol Trace Elem Res 2006; 112: 221-232.

[56] Buonocore G, Perrone S, Longini M, Paffetti P, Gatti MG, Bracci R. Non protein bound iron as predictive marker of neonatal brain damage. Brain 2003; 126: 1-7.

[57] Young C, Sharma R, Handfield M, Mai V, Neu J. Biomarkers for infants at risk for necrotizing enterocolitis: clues to prevention? Pediatr Res 2009; 65(5 Pt 2): 91R-97R.

[58] Perrone S, Tataranno ML, Negro S, *et al*. Early identification of the risk for free radical-related diseases in preterm newborns. Early Hum Dev 2010; 86(4): 241-4.

[59] Perrone S, Vezzosi P, Longini M, *et al*. Biomarkers of oxidative stress in babies at high risk for retinopathy of prematurity. Front Biosci (Elite Ed) 2009; 1: 547-52.

[60] Ozdemir R, Yurttutan S, Sari FN, *et al*. All-trans-retinoic acid attenuates intestinal injury in a neonatal rat model of necrotizing enterocolitis. Neonatology 2013;104(1): 22-7.

[61] Rao J, Qian X, Wang P, *et al*. All-trans retinoic acid preconditioning protects against liver ischemia/reperfusion injury by inhibiting the nuclear factor kappa B signaling pathway. J Surg Res 2013; 180(2): e99-e106.

[62] Darlow BA, Graham PJ. Vitamin A supplementation to prevent mortality and short- and long-term morbidity in very low birthweight infants. Cochrane Database Syst Rev 2011; (10): CD000501.

[63] Weinberg ED. Human lactoferrin: a novel therapeutic with broad spectrum potential. J Farm Farmacol 2001; 53: 1303-10.

[64] Leibman A, Aisen P. Distribution of iron between the binding sites of transferrin in serum: methods and results in normal human subjects. Blood 1979; 53(6): 1058-65.

[65] Bullen JJ, Rogers HJ, Leigh L. Iron-binding proteins in milk and resistance to Escherichia coli infection in infants. Br Med J 1972; 1(5792): 69-75.

[66] Sanchez L, Calvo M, Brock J H. Biological role of lactoferrin. Arch Dis Child 1992; 67: 657-61.

[67] Baldwin DA, Jenny ER, Aisen P. The effect of human serum transferrin and milk lactoferrin on hydroxyl radical formation from superoxide and hydrogen peroxide. J Biol Chem 1984; 259: 13391-4.

[68] Weinberg ED. The therapeutic potential of lactoferrin. Expert Opin Investig Drugs 2003; 12: 841-51.

[69] Lönnerdal B. Nutritional and physiologic significance of human milk proteins. Am J Clin Nutr 2003; 77: 1537S-43S.

[70] Manzoni P, Rinaldi M, Cattani S, *et al*. Bovine lactoferrin supplementation for prevention of late-onset sepsis in very low-birthweight neonates: a randomized trial. JAMA 2009; 302: 1421-8.

[71] Pammi M, Abrams SA. Oral lactoferrin for the prevention of sepsis and necrotizing enterocolitis in preterm infants. Cochrane Database Syst Rev 2011; (10): CD007137.

Received: ????? 0?, 201? Revised: ?????? ??, 201? Accepted: ?????? ??, 201?