Necrotizing Enterocolitis in Preterm Infants is Related to Enteral Feeding, But the Mechanisms Remain Uncertain and Have Changed Over Time

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Abstract Although the pathophysiology of necrotizing enterocolitis (NEC) remains incompletely understood, recent evidence supports the important role of aberrant bacterial colonization and activated pro-inflammatory signaling in the high-risk premature infant (Neu and Walker, *N Engl J Med* 364:255–264, 2011). Nonetheless, most clinicians surmise that enteral feeding significantly contributes to the initiation of intestinal injury in NEC, and multiple studies have provided insight into the impact of feeding on intestinal health and homeostasis (Meinzen-Derr et al., *J Perinatol* 29:57–62, 2009). In this report, the impact of feeding on NEC will be reviewed, and it is suggested that enteral feedings can have a protective role in the disease, while conversely in other situations, enteral feedings might initiate changes contributing to this devastating condition. It is concluded that our understanding of enteral feeding and NEC has changed over time, and new insights continue to provide clues to the pathogenesis of this challenging condition.

Keywords Necrotizing enterocolitis · Microbiome · Cow's milk protein allergy · Inflammation · Osmolar stress

Introduction

For many years, the pathophysiology of NEC was known to include four key risk factors: prematurity, bacterial colonization, intestinal ischemia, and enteral feeding [1],

[2]. While prematurity may be the most consistent risk factor, recent information suggests that an altered intestinal microbiome and an activated pro-inflammatory signaling response contribute to the development of intestinal necrosis and NEC in a compromised immune-deficient host [3, 4]. In this context, the importance of enteral feeding in premature infants has been extensively studied, yet there remains conflicting data on the impact of feeding on the development of the disease. In some instances, feedings appear to protect against the initiation of NEC, and in others, feedings clearly promote changes that may contribute to the disease. In this review, we will discuss the current theory regarding the pathophysiology of NEC, consider pathomechanisms whereby feedings might initiate the disease, and then review mechanisms in which feeding can protect against intestinal injury and NEC. Finally, we will summarize the balance of these effects, and describe how our understanding of these interactions has changed over the last several years.

Pathophysiology of NEC

Despite the fact that NEC remains a poorly understood disease, there is accumulating evidence to suggest that the intestinal microbiome contributes to the initiation of intestinal necrosis (see Fig. 1). Previous studies using culture-based methodology and more recent investigation using molecular approaches have shown that the microbiome of the preterm gut differs significantly from that of the full-term infant [3, 5, 6]. In these studies, the preterm infant has much less bacterial diversity, many fewer symbiotic organisms including *Bifidobacteria* and *Lactobacillus* species, and increased numbers of pathogenic organisms. Furthermore, recent studies have shown that preterm

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Fig. 1 The pathophysiology of NEC

infants who go on to develop NEC have different microbial patterns compared to preterm infant controls, with studies identifying decreased amounts of bacterial diversity in the NEC patients, and a possible ‘bloom’ of pathogenic organisms (particularly *Proteobacteria* species) prior to the diagnosis of the disease.

Many factors might contribute to the altered microbiome observed in premature infants, and include invasive catheters, lack of skin-to-skin interaction with their mothers, the abnormal neonatal intensive care unit environment, delayed enteral feeding, delayed oral feeding and contact with the breast, and the abnormal host immune responses in the immunodeficient preterm gut. Once an abnormal intestinal microbiome is established, and without enough anti-microbial and anti-inflammatory biofactors from early lack of adequate human milk feeding, microbial ligands can bind to human toll-like receptors (TLRs), particularly TLR2 (for gram positive organisms) and TLR4 (for gram negative lipopolysaccharides), and initiate an aggressive pro-inflammatory response [7]. This inflammatory response has been suggested to play an important role in the initiation and development of NEC, and in preterm infants (and young animals) perpetuates in part due to inadequate anti-inflammatory protection [8]. Multiple deficiencies in anti-inflammatory activity have been identified, including decreased endogenous platelet activating factor-acetylhydrolase (PAF-AH) activity leading to prolonged exposure to the potent phospholipid pro-inflammatory compound PAF [9•], un-suppressed activation of the transcription

Table 1 Enteral feedings: how might they promote the development of NEC?

1. Alteration of microbiome
2. Alterations in intestinal blood flow
3. Hyperosmolar stress
4. Cow’s milk protein
5. Stimulation of pro-inflammatory responses

factor nuclear factor kappa beta ($\text{NF}\kappa\text{B}$) due to down-regulated I kappa beta ($\text{I}\kappa\text{B}$) [10], and increased interleukin-8 (IL-8) signaling [11]. Following the prolonged activation of a local pro-inflammatory response, gut hypoperfusion ensues, and the resulting pathologic events culminate in the final common pathway of NEC.

Role of Feeding on the Pathophysiology of NEC

Many studies have investigated the contributions of feeding to the pathophysiology of NEC. While the data are difficult to interpret, some have suggested that feedings increase the risk of disease, while others seem to indicate that feedings protect against NEC. Mechanisms have been identified in which feedings increase the risk of disease (see Table 1), and include (1) perturbations in the microbiome, (2) alterations in intestinal blood flow, (3) hyperosmolar stress, (4) cow’s milk protein effects, and (5) activation of pro-inflammatory signaling responses.

Table 2 Enteral feeding: how might they decrease the risk for NEC?

1. Human milk bio-active factors (leukocytes, growth factors, IgA, lactoferrin, oligosaccharides, etc.)
2. Prevention of villous atrophy and apoptosis
3. Stimulation of peristalsis
4. Promotion of commensal bacterial colonization
5. Modification of untoward immune responses

Additional mechanisms have been described whereby feedings help protect against NEC (see Table 2), such as (1) the provision of human milk bioactive factors (e.g., leukocytes, immunoglobulins, polyunsaturated fatty acids, lactoferrin, transforming growth factor beta, PAF-AH, etc.), (2) prevention of villous atrophy and apoptosis, (3) stimulation of peristalsis, (4) promotion of commensal bacterial colonization, and (5) modification of host immune responses. In this report, data will be reviewed that alter key mechanisms in this NEC cascade. The effects on the microbiome as related to human milk factors will not be discussed.

Effect of Enteral Feedings on Intestinal Blood Flow

Intestinal blood flow perturbations have been associated with the development of NEC in multiple animal and human studies [12]. It has been shown that intestinal perfusion is altered in neonates, and following circulatory stress these changes are more pronounced. Multiple chemical mediators regulate intestinal blood flow, and nitric oxide and endothelin have been shown to play an important role in neonatal perfusion abnormalities [13, 14]. Less is known about the impact of enteral feeding on neonatal intestinal blood flow and NEC. In one report, Szabo et al. showed that in hypoxic piglets, appropriate increases in flow following feedings were delayed and suppressed compared to control animals, suggesting that this abnormal circulation could contribute to NEC following enteral feedings [15]. While it remains difficult to clearly elucidate whether feedings might contribute to NEC due to altered gut blood flow, there was a provocative study published 25 years ago by Cassady et al. that evaluated the impact of early surgical ligation for the PDA in babies born weighing less than 1,000 g [16]. In a randomized, prospective trial, the authors showed that patients with early (first day of life) PDA ligation developed less NEC than patients whose PDA was treated with standard care (early indocin with surgical ligation after 1 week if PDA still present). Of interest, patients who had enteral feedings begun in the first 14 days of life had the majority of NEC compared to minimal incidence of disease in patients that

began feedings later than 14 days. While this suggests that enteral feedings may contribute to altered intestinal blood flow and NEC in high-risk premature infants, this issue is difficult to prove, and remains somewhat controversial.

Does Hyperosmolar Stress From Enteral Feedings Contribute to NEC?

Many years ago, animal studies were done to suggest that hyperosmolar feedings altered intestinal architecture. Kamada et al. showed that feeding a solution of 600 mOsm led to epithelial necrosis in rat jejunum [17]. Subsequently, Book et al. studied 16 premature infants and compared feedings with routine premie formula versus elemental formula (Pregestimil) [18]. They found a much higher incidence of NEC in those fed Pregestimil vs preterm formula (87 vs 25 %), and suggested that the high osmolality may have contributed to these findings (650 mOsm/L for Pregestimil vs. 350 mOsm/L in preterm formula). More recently several investigators have measured the osmolality of feedings, and described values when a variety of supplements and medications were added to the routine feed. While the osmolality of various formula and milk preparations range from 300 to 400 mOsm/kg H₂O, the addition of diuretics, caffeine, and mineral supplements can increase the mOsm to 1,000–1,200 [19]. Nonetheless, it is surmised that the actual osmolality as measured in vitro using the techniques in these studies is significantly higher than the true osmolality delivered to the distal intestine in vivo, in part due to transport of high osmolar compounds across the intestinal lumen. In summary, it seems plausible that high osmolar feedings can initiate intestinal injury, however, it seems unlikely that this mechanism significantly contributes to the pathogenesis of NEC in premature infants.

Does Cow's Milk Protein Contribute to NEC?

Almost 40 years ago, Powell et al. described the unique effect of cow's milk protein and soy protein on the development of enterocolitis in a 32 week, 1,600 g premature infant [20]. During intervals when this patient was fed these protein components, symptoms of diarrhea and bloody stools were observed, and the diagnosis of NEC was suspected. Nonetheless, the symptoms improved with elemental, non-protein containing formula, and it was concluded that the condition was an allergic enterocolitis associated with cow's milk or soy protein. Many subsequent papers described similar observations, and the condition of food protein-induced enterocolitis syndrome (FPIES) was established, and shown to occur even in

premature infants. Morita et al. recently published observations from their newborn nurseries in Japan, and described a series of premature and full-term infants afflicted by FPIES [21•]. Of note, while most of the patients were fed cow's milk protein preparations, some were exclusively on human milk, and yet the patients had bloody stools and a significant amount of stool eosinophilia.

While there are multiple reports suggesting that cow's milk protein (CMP) can lead to allergic gastrointestinal symptoms and signs, few studies support the hypothesis that CMP can cause full-blown NEC. In one study, it was shown that CMP was associated with T cell helper type I and II cytokine responses in patients with NEC [22]. In this study, peripheral blood mononuclear cells were collected from premature infant controls and those with active NEC, and following β -lactoglobulin stimulation, the patients with NEC had much higher elevations of interferon gamma (IFN- γ) and interleukin 4 (IL-4) compared to preterm controls. Nonetheless, while this association is of interest, there remains no clear evidence that CMP can initiate or cause the development of NEC.

It remains controversial whether CMP components that are in human milk fortifier can be deleterious for the growing premature infant. Based on the Cochrane Review by Kuschel and Harding, after reviewing studies, they concluded that there remains no evidence to implicate CMP-containing fortifier as a contributor to neonatal NEC [23]. In two recent studies, it was suggested that human-milk based fortifier resulted in less NEC compared to CMP-based preparations [24, 25]. Nonetheless, these studies were underpowered to identify a difference in NEC, the incidence of NEC was uncharacteristically high in the CMP cohort, and the CMP-fortified group received much less human milk compared with the human-milk-based fortifier group. Further studies may be needed to clarify the importance of CMP in neonatal NEC, but based on the current evidence, the data do not support the cause-effect relationship between CMP and NEC.

Does Feeding Increase the Risk of NEC by Activating the Inflammatory Response?

There have been multiple reports suggesting that the activation of the pro-inflammatory response with impaired anti-inflammatory down-regulation contributes the development of NEC. Of interest, some data suggest that enteral feedings can initiate this pro-inflammatory response in certain situations. Sheifele and colleagues measured blood endotoxemia in a series of premature infants, and showed that prefeeding, patients had a 13 % incidence of endo-

toxinemia while after feedings were established, the incidence increased to 56 % ($p < 0.001$) [26]. Of interest, 78 % of patients with NEC had measurable endotoxin in the blood, and these data suggest that feeding alone can allow for endotoxin translocation across the intestinal mucosa. It has been clearly shown that endotoxin results in inflammatory signaling by engaging and activating the human toll receptor 4 (TLR4), and following this ligand binding, a complex series of molecular changes occur leading to the production and release of pro-inflammatory cytokines [27]. Therefore, it can be inferred that feeding can activate the inflammatory response, in part by activating and releasing endotoxin and stimulating the TLR4 pathway.

Other studies support the potential activation of the inflammatory response by enteral feeding. Amer and colleagues measured stool levels of platelet activating factor (PAF), a potent endogenous phospholipid mediator that is present in most cells and tissues [28]. PAF has previously been shown to be an important mediator associated with NEC, particularly in rodent studies in which PAF receptor antagonists and the PAF-degrading enzyme PAF-acetylhydrolase reduce the risk of disease [29, 30]. Amer et al. found that stool PAF content increased significantly 3 days following the initiation of enteral feeding compared to pre-feeding levels, and in patients who developed NEC, the stool PAF content was significantly higher. These studies imply that feeding alone can increase intestinal luminal PAF levels, and these increases, at least in animals, have been associated with the development of NEC, with mechanisms including PAF's effect on (1) apoptosis, (2) increased TLR4 expression, (3) mucosal permeability, (4) bacterial translocation, and (5) secondary pro-inflammatory signaling [31•]. Overall, these data support the hypothesis that enteral feeding can contribute to the activation of pro-inflammatory signaling that could predispose the preterm infant for NEC.

Enteral Feedings: How Might They Prevent the Development of NEC?

As discussed above, multiple mechanisms are identified that might alter intestinal homeostasis and host defense, and ultimately protect against NEC. Key mechanisms include the rich supply of bioactive factors in breast milk, and feeding effects that increase intestinal microbial diversity, but since these topics are discussed in detail in accompanying papers, they will not be mentioned here. Additional potential mechanisms include feeding effects on gut atrophy and apoptosis, peristalsis, and immune host defense.

Enteral Feeding Reduces Intestinal Atrophy and Apoptosis

It has been postulated that enteral feeding reduces or prevents intestinal atrophy and apoptosis (programmed cell death) of intestinal epithelial cells, as animals and patients on prolonged total parental nutrition (TPN) without enteral nutrition develop these pathologic changes. In enterally fasted animals on prolonged intravenous TPN, many investigators have shown evidence of mucosal atrophy and blunted villous height. In adult rats with delayed enteral feedings, Aydin et al. identified progressive loss of villous height, loss of villous count, and increasing amounts of epithelial apoptosis with increasing lengths of time spent without enteral feeding [32].

To evaluate whether apoptosis might contribute to NEC, Jilling et al. studied a neonatal rat model of NEC using formula feeding and asphyxia, and showed that 80 % of animals developed apoptosis by 48 h of life, while 47 % had NEC by 72 h. Using a compound that inhibits caspase enzyme activation (a critical, rate limiting enzyme responsible for apoptosis), they showed that apoptosis decreased to 25 % at 48 h and NEC was observed in only 23 % of animals by 72 h of life [33]. These results support that apoptosis plays an important role in the development of NEC, and suggests that feeding can prevent NEC in part by influencing epithelial cell programmed cell death.

Feeding Influences Intestinal Motor Complexes

Peristalsis is an important function of healthy intestine as it propulses nutritional material down the intestinal tract, and prepares waste to be expelled. Without peristalsis, material accumulates, and it is hypothesized that bacteria can aggregate in larger quantities and initiate inflammatory responses. Peristalsis is an intricate process controlled by a rich neuronal network in the intestine that signals thru intestinal motor complexes (IMC). Owens and colleagues studied neonatal dogs and showed that enteral feedings contribute to normal IMC's, and allow for normal peristalsis [34]. Their study demonstrated that enterally-fasted dogs displayed abnormal IMC's and had minimal peristalsis, while animals fed only 10 % of nutritional requirements via the enteral route had improved peristaltic function. Since decreased peristalsis contributes to bacterial overgrowth, which can contribute to pro-inflammatory signaling and NEC, it is postulated that enteral feeds may protect against NEC, in part via the effects on these intestinal motor complexes. Definitive evidence for this mechanism will require additional investigation.

Does Feeding Reduce NEC by Modulating Immune Responses?

Human milk contains multiple bioactive factors that have been discussed elsewhere in this compendium, and these factors all influence the development of immune responses in high-risk premature infants. There are a myriad of studies that identify specific milk components playing important roles in the NEC cascade, and in this section, we will highlight one example—the impact of transforming growth factor beta (TGFβ) on intestinal inflammation and necrosis.

TGFβ is a potent growth factor that is present in human milk in significant quantities, and has been shown to contribute to intestinal maturation and inflammation in a variety of experimental systems [35]. During fetal life, TGFβ is present in amniotic fluid and interaction with TGFβ receptors promote normal intestinal growth and development. In addition, in fetal life, it has been shown that murine intestinal macrophages respond to lipopolysaccharide (LPS) exposure with a significant rise in tumor necrosis factor alpha (TNFα) synthesis and secretion [36]. As these murine macrophages approach full-term, the effect wanes, and post-natally acquired murine macrophages do not respond to LPS with a rise in TNFα production, a process known as inflammatory anergy or tolerance. Studies using cell culture methodology by Smythies and colleagues in 2005 showed that anti-TGFβ antibodies returned LPS-induced TNF production to control values by blocking TGFβ effects that were present in the extracellular matrix accounting for this anergy [37]. These studies suggested that TGFβ downregulated this LPS-induced TNF response, and they and others have shown that this effect likely involved the down-regulation of human toll-like receptor 4 (TLR4) expression on intestinal macrophages. Finally, Maheshwari et al. demonstrated that TGFβ supplementation reduced the risk of NEC in a neonatal mouse model of disease, suggesting that TGFβ and macrophages play a role in the pathophysiology of feeding-associated NEC [36].

Summary

In summary, enteral feedings in premature infants are critical in promoting normal growth and development. Nonetheless, ancient dogma suggested that these feedings put the preterm infant at increased risk for NEC, and that clinicians should feed very cautiously with delayed initiation rates and small volume increases. Over the years, there have been many experimental studies supporting the notion that feedings could promote the development of NEC, and a variety of mechanisms have been suggested consistent

with those findings. Most of these implied mechanisms remain speculative, as there is little confirmatory evidence to support the role of cow's milk protein, hyperosmolar feedings, and perturbed microbiome development as a direct association with enteral feedings. More recently, several mechanisms have been described whereby enteral feedings could reduce the risk for NEC. Although human milk bioactive factors are clearly superior to neonatal formula preparations, particularly on stimulating protective immune responses, enteral feedings in general may reduce NEC by influencing peristalsis, atrophy and apoptosis, and even the development of the intestinal microbiome. On balance, it seems that enteral feedings (particularly of human milk) may reduce the overall risk for NEC, but these should be given judiciously, as feeding regimens and volume changes could contribute to altered intestinal responses. More studies will be required to confirm these associations, and provide a clearer framework for feeding premature infants, an essential component to care for these fragile infants.

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