Jay and Margie Grosfeld Lecture

Roles of nitric oxide and intestinal microbiota in the pathogenesis of necrotizing enterocolitis☆

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ABSTRACT

Necrotizing enterocolitis remains one of the most vexing problems in the neonatal intensive care unit. Risk factors for NEC include prematurity, formula feeding, and inappropriate microbial colonization of the GI tract. The pathogenesis of NEC is believed to involve weakening of the intestinal barrier by perinatal insults, translocation of luminal bacteria across the weakened barrier, an exuberant inflammatory response, and exacerbation of the barrier damage by inflammatory factors, leading to a vicious cycle of inflammation-inflicted epithelial damage. Nitric oxide (NO), produced by inducible NO synthase (iNOS) and reactive NO oxidation intermediates play a prominent role in the intestinal barrier damage by inducing enterocyte apoptosis and inhibiting the epithelial restitution processes, namely enterocyte proliferation and migration. The factors that govern iNOS upregulation in the intestine are not well understood, which hampers efforts in developing NO/iNOS-targeted therapies. Similarly, efforts to identify bacteria or bacterial colonization patterns associated with NEC have met with limited success, because the same bacterial species can be found in NEC and in non-NEC subjects. However, microbiome studies have identified the three important characteristics of early bacterial populations of the GI tract: high diversity, low complexity, and fluidity. Whether NEC is caused by specific bacteria remains a matter of debate, but data from hospital outbreaks of NEC strongly argue in favor of the infectious nature of this disease. Studies in *Cronobacter muytjensii* have established that the ability to induce NEC is the property of specific strains rather than the species as a whole. Progress in our understanding of the roles of bacteria in NEC will require microbiological experiments and genome-wide analysis of virulence factors.

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Necrotizing enterocolitis (NEC), a severe inflammation of the small intestine, is the most common and most lethal disease affecting the GI tract of the premature infant. Despite aggressive medical and surgical treatment, the mortality rate for NEC is typically 15%–30% in the very low birth weight neonates and approaches 100% in cases involving pan-necrosis [1,2]. Unfortunately, the incidence of NEC continues to rise because recent advances in neonatology have resulted in the survival of infants born at 23 weeks gestation [3].

1. Pathogenesis of NEC

According to the currently accepted pathogenetic scenario, perinatal insults such as hypoxia, hypothermia, and enteral feeding with formula compromise the barrier function of the intestinal epithelium [4]. At the same time, bacterial colonization of the neonate's GI tract occurs [5–7]. Some luminal bacteria and their components, such as lipopolysaccharide (LPS), CpG DNA, or flagellin, translocate across the compromised barrier and engage immunocytes, eliciting the production of inflammatory factors including proinflammatory cytokines, NO and peroxynitrite, and inflammatory prostanoids [8–12]. These proinflammatory stimuli damage the barrier by their effects on the epithelial tight junctions [13]. They also increase the rate of enterocyte cell death and decrease the rates of enterocyte proliferation and migration [14]. Increased barrier damage and decreased epithelial restitution further compromise the mucosal barrier, leading to more bacterial translocation, more inflammation, and more epithelial injury, which, if unchecked, may culminate

in intestinal perforation, fulminant sepsis and death (Fig. 1). Although this scenario is broadly accepted, the specific details at each step remain largely unknown. In this review we will examine the roles of inflammatory NO and opportunistic pathogens in the pathogenesis of NEC.

2. Role of nitric oxide

Nitric oxide is known to play a paradoxical role in intestinal physiology. NO is produced from arginine in a reaction catalyzed in the intestine mainly by two NO synthases (NOS), endothelial NOS or eNOS, and inducible NOS, or iNOS. eNOS is constitutively expressed in the intestinal microcapillaries at low levels and is responsible for the low background levels of NO. Low levels of NO regulate vascular tone and mucosal blood flow in a cyclic GMP- and neuron-dependent manner. Constitutively low production of NO is also required for the maintenance of mucosal capillaries [15] and mucosal homeostasis [16]. NO may protect from oxidative stress by scavenging oxygen radicals [17]. eNOS-derived NO promotes leukocyte adhesion to the endothelium, facilitating leukocyte recruitment [18]. These are all beneficial effects. iNOS is upregulated during inflammation and is responsible for high levels of NO, which dramatically increase blood flow by dilating the capillaries. Our lab was the first to demonstrate that sustained upregulation of iNOS in the intestine caused by LPS, can lead to gut barrier failure [12]. Indeed, high levels of NO seen during inflammation exert detrimental effects on the gut barrier leading to increased bacterial translocation [19,20], impaired mitochondrial function [21] and epithelial restitution [22], as well as decreased leukocyte recruitment by the endothelium [23,24]. NO readily reacts with the superoxide ion to form peroxynitrite, a reactive oxygen and nitrogen species that is highly toxic to epithelial cells [25]. Peroxynitrite may damage the epithelium in multiple ways. It may induce enterocyte apoptosis and inhibit epithelial restitution processes including enterocyte proliferation and migration [26–28]. We were the first to demonstrate increased expression of iNOS mRNA and protein in the intestinal mucosa during NEC [29]. iNOS upregulation is accompanied by increased rate of enterocyte apoptosis. The latter co-localizes with iNOS expression and nitrotyrosine immunoreactivity, a molecular footprint of peroxynitrite, in enterocytes. iNOS expression decreased at the time of stoma closure when the acute inflammation had subsided [25]. Thus, on the one hand NO plays an important role in intestinal homeostasis, but on the other hand, high levels of NO contribute to the epithelial damage seen in NEC.

3. Regulation of iNOS expression by bacteria

Regulation of the iNOS gene in response to bacteria and their pathogen-associated molecular patterns has been most extensively studied in macrophages and neutrophils, the specialized cells of the innate immune system, in the C57Bl/6 strain of mice. In these cells, iNOS is induced by pathogenic bacteria such as *Listeria monocytogenes*, or bacterial components such as LPS. This induction involves signaling via pattern recognition Toll-like receptors, activation of the transcription factor nuclear factor kappa B (NF-kB), binding of NF-kB to the iNOS promoter, and transcriptional induction of the iNOS gene [30,31]. Toll-like receptor ligands alone cause only moderate induction of iNOS in macrophages; full-scale induction requires costimulation with type I or type II interferons [32]. These interferons are produced by natural killer cells and T cells in response to stimulation with Toll-like receptor ligands. Interferons act via their cognate receptors on macrophages to activate signal transduction activators of transcription (STATs) and interferon response factors (IRFs), which form transcription activation complexes on the iNOS promoter at distinct locations from NF-kB-binding sites [32]. Thus, efficient induction of iNOS in mouse macrophages requires synergy between macrophages and T cells, between Toll-like receptor ligands and interferons, and between distinct transcription activation complexes acting on the iNOS promoter.
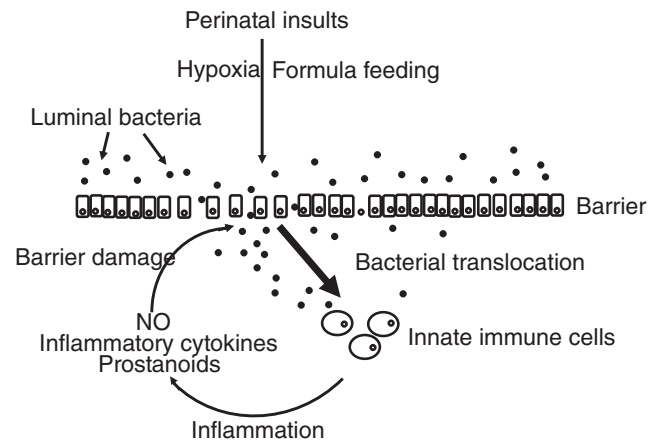

Fig. 1. Pathogenesis of NEC. Perinatal insults of prematurity including hypoxia, formula feeding, and colonization with opportunistic pathogens compromise the gut barrier, leading to bacterial translocation. After crossing the barrier, bacteria engage the innate immune cells of the lamina propria and elicit an inflammatory response by stimulating production of nitric oxide, inflammatory cytokines, and inflammatory prostanoids. These inflammatory factors further compromise the gut barrier, increasing bacterial translocation and exacerbating inflammation. A vicious circle of inflammation-inflicted barrier damage and bacterial translocation culminates in intestinal necrosis.

Findings in C57Bl/6 mice may not be universally applicable. Macrophages from BALB/c mice produce much less iNOS upon stimulation with LPS and interferon compared to those from C57Bl/6 mice [33]. Numerous studies failed to detect iNOS induction in human macrophages/monocytes of various tissue origin upon stimulation ex vivo, although expression of iNOS was sometimes detected in macrophages isolated from patients with a variety of inflammatory disorders [34]. Similarly, little or no iNOS induction was observed in rat, bovine, or porcine macrophages [35,36]. It has been suggested that stimuli other than LPS/interferon are required to induce iNOS in macrophages of species other than mice [37]. Indeed, induction of iNOS by *Leischmania* is strongly potentiated by IL-1β [38]. Differences in organization of the iNOS promoter between mice and other vertebrates [39] may reflect the roles of alternative transcription factors and signaling pathways.

Another possibility is that cell types other than macrophages could be sources of NO during NEC. iNOS expression is not confined to macrophages, it has been detected in a broad array of tissues and cell types [40]. Of interest to mucosal pathophysiology, iNOS is expressed in intestinal smooth muscle cells [41,42], in endothelial cells of the intestinal capillaries [23,24], and in enterocytes [43,44]. Moreover, the epithelium is the major site of iNOS expression and NO production during intestinal inflammatory disorders [45,46]. We have shown that experimental NEC induced by the opportunistic pathogen *Cronobacter muytjensii* in rats depends on the upregulation of intestinal iNOS [47]. Although the intestinal epithelium may be a major source of NO in the pathogenesis of NEC, the molecular mechanisms of mucosal iNOS induction in this disease remain unclear.

4. Microbiota in NEC

Current evidence suggests that bacterial colonization of the gut is a key prerequisite for the pathogenesis of NEC. Indeed, germ-free rats [48] or mice [49] do not develop NEC. Over the last 20 years there have been numerous attempts to identify specific bacteria that contribute to the development of NEC. These studies compared bacterial populations found in patients with NEC to those found in healthy individuals. Although some studies reported an association between the prevalence of proteobacteria [50–55], clostridia [56], staphylococci [57], or decreased bacterial species diversity [53,55] with the development of NEC, other studies failed to corroborate these findings. The same bacterial species that were found in NEC patients were also found in healthy

individuals [58–62]. These observations led some to conclude that NEC is not caused by any specific pathogen [63]. Thus, the question of whether NEC is associated with specific bacteria or specific patterns of microbiota remains unanswered.

5. Characteristics of early microbiota

Although studies of the neonatal gut microbiome failed to establish colonization patterns pertinent to NEC, they nevertheless revealed important differences between adult and early postnatal bacterial populations. Adult populations are very complex and feature more than one thousand species; they are relatively uniform, with the proportion of different groups and total bacterial load varying little among individuals. In addition, they are stable, with different groups of bacteria displaying little change over time [64–67]. By contrast, early postnatal populations are very simple, featuring typically 1–5 species; they are highly diverse with regard to total bacterial load and species composition, and they are transient, with different groups of bacteria rapidly succeeding one another [6]. Fig. 2 presents an example of interindividual diversity of the neonatal microbiota. In a litter of 4 day-old formula-fed rats, the total bacterial load and composition within each animal were quite different. The total bacterial load in animal 9 is almost 4 orders of magnitude higher than that in animal 4. In animals 1, 4, and 9 the predominant colonizer is *Shigella* spp., while in animals 2, 3, 5, 6, and 7 it is the bacterium S1-91. *Enterococcus faecalis* is the predominant colonizer in animal 8. Thus, even among littermates kept in the same environment, the initial bacterial population may be quite dissimilar (Bell BA, Ford HR, unpublished).

From the results of multiple clinical studies, the most common first colonizers are not surprisingly normal human commensals from various bodily compartments, such as intestinal *Escherichia coli* and *Enterococcus*, skin *Staphylococcus*, oral cavity *Streptococcus*, or vaginal *Lactobacillus*. Less common commensals, including intestinal *Enterobacter*, *Shigella*, and *Salmonella*, or respiratory tract commensals such as *Haemophilus*, *Klebsiella*, or *Pseudomonas* are also found, although less frequently. Surprisingly, many species of bacteria from the environment – bacteria from domestic animals, plants, soil, or water – can also act as predominant first colonizers, although colonization with these species is relatively infrequent.

Thus far, more than a hundred bacterial species have been identified as first colonizers, although this might be just the tip of the iceberg. It is apparent that a very broad array of bacteria is capable of colonizing the neonate's intestine [6].

6. Hospital outbreaks of NEC

Although some believe that NEC is not caused by specific pathogens, the extensive body of evidence regarding hospital outbreaks of NEC argues that NEC may have an infectious etiology. NEC is not a very common disease, therefore whenever a cluster of similar cases occurs in a hospital environment, it is assumed that a common pathogen is involved. Although a causal relationship between a given pathogen and NEC has been rigorously proven only in a few cases, the putative pathogens were tentatively identified or suspected based on their presence in patients, but not in healthy subjects (Table 1). The conclusion from various hospital outbreaks is that the relationship between NEC and putative microbial pathogens is not as stringent as, for example, between cholera and *Vibrio cholera*. In fact, NEC can be caused by a broad variety of pathogens of bacterial, viral, or fungal origin [6,68].

7. Identification of NEC pathogens

Clinical and experimental studies of NEC pathogens have been largely inconclusive because the ability of pathogenic microbes to cause NEC is rarely proven with rigor. To unambiguously identify a pathogen one has to satisfy Koch's three postulates. First, the microbe must be isolated from a diseased individual and grown in pure culture. Second, the ability of the isolated microbial strain to cause the disease must be proven by introduction into a healthy individual. Third, the microbe must be reisolated from the inoculated, diseased experimental host and confirmed to be identical to the originally isolated strain. All three postulates can be satisfied using animal models of NEC. So far, only a few strains of bacteria have been proven in this rigorous manner to be true NEC pathogens. They include strains of *Clostridium butyricum* and *Clostridium perfringens* [69], *Helicobacter hepaticus* MU 94-1 [70], and *C. muytjensii* 51329 [71].
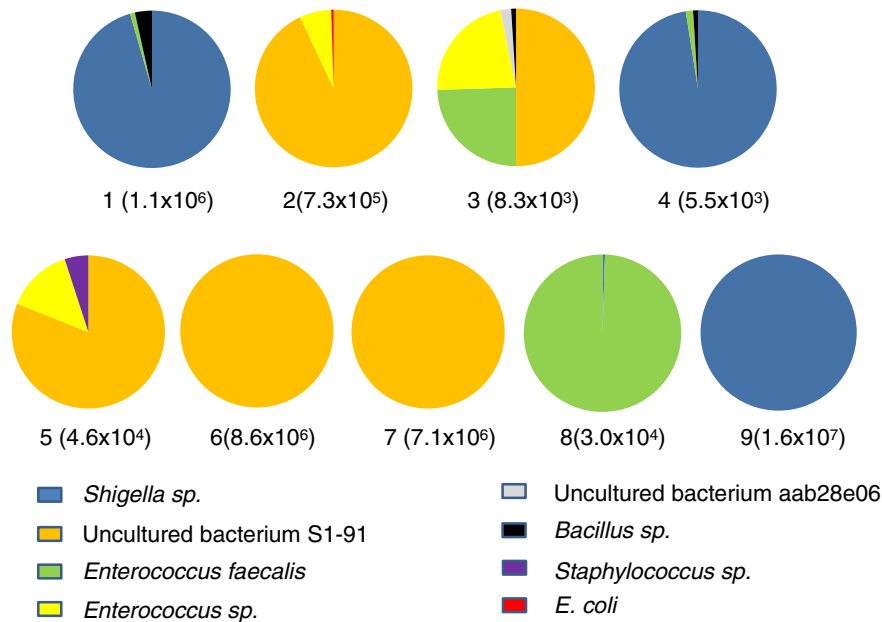

Fig. 2. An example of diverse early intestinal microbiota in a litter of rats. The neonates were separated at birth and formula-fed for 4 days. Equal samples of the small intestine were serially diluted and plated on blood agar. Following 2-day incubation at 37 °C, the emerging bacterial colonies were classified and enumerated. Bacterial species were determined by sequencing a variable region of the 16S rRNA gene. Each pie chart represents an individual littermate. Total loads of bacteria in cfu/ml are given below each pie chart.

Table 1
Infectious agents associated with hospital cases of NEC.

Gram⁻ bacteria	Gram⁺ bacteria	Viruses	Fungi
Cronobacter muytjensii	*Enterococcus faecalis*	Cytomegalovirus	*Candida* spp.
Pseudomonas aeruginosa	*Staphylococcus aureus*	Rotavirus	*Aspergillus* spp.
Klebsiella pneumoniae	*Staphylococcus epidermidis*	Adenovirus	*Absidia* spp.
Enterobacter spp.	*Clostridium difficile*	Coronavirus	*Mucor* spp.
Escherichia coli O157:H7	*Clostridium perfringens*	Echovirus 7	
Salmonella spp.	*Clostridium butyricum*	Torovirus	
Shigella spp.		Astrovirus	
		Herpesvirus	

8. Studies in *C. muytjensii*

One of the best studied opportunistic pathogens associated with NEC is *C. muytjensii*, previously known as *Enterobacter sakazakii* or *Cronobacter sakazakii*. This is a common food spoilage bacteria frequently found in dairy products, sometimes in baby formula. *C. muytjensii* has been identified as an opportunistic pathogen associated with multiple hospital outbreaks of meningitis, sepsis, and NEC in preterm neonates [72]. When live *C. muytjensii* 51329 was introduced in formula that was gavage-fed to neonatal rats, NEC scores significantly increased in these animals compared to neonates fed formula alone. One could argue that introduction of any bacteria regardless of the species could have the same effect. However, introduction of the same number of colony-forming units of a commensal strain of *E. coli* did not cause any appreciable change in histology scores. Therefore, the effect appears to depend on bacterial species [71]. Interestingly, *C. muytjensii* 51329 was found attached to the outer surface of the villus epithelium, suggesting that these bacteria are capable of binding enterocytes. *C. muytjensii* 51329 also dramatically increased the levels of enterocyte apoptosis. In the absence of bacteria, apoptosis was largely confined to the villus tips where the enterocytes are continuously shed. However, in the presence of *C. muytjensii* 51329, the apoptotic events were evenly spread over the surface of the villi [71]. Thus, *C. muytjensii* 51329 might damage the epithelium by attaching to enterocytes and causing enterocyte apoptosis. The fact that *C. muytjensii* 51329 significantly increases NEC scores satisfies the second Koch postulate – that introduction of a cultured microbe into a healthy subject should cause the disease. The intestinal titers of *C. muytjensii* 51329 increased more than 5 logs in 3 days following introduction of this strain to neonatal rats. Thus, the same strain was recovered from NEC rats following the introduction of *C. muytjensii* 51329, which satisfies the third Koch postulate. These data also demonstrate that *C. muytjensii* 51329 is capable of colonizing the GI tract of the neonate [73].

To determine whether the pathogenic properties of *C. muytjensii* are strain-specific, multiple independent clinical, environmental, and food origin isolates of this species were examined for their ability to bind to intestinal epithelial cells. The strains that efficiently bound to enterocytes, caused enterocyte apoptosis and disrupted the epithelial barrier were exclusively clinical isolates. Therefore, the pathogenic affinity of *C. muytjensii* for the intestinal epithelium is strain-specific rather than species-specific [74]. This finding may shed some light on the limited success of microbiome studies in NEC. Most of these studies identified bacteria by species, whereas virulence within a species may be strain-specific. Detailed whole genome analysis and microbiological studies may be necessary to distinguish between pathogenic and nonpathogenic strains of the same species.

9. Conclusions and perspective

The pathogenesis of NEC is still incompletely understood. Nitric oxide and its oxidation intermediates play a prominent role in the epithelial barrier damage by increasing enterocyte apoptosis, decreasing enterocyte proliferation and impairing enterocyte migration. iNOS, the enzyme responsible for the dramatic elevation of NO during intestinal inflammation, is regulated by inflammatory factors and pathogens via a complex network of signaling cascades including Toll-like and cytokine receptors, intracellular signaling mediators, and transcription factors. Although much is known about iNOS gene regulation in mouse macrophages, this information may be of limited value for understanding iNOS regulatory networks in other cell types and species. Particularly, iNOS regulation in the intestine remains largely unknown, and efforts in this direction are necessary for the design of iNOS-targeted NEC therapies.

The key role of bacteria in the pathogenesis of NEC is universally recognized. However, whether NEC is caused by specific bacteria remains a matter of debate. Early intestinal microbiomes are highly diverse, simple, and fluid, as opposed to adult populations, which are relatively uniform, complex, and stable. A broad range of bacteria may act as predominant first colonizers. Total microbial load and species composition in early microbiomes may be, to a large extent, a matter of chance. Some of the first colonizers may act as opportunistic pathogens, while others may be innocuous or even protective. Proteobacteria, a group of Gram-negative bacteria that includes such potentially pathogenic genera as *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, and *Pseudomonas*, are often found in NEC patients. Studies in *C. muytjensii* clearly demonstrated that the ability to act as an NEC pathogen is the property of individual strains rather than a species as a whole. Understanding the roles of bacteria in the pathogenesis of NEC will require microbiological experiments and genome-wide analysis of virulence.

Acknowledgments

References

[1] Dominguez KM, Moss RL. Necrotizing enterocolitis. Clin Perinatol 2012;39:387–401.
[2] Yee WH, Soraisham AS, Shah VS, et al. Incidence and timing of presentation of necrotizing enterocolitis in preterm infants. Pediatrics 2012;129:e298–304.
[3] Patel RM, Kandefer S, Walsh MC, et al. Causes and timing of death in extremely premature infants from 2000 through 2011. N Engl J Med 2015;372:331–40.
[4] Petrosyan M, Guner YS, Williams M, et al. Current concepts regarding the pathogenesis of necrotizing enterocolitis. Pediatr Surg Int 2009;25:309–18.
[5] Carlisle EM, Morowitz MJ. The intestinal microbiome and necrotizing enterocolitis. Curr Opin Pediatr 2013;25:382–7.
[6] Grishin A, Papillon S, Bell B, et al. The role of the intestinal microbiota in the pathogenesis of necrotizing enterocolitis. Semin Pediatr Surg 2013;22:69–75.
[7] Carlisle EM, Poroyko V, Caplan MS, et al. Gram negative bacteria are associated with the early stages of necrotizing enterocolitis. PLoS One 2011;6:e18084.
[8] Chokshi NK, Guner YS, Hunter CJ, et al. The role of nitric oxide in intestinal epithelial injury and restitution in neonatal necrotizing enterocolitis. Semin Perinatol 2008;32:92–9.
[9] Ford HR, Avanoglu A, Boechat PR, et al. The microenvironment influences the pattern of bacterial translocation in formula-fed neonates. J Pediatr Surg 1996;31:486–9.
[10] Hackam DJ, Afrazi A, Good M, et al. Innate immune signaling in the pathogenesis of necrotizing enterocolitis. Clin Dev Immunol 2013;2013:475415.
[11] Lugo B, Ford HR, Grishin A. Molecular signaling in necrotizing enterocolitis: regulation of intestinal COX-2 expression. J Pediatr Surg 2007;42:1165–71.
[12] Sorrells DL, Friend C, Koltuksuz U, et al. Inhibition of nitric oxide with aminoguanidine reduces bacterial translocation after endotoxin challenge in vivo. Arch Surg 1996;131:1155–63.
[13] Anand RJ, Leaphart CL, Mollen KP, et al. The role of the intestinal barrier in the pathogenesis of necrotizing enterocolitis. Shock 2007;27:124–33.

[14] Kandasamy J, Huda S, Ambalavanan N, et al. Inflammatory signals that regulate intestinal epithelial renewal, differentiation, migration and cell death: Implications for necrotizing enterocolitis. Pathophysiology 2014;21:67–80.

[15] Kolb E. Current knowledge on the formation of nitric oxide in endothelial cells of blood vessels, in nerve cells and macrophages as well as its significance in vascular dilatation, information transmission and damage of tumor cells. Z Gesamte Inn Med 1991;46:431–6.

[16] Vallance BA, Dijkstra G, Qiu B, et al. Relative contributions of NOS isoforms during experimental colitis: endothelial-derived NOS maintains mucosal integrity. Am J Physiol Gastrointest Liver Physiol 2004;287:G865–74.

[17] Krauss H, Sosnowski P, Biczysko M, et al. Effects of L-arginine and NG-nitro L-arginine methyl ester (L-NAME) on ischemia/reperfusion injury of skeletal muscle, small and large intestines. Chin J Physiol 2011;54:7–18.

[18] Hierholzer C, Kalff JC, Billiar TR, et al. Induced nitric oxide promotes intestinal inflammation following hemorrhagic shock. Am J Physiol Gastrointest Liver Physiol 2004;286:G225–33.

[19] Du Plessis J, Vanheel H, Janssen CE, et al. Activated intestinal macrophages in patients with cirrhosis release NO and IL-6 that may disrupt intestinal barrier function. J Hepatol 2013;58:1125–32.

[20] Hackam DJ. Danger at the doorstep: regulation of bacterial translocation across the intestinal barrier by nitric oxide. Crit Care Med 2011;39:2189–90.

[21] Erusalimsky JD, Moncada S. Nitric oxide and mitochondrial signaling: from physiology to pathophysiology. Arterioscler Thromb Vasc Biol 2007;27:2524–31.

[22] Cetin S, Leaphart CL, Li J, et al. Nitric oxide inhibits enterocyte migration through activation of RhoA-GTPase in a SHP-2-dependent manner. Am J Physiol Gastrointest Liver Physiol 2007;292:G1347–58.

[23] Benjamim CF, Silva JS, Fortes ZB, et al. Inhibition of leukocyte rolling by nitric oxide during sepsis leads to reduced migration of active microbicidal neutrophils. Infect Immun 2002;70:3602–10.

[24] Binion DG, Rafiee P, Ramanujam KS, et al. Deficient iNOS in inflammatory bowel disease intestinal microvascular endothelial cells results in increased leukocyte adhesion. Free Radic Biol Med 2000;29:881–8.

[25] Ford HR. Mechanism of nitric oxide-mediated intestinal barrier failure: insight into the pathogenesis of necrotizing enterocolitis. J Pediatr Surg 2006;41:294–9.

[26] Guner YS, Ochoa CJ, Wang J, et al. Peroxynitrite-induced p38 MAPK pro-apoptotic signaling in enterocytes. Biochem Biophys Res Commun 2009;384:221–5.

[27] Potoka DA, Upperman JS, Zhang XR, et al. Peroxynitrite inhibits enterocyte proliferation and modulates Src kinase activity in vitro. Am J Physiol Gastrointest Liver Physiol 2003;285:G861–9.

[28] Wendel M, Heller AR. Mitochondrial function and dysfunction in sepsis. Wien Med Wochenschr 2010;160:118–23.

[29] Nadler EP, Dickinson E, Knisely A, et al. Expression of inducible nitric oxide synthase and interleukin-12 in experimental necrotizing enterocolitis. J Surg Res 2012;92:71–7.

[30] Kleinert H, Schwarz PM, Forstermann U. Regulation of the expression of inducible nitric oxide synthase. Biol Chem 2003;384:1343–64.

[31] Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem 1994;269:4705–8.

[32] Farlik M, Reutterer B, Schindler C, et al. Nonconventional initiation complex assembly by STAT and NF-kappaB transcription factors regulates nitric oxide synthase expression. Immunity 2010;33:25–34.

[33] Mills CD, Kincaid K, Alt JM, et al. M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol 2000;164:6166–73.

[34] Thomas AC, Mattila JT. "Of mice and men": arginine metabolism in macrophages. Front Immunol 2014;5:479.

[35] Kapetanovic R, Fairbairn L, Beraldi D, et al. Pig bone marrow-derived macrophages resemble human macrophages in their response to bacterial lipopolysaccharide. J Immunol 2012;188:3382–94.

[36] Zelnickova P, Matiasovic J, Pavlova B, et al. Quantitative nitric oxide production by rat, bovine and porcine macrophages. Nitric Oxide 2008;19:36–41.

[37] Weinberg JB. Nitric oxide production and nitric oxide synthase type 2 expression by human mononuclear phagocytes: a review. Mol Med 1998;4:557–91.

[38] Lima-Junior DS, Costa DL, Carregaro V, et al. Inflammasome-derived IL-1beta production induces nitric oxide-mediated resistance to Leishmania. Nat Med 2013;19:909–15.

[39] Zhang X, Laubach VE, Alley EW, et al. Transcriptional basis for hyporesponsiveness of the human inducible nitric oxide synthase gene to lipopolysaccharide/interferon-gamma. J Leukoc Biol 1996;59:575–85.

[40] Mattila JT, Thomas AC. Nitric oxide synthase: non-canonical expression patterns. Front Immunol 2014;5:478.

[41] Mahavadi S, Nalli AD, Kumar DP, et al. Cytokine-induced iNOS and ERK1/2 inhibit adenylyl cyclase type 5/6 activity and stimulate phosphodiesterase 4D5 activity in intestinal longitudinal smooth muscle. Am J Physiol Cell Physiol 2014;307:C402–11.

[42] Wu J, Chitapanarux T, Chen Y, et al. Intestinal myofibroblasts produce nitric oxide in response to combinatorial cytokine stimulation. J Cell Physiol 2013;228:572–80.

[43] Meyerhoff RR, Nighot PK, Ali RA, et al. Characterization of turkey inducible nitric oxide synthase and identification of its expression in the intestinal epithelium following astrovirus infection. Comp Immunol Microbiol Infect Dis 2012;35:63–9.

[44] Nordone SK, Gookin JL. Lymphocytes and not IFN-gamma mediate expression of iNOS by intestinal epithelium in murine cryptosporidiosis. Parasitol Res 2010;106:1507–11.

[45] Kolios G, Rooney N, Murphy CT, et al. Expression of inducible nitric oxide synthase activity in human colon epithelial cells: modulation by T lymphocyte derived cytokines. Gut 1998;43:56–63.

[46] Singer II, Kawka DW, Scott S, et al. Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. Gastroenterology 1996;111:871–85.

[47] Hunter CJ, Williams M, Petrosyan M, et al. Lactobacillus bulgaricus prevents intestinal epithelial cell injury caused by Enterobacter sakazakii-induced nitric oxide both in vitro and in the newborn rat model of necrotizing enterocolitis. Infect Immun 2009;77:1031–43.

[48] Musemeche CA, Kosloske AM, Bartow SA, et al. Comparative effects of ischemia, bacteria, and substrate on the pathogenesis of intestinal necrosis. J Pediatr Surg 1986;21:536–8.

[49] 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.

[50] Carlisle EM, Poroyko V, Caplan MS, et al. Gram negative bacteria are associated with the early stages of necrotizing enterocolitis. PLoS One 2011;6:e18084.

[51] Jenke AC, Postberg J, Mariel B, et al. S100A12 and hBD2 correlate with the composition of the fecal microflora in ELBW infants and expansion of E. coli is associated with NEC. Biomed Res Int 2013;2013:150372.

[52] Mai V, Young CM, Ukhanova M, et al. Fecal microbiota in premature infants prior to necrotizing enterocolitis. PLoS One 2011;6:e20647.

[53] Stewart CJ, Marrs EC, Nelson A, et al. Development of the preterm gut microbiome in twins at risk of necrotising enterocolitis and sepsis. PLoS One 2013;8:e73465.

[54] Torrazza RM, Ukhanova M, Wang X, et al. Intestinal microbial ecology and environmental factors affecting necrotizing enterocolitis. PLoS One 2013;8:e83304.

[55] Wang Y, Hoenig JD, Malin KJ, et al. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. ISME J 2009;3:944–54.

[56] Cilieborg MS, Boye M, Molbak L, et al. Preterm birth and necrotizing enterocolitis alter gut colonization in pigs. Pediatr Res 2011;69:10–6.

[57] Stewart CJ, Marrs EC, Magorrian S, et al. The preterm gut microbiota: changes associated with necrotizing enterocolitis and infection. Acta Paediatr 2012;101:1121–7.

[58] Brower-Sinning R, Zhong D, Good M, et al. Mucosa-associated bacterial diversity in necrotizing enterocolitis. PLoS One 2014;9:e105046.

[59] Millar MR, Linton CJ, Cade A, et al. Application of 16S rRNA gene PCR to study bowel flora of preterm infants with and without necrotizing enterocolitis. J Clin Microbiol 1996;34:2506–10.

[60] Normann E, Fahlen A, Engstrand L, et al. Intestinal microbial profiles in extremely preterm infants with and without necrotizing enterocolitis. Acta Paediatr 2013;102:129–36.

[61] Smith B, Bode S, Petersen BL, et al. Community analysis of bacteria colonizing intestinal tissue of neonates with necrotizing enterocolitis. BMC Microbiol 2011;11:73.

[62] Ullrich T, Tang YW, Correa H, et al. Absence of gastrointestinal pathogens in ileum tissue resected for necrotizing enterocolitis. Pediatr Infect Dis J 2012;31:413–4.

[63] Peter CS, Feuerhahn M, Bohnhorst B, et al. Necrotising enterocolitis: is there a relationship to specific pathogens? Eur J Pediatr 1999;158:67–70.

[64] Ohland CL, Jobin C. Microbial activities and intestinal homeostasis: a delicate balance between health and disease. Cell Mol Gastroenterol Hepatol 2015;1:28–40.

[65] Sankar SA, Lagier JC, Pontarotti P, et al. The human gut microbiome, a taxonomic conundrum. Syst Appl Microbiol 2015;38:276–86.

[66] Scott KP, Antoine JM, Midtvedt T, et al. Manipulating the gut microbiota to maintain health and treat disease. Microb Ecol Health Dis 2015;26:25877.

[67] Silva MJ, Carneiro MB, dos Anjos Pultz B, et al. The multifaceted role of commensal microbiota in homeostasis and gastrointestinal diseases. J Immunol Res 2015;2015:321241.

[68] Coggins SA, Wynn JL, Weitkamp JH. Infectious causes of necrotizing enterocolitis. Clin Perinatol 2015;42:133–54.

[69] Butel MJ, Roland N, Hibert A, et al. Clostridial pathogenicity in experimental necrotising enterocolitis in gnotobiotic quails and protective role of bifidobacteria. J Med Microbiol 1998;47:391–9.

[70] Dvorak K, Coursodon-Boyiddle CF, Snarrenberg CL, et al. Helicobacter hepaticus increases intestinal injury in a rat model of necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 2013;305:G585–92.

[71] Hunter CJ, Singamsetty VK, Chokshi NK, et al. Enterobacter sakazakii enhances epithelial cell injury by inducing apoptosis in a rat model of necrotizing enterocolitis. J Infect Dis 2008;198:586–93.

[72] Hunter CJ, Petrosyan M, Ford HR, et al. Enterobacter sakazakii: an emerging pathogen in infants and neonates. Surg Infect (Larchmt) 2008;9:533–9.

[73] Emami CN, Mittal R, Wang L, et al. Role of neutrophils and macrophages in the pathogenesis of necrotizing enterocolitis caused by Cronobacter sakazakii. J Surg Res 2012;172:18–28.

[74] Liu Q, Mittal R, Emami CN, et al. Human isolates of Cronobacter sakazakii bind efficiently to intestinal epithelial cells in vitro to induce monolayer permeability and apoptosis. J Surg Res 2012;176:437–47.