Transition from parenteral to enteral nutrition induces immediate diet-dependent gut histological and immunological responses in preterm neonates

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Running head: Temporal development of NEC in preterm neonates

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Abstract

Necrotizing enterocolitis (NEC) in preterm infants develops very rapidly from a mild intolerance to enteral feeding into intestinal mucosal haemorrhage, inflammation and necrosis. We hypothesized that immediate feeding-induced gut responses precede later clinical NEC symptoms in preterm pigs. Fifty-six preterm pigs were fed total parenteral nutrition (TPN) for 48 h followed by enteral feeding for 0, 8, 17 or 34 h with either colostrum (COLOS, n=20) or formula (FORM, n=31). Macroscopic NEC lesions were detected in FORM pigs throughout the enteral feeding period (20/31, 65%), while most COLOS pigs remained protected (1/20, 5%). Just 8 h of formula feeding induced histopathological lesions, as evidenced by capillary stasis and necrosis, epithelial degeneration, edema and mucosal hemorrhage. These immediate formula-induced changes were paralleled by decreased digestive enzyme activities (lactase and DPPIV), increased nutrient fermentation and altered expression of innate immune defense genes such as interleukins (IL-1α, IL-6, IL-18), nitric oxide synthetase (NOS), tight junction proteins (claudins), Toll like receptors (TLR-4), and tumor necrosis factor (TNF-α). In contrast, the first hours of colostrum feeding induced no histopathological lesions, increased maltase activity and induced changes in gene expressions related to tissue development. Total bacterial density was high after two days of parenteral feeding, and not significantly affected by diet (colostrum, formula) or length of enteral feeding (8-34 h), except that a few bacterial groups (Clostridium, Enterococcus, Staphylococcus species) increased with time. We conclude that a switch from parenteral to enteral nutrition rapidly induces diet-dependent histopathological, functional and proinflammatory insults to the immature intestine. Great care is required when introducing enteral feeds to TPN-fed preterm infants, particularly using formula, because early feeding-induced insults may predispose to NEC lesions that are difficult to revert by later dietary or medical interventions.
Introduction

Necrotizing enterocolitis (NEC) is a serious inflammatory disease of the gastrointestinal tract (GIT) and the most common gastrointestinal emergency in newborn infants (14). Morbidity and mortality rates associated with NEC are significant (15), rendering NEC one of the greatest challenges to neonatologists and paediatric surgeons. Advances in obstetric and neonatal care have improved survival rates for smaller, more immature infants, and as increasing numbers of very low birth weight infants survive the neonatal period, the population at risk for NEC increases (18). Despite advances in neonatal intensive care management and surgery, diagnosis and management of NEC remains complex and difficult.

The exact etiology of NEC remains unclear, but predisposing factors include bacterial colonization and enteral feeding (18). A feeding-induced ischemic insult may damage the mucosal barrier, leading to increased intestinal permeability and in severe cases bacterial translocation. Regardless, little is known about the temporal sequence of events by which enteral feeding and bacterial colonization lead to decreased intestinal defenses and predispose infants to NEC. Until now, NEC research into both infants and animal models has focused on the end stage NEC pathology, but in order to identify preventive interventions we need to better characterize the early events in disease progression and their relation to diet. We hypothesized that abrupt introduction of a suboptimal diet induces rapid intestinal responses that precede later clinical symptoms of NEC in preterm neonates. In the preterm pig model of NEC, clinical symptoms normally develop 1-2 days after the start of enteral feeding and only very few pigs were diagnosed with NEC before 24 h of feeding in an earlier study on 435 preterm piglets (3). On the other hand, important digestive functions, such as glucose uptake capacity, are negatively affected already few hours after formula feeding (31), even using a slow parenteral to enteral transition with small feeding volumes (5). How this relates to other structural or functional changes is unknown. Using clinical, histopathological,
functional, microbiological, and immunological indices, we characterize the temporal and diet-related development of NEC in preterm pigs, with the intention to identify early tissue markers of NEC and thus, targets for prevention.

Materials and Methods

Animals and their treatment

Fifty-six preterm piglets were obtained from 6 litters delivered by caesarean section at 106 d of gestation (Danish Landrace × Yorkshire × Duroc, Askelygaard Farm, Roskilde, Denmark; term = 116 ± 2 d). Caesarean section, rearing in incubators, and catheterization of piglets was performed as previously described (3). The National Committee on Animal Experimentation (Denmark) approved all procedures.

The total parenteral nutrient solution (TPN) used was prepared as previously described, and was formulated to meet the nutrient requirements of the preterm pig. The enteral formula diet was made from 3 commercially available products used for feeding infants 0-2 years of age (per liter of water: 80 g Pepdite 2-0, 70 g Maxipro, and 75 ml Liquigen-MCT, all products kindly donated by Nutricia, Allerød, Denmark), as previously described (23). The energy and protein concentrations of the formula were designed to match the composition of porcine milk during lactation. Porcine colostrum was collected manually from sows (Danish Landrace × Yorkshire × Duroc, Askelygaard Farm) within 6 h of farrowing and stored at -20°C until used.

For the first 48 h, TPN was infused continuously and passive immunity was provided by administration of adult porcine plasma via the arterial catheter using automatic infusion pumps (Infusomat Secura, Braun), as previously described (26). After 48 h of TPN, five pigs were euthanized as a reference sample for later comparison of the effects of enteral feeding (TPN group).
The remaining pigs were randomly assigned to receive either enteral formula (FORM) or porcine colostrum (COLOS) at 15 ml/kg body weight/3 h and parenteral nutrition was disconnected. Pigs allocated to each of these feeding regimes were randomly selected to be euthanized at 8 ± 1 h (FORM, n = 8; COLOS, n = 6), 17 ± 1 h (FORM, n = 10; COLOS, n = 7), and 34 ± 4 h (FORM, n = 13; COLOS, n = 7).

**Tissue collection and NEC evaluation**

Throughout the experiment, potential clinical signs of NEC (feeding intolerance/vomiting, abdominal distension, haemorrhagic diarrhoea, and respiratory distress) were recorded every 3 h. At euthanasia (sodium pentobarbital, 200 mg/kg BW, intra-arterially through the umbilical catheter) the gastrointestinal tract (GIT) was macroscopically evaluated for NEC lesions, and tissue samples were collected and stored as previously described (26). Gastrointestinal organs were removed and placed on a cooled metal plate, and five regions (stomach, proximal, middle, and distal small intestine, and colon) were evaluated for NEC lesions according to a macroscopic NEC scoring system where: 1 = absence of lesions, 2 = local hyperaemia, inflammation and edema, 3 = hyperaemia, extensive edema and local hemorrhage, 4 = extensive hemorrhage, 5 = local necrosis and pneumatosis intestinalis, 6 = extensive necrosis and pneumatosis intestinalis. NEC was defined as a score of minimum 3 in at least one region (28). Together with the colon, the distal small intestine is the area most frequently affected by NEC lesions in preterm pigs, although lesions may occur throughout the gut from the stomach to the rectum in severe cases.

Two cm tissue samples (2 cm) of the distal small intestine were snap-frozen in liquid nitrogen for gene expression analyses. For analyses of mucosal morphometry and fluorescence in situ hybridization (FISH), sections were cut from the middle part of the distal segment and fixed in formalin, and embedded in paraffin. Sections of 3 µm were cut and mounted on glass slides,
deparaffinated in xylene, and dehydrated in 100% ethanol before FISH analysis (see below) as previously described (3). Sections were stained for histopathological evaluation by hematoxylin and eosin stain (H&E), phosphotungstic acid hematoxylin staining (PTAH) and periodic acid Schiff reaction (PAS). Histo-morphological measurements were made on scanning images (ArrayWoRx Biochip Reader, Applied Precision, Issaquah, WA, USA) and analyzed using SoftWoRx Explorer version 1.1 (Applied Precision) One representative cross section was selected from each pig and 10 full length villi and 10 crypts were measured.

The sections of the distal intestine were used to make an additional combined histopathological and macroscopic lesion evaluation. In this scoring system, points for the presence of one or more of eight NEC characteristics (submucosal edema, vacuolization of the enterocytes, congestion of vessels, regional villi sloughing, loss of villi, hemorrhage, picnotic nuclei and infiltration of inflammatory cells) were combined with points given for the extent of damage, as indicated by hemorrhage and/or necrosis in stomach, three intestinal sites and colon (0: no damage; 1: occasional areas of violaceous mucosa with 0-25% affected; 2: multiple areas of violaceous mucosa with 25-50% affected; 3: severe hemorrhagic mucosa with 50-75% affected; 4: extensive hemorrhage mucosa with >75% affected, with or without areas of necrosis). A cumulative lesion score was calculated by summing the macroscopic score for each region with the histopathological score for the distal intestinal tissue section, resulting in a 6-38 score range.

**Brush border enzyme activities, short chain fatty acids and microbiology**

Activities of brush-border peptidases [aminopeptidase A (ApA), aminopeptidase N (ApN), dipeptidylpeptidase IV (DPPIV)] and disaccharidases (lactase, maltase, and sucrase) were all measured as previously described (24). The concentrations of 15 different short chain fatty acids (SCFAs) in samples of stomach contents were measured by gas chromatography as described
previously (5). The limited sample volume obtained from colon in many piglets was first used for microbiological measurements, thus preventing measurements of colonic SCFA concentrations.

The bacterial abundance and location of bacteria adhering to the mucosa was investigated using specific bacterial oligonucleotide probes and FISH on formalin-fixed sections of distal small intestine as previously described (28). For hybridization, a general probe targeting the bacterial Domain (EUB338, systematic name: S-D-bact-0338-a-A-18, sequence: 5’-GCTGCCTCCGTAGGAGT-3’) and an unspecific control probe (non-EUB338, systemic name: S-*-non-0338-a-S-18, sequence: 5’-CGACGGAGGGCATCCTCA-3’) were used (1). Additionally, the bacterial assemblage of the colon contents was analyzed by terminal restriction fragment length polymorphism (T-RFLP) as previously described with minor modifications (26). For T-RFLP analyses bacterial DNA was extracted and purified using QIAamp DNA Mini Kit (Qiagen, Ballerup, DK) as described by the manufacturer, and the PCR products were analysed on an automatic sequence analyzer (Applied Biosystems Genetic Analyzer 3130/3130xl, Nærum, Denmark).

**RNA extraction for gene expression analysis**

Snap-frozen intestinal tissue samples were thawed in RNAlater-ICE (Ambion, Nærum, Denmark). Total RNA was extracted using RNeasy Midi Kit with TRIZOL reagent (Qiagen) as described in the manufacturer’s protocol. All RNA samples were treated with RNase-free DNase Set (Qiagen) according to the manufacturer's instructions. Quality of extracted total RNA was estimated by Agilent 2100 bioanalyzer, using the Nanochip 6000 (Agilent Technologies, Nærum, Denmark). A RNA integrity number (RIN) was assigned to each sample, indicating the integrity of the extracted RNA. Only samples with a RIN greater than or equal to 7.0 were used for further gene expression analysis.
Immune focused microarrays

Transcriptional profiles of FORM and COLOS pigs after 8 h of enteral feeding were compared with TPN fed control pigs using a porcine low density microarray as previously described (29). Briefly, these microarrays contain 373 different oligonucleotide probes representing more than 200 different immune related genes and 8 different array control oligonucleotides (ArrayControl; Ambion). Detailed description of this microarray experiment can be found at NCBI’s Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/geo) using the platform accession number GPL6849. The microarray analysis was conducted using a dual-channel reference design co-hybridizing 15 samples (five animals randomly selected from each group) against a common reference, prepared by mixing equal amounts of total distal small intestinal RNA from all samples. The samples and reference pool were labelled with Oyster 550 and 650, respectively.

3DNA Array 900 expression array detection kits (Genisphere, Hatfield, PA) were used for the labeling and cDNA synthesis of RNA as previously described with minor modifications (29). Briefly, two times 25 µl cDNA (sample and reference), 1 µl salmon sperm DNA (10 µg/µl) and 51 µl 2 × formamide-based hybridization buffer (3DNA Array 900, Genisphere) was mixed, and 85 µl of this solution was applied under a 25 x 60 mm LifterSlip (Erie Scientific Company, Portsmouth, NH) carefully avoiding air bubbles. Slides were incubated at 42°C in a water bath over night. Wash buffer 1 (3DNA Array 900, Genisphere) was preheated to 42°C for the post cDNA hybridization wash. For 3DNA hybridization reaction mixes; 3 µl of each Capture reagent (3DNA Array 900, Genisphere), 42 µl SDS-based hybridization buffer (3DNA Array 900, Genisphere), 35 µl milliQ water, and 1 µl salmon sperm DNA (10 µg/µl) was mixed to a final volume of 84 µl. Eighty microliter 3DNA hybridization mix was applied to each slide and incubated in darkness in a water bath at 62°C for 4 h. Wash buffer 1 was preheated to 65°C for the post 3DNA hybridization wash.
Slides were scanned using a CCD-based imaging system (ArrayWoRx Biochip Reader auto, Applied Precision).

Microarray image processing and spot finding were performed using GenePixPro 6.0 (Molecular Devices Corporation, Sunnyvale, CA). Low intensity spots were flagged if they did not have at least 45% of their feature pixels more than two standard deviations above background. Incorrectly placed spots were flagged manually. Data was saved to Acuity 4.0 (Molecular Devices Corporation) and normalized using the LOWESS method (37). A data set was created in Acuity consisting of all un-flagged, normalized data points. Data points that were not represented in at least 70% of the microarrays were deleted. Differently expressed genes were determined using ANOVA and t-test, and P-values <0.05 were considered statistically significant. Raw and processed data files can be accessed through Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/), accession number GSE13516.

**Quantitative PCR**

RT-qPCR analysis was performed on distal small intestinal tissue samples. Extracted RNA from the samples was converted to first-strand cDNA by reverse transcription of 1 µg RNA using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions (total volume: 20 µl). The cDNA was stored at -20°C until further use. RT-qPCR was performed on the 7900HT Fast Real-Time PCR System (Applied Biosystems). Briefly, TaqMan 2 × Fast Universal PCR Master Mix (Applied Biosystems) was mixed with 50 ng template cDNA and gene specific primers and TaqMan (5' 6Fam/3' MGB) probes (reaction volume: 20 µl). Cycling conditions were as follows: 20 s at 95°C, followed by 40 cycles with denaturation for 1 s at 95°C and annealing/elongation for 60 s at 60°C. All reactions were performed in triplicate. All RT-qPCR primers were designed using PrimerExpress software v2.0 (Applied Biosystems) and were
synthesized at Applied Biosystems (Supplementary Data Table 2). Sequences used for primer/probe
design were obtained from public databases (GenBank, NCBI). BLAST searches were performed to
confirm the total gene specificity of the primer sequences and to show the absence of
polymorphisms at the primer site.

The stability of the 5 acknowledged reference genes, beta-2-microglobulin (B2M), beta
actin (ACTB), phosphoglycerate kinase 1 (PGK1), 18S rRNA subunit (RPS18) and TATA box
binding protein (TBP) were examined using geNorm (34), a Visual Basic Applications (VBA) for
Microsoft Excel. The gene expression normalization factor (NF) based on the geometric mean of
the relative concentration of the best reference genes was calculated for all samples using geNorm.
Normalization was obtained by dividing each sample value of the gene of interest by its
Corresponding normalization factor. To compare the fold change in gene expression between the
treatment groups normalized gene expression values for the treatment groups were compared
relative to TPN pigs, which was set to one.

Interleukin-18 (IL-18) expression was further investigated by measuring the IL-18 protein
levels in distal small intestine tissue homogenates (2) using a porcine IL-18 ELISA kit (Bender
MedSystems, Burlingame, CA). Only samples from TPN, 8 h, and 17 h were analyzed, since we
were interested mainly in the early events of disease progression.

**Statistical analysis**

All data, except those from the microarray, were analysed using a mixed linear model in SAS
(SAS/STAT version 8.1, SAS Institute, Cary, NC). Treatment (FORM and COLOS), time (TPN, 8,
17, and 34 h) and intestinal region (proximal, middle, and distal) were considered as fixed effects,
and pig and litter were included as random effects. When no significant effect of region, time, or
treatment was detected, data were pooled. The results in tables and figures are given as the least
square means (LSmeans) ± SEM and differences between two means were tested by the least significant difference test, and effects of increasing feeding time by regression analyses, unless otherwise indicated. NEC incidence was analysed using Fisher’s exact test, with subsequent pairwise comparisons. For T-RFLP data, comparisons between individual terminal restriction fragments (T-RFs), a two-tailed Monte Carlo Estimates Mann-Whitney U-test and Kruskal-Wallis tests were used. T-RFs smaller than 60 bp and larger than 800 bp were excluded because these were outside the range of the standards. Identification of specific bacteria characterized by T-RFs was done in silico by inserting primer sequences and restriction enzymes in the MiCA home page (http://mica.ibest.uidaho.edu/digest.php) using the RDPII database (Release 9, Update 37, Bacterial SSU 16S rRNA; 7). Regression analyses were used to estimate the specific effect of length of feeding (8, 17 or 34 h) on various parameters. P<0.05 was used as the critical level of significance for all statistical evaluations.

Results

Clinical observations, NEC and histopathology

TPN pigs lost an average of 15±22 g body weight (BW) from birth to two days, while COLOS pigs gained weight (67±10 g, across all time points) with intermediate values in FORM pigs (40±8 g, across all time points) (all P<0.05). Independent of diet type, the relative wet weight of the small intestine (per kg body weight) increased immediately after feeding (from 16.2±0.9 to 23.4±1.0, 26.9±0.8 and 37.2±1.8 g/kg at the 8, 17 and 34 h time points, respectively, all P<0.05, Figure 1). This increase in weight was associated with a relatively lower and slower increase in intestinal length that was significant at 17 h and 34 h of feeding, relative to TPN (314±6 and 387±11 versus 264±15 cm, both P<0.05, Figure 1).
NEC lesions were not observed before enteral feeding was initiated, and at 8, 17, and 34 h of feeding, FORM pigs had a significantly higher incidence of NEC lesions [63% (5/8), 80% (8/10), 54% (7/13), respectively], compared with COLOS pigs [0% (0/6), 14% (1/7), and 0% (0/7), respectively] (P<0.05). Scoring of the lesions according to the combined macroscopic and microscopic evaluation system gave a score of 6.6±3.0 before enteral feeding (TPN group), with similar values in all groups of COLOS pigs (6.0±2.8, 7.1±2.6, 7.7±2.6 at 8, 17 and 34 h, respectively). FORM pigs had significantly higher scores at all three time points, compared with TPN and COLOS pigs (16.8±2.4, 14.8±2.1 and 17.3±1.9 at 8, 17 and 34 h of feeding, respectively, all P<0.05). Morphological measurements on sections from the distal small intestine from healthy pigs showed that 34 h of enteral feeding resulted in increased villous height (+24-43%, P<0.05), relative to the groups with shorter feeding periods or no enteral feeding at all, which were similar (Figure 1). There were no differences between diets except that FORM pigs showed increased crypt depth 34 h after feeding (+11%, P<0.05). Pigs diagnosed with NEC (n=17) had significantly lower villous height (547±51 µm) compared with healthy FORM pigs (n=13, 719±60 µm, P<0.05).

In TPN pigs, the distal small intestine had a normal mucosal morphology with long, contracted villi and short crypts. The villous epithelium consisted of vacuolated fetal-type enterocytes characterised by multiple small to medium size, empty cytoplasmatic vacuoles and a central to apical located nucleus (Fig. 2A). Goblet cells were identified on the villi and in the crypts, and low densities of bacterial colonies were detected on the villous epithelium by the FISH analyses (Fig 2AFISH). In healthy pigs fed colostrum for 8 h, the cytoplasmatic vacuoles were intensely and homogenously stained by absorbed protein and the villous surface was covered with a colostrum-related biofilm. Various densities of bacteria of different morphologies were commonly seen along the villous epithelium of the COLOS intestines (picture not shown). Preterm pig intestines that remained healthy after 8 h of formula feeding (clinical NEC score of 1) showed enlarged, empty
vacuoles that displaced the nucleus to the basolateral or apical side of the distended enterocytes (Fig. 2C). Neither the healthy FORM, nor the COLOS intestines, showed any change in the cytological appearance of the villous enterocytes, or in total bacterial density detected by FISH, following increased feeding time (8, 17 or 34 h).

Preterm pigs with moderate macroscopic NEC lesions after 8 h of formula feeding (clinical NEC scores 2-3), showed multiple villi with abundant stasis of the capillaries and venuoles, cleft formation with degeneration, and separation of the epithelium from the lamina propria, extravasations of erythrocytes, and even necrosis and disappearance of stromal cells and the apical capillaries (Fig. 2D). Interstitial edema and focal hemorrhage was seen in the submucosa together with increased number of mononuclear cells, lymphocytes and eosinophilic granulocytes in the lamina propria and submucosa. There was a moderate amount of bacteria colonizing the villous epithelium but no signs of bacterial invasion (Fig. 2D). At higher magnification (x 40), the degenerated epithelial cells were slightly swollen, showed pyknotic nuclei (Fig. E) and were detached from the basement membrane. The adjacent capillaries and stromal cells were undergoing necrosis. Further down the villi the epithelium appeared unaffected with normal size nuclei.

Preterm pigs with severe macroscopic NEC lesions after 8 h of formula feeding (clinical NEC scores 4-6) showed even more advanced lesions with extensive stasis of villi vessels, diffuse hemorrhage within lamina propria, submucosa and muscularis, and finally, necrosis and extensive loss of villi (Fig. 2F and 2G), with or without damage to the crypt epithelium. Cellular inflammatory reactions were generally not apparent in pigs fed formula for 8 h, but present in some of the pigs fed formula for 34 h with NEC. Such intestines showed cellular infiltration (Fig. H), crypts of variable size, massive congestion of mucosal vessels, diffuse hemorrhage within the lamina propria and most of the villi were atrophic and mainly covered by leukocytotic debris.
Interstitial edema was found in the submucosa and subserosa while macrophages containing hemosiderin granules were absent.

**Brush border enzymes, short chain fatty acids and microbiology**

Relative to TPN pigs, ApA increased with time for both COLOS and FORM pigs (P<0.05, Figure 3). Similarly, ApN and DPPIV activities increased over time relative to TPN pigs, however the increase was more significant for COLOS pigs compared with FORM pigs (P<0.05). Lactase activity decreased after formula feeding, remaining significantly lower than in TPN and COLOS pigs at all time points (P<0.05). Maltase activity increased over time in COLOS pigs and was significantly higher than in FORM pigs across all time points (P<0.05). Irrespective of diet and feeding length, sucrase activity was higher after than before enteral feeding (Figure 3).

The total concentration of short chain fatty acids were low in TPN pigs (<2 μmol/g contents), but increased rapidly after enteral feeding due to bacterial nutrient fermentation, especially for the formula group with 80±16 and 106±17 μmol/g at 8 and 17 h respectively, and lower concentrations in the corresponding colostrum groups (17±3 and 32±6 μmol/g, respectively, both P<0.05). The majority of the diet-related differences were explained by differences in lactate concentration (29±9 and 44±10 after formula versus 7±2 and 21±6 μmol/g after colostrum at 8 and 17 h, respectively, P<0.05) and to the octanoic acid delivered by the formula itself (contributing 40-50 μmol octanoic acid/g contents in formula-fed pigs).

Before feeding, TPN pigs with a retained meconium plug had a low bacterial diversity in the colon contents (55 T-RFs, n=2), but a high total bacterial density, as evaluated by the total intensity of T-RF bands. The total intensity decreased after 8 h of feeding (from 37847±3225 to 26599±6040 density units, P<0.01) after which density values remained similar (P>0.05) for all three time points for both diets groups (colostrum, formula), and with no effect of disease status.
(NEC or healthy, all P>0.05). Figure 4 shows how the intensity distribution for the dominant T-RFs bands as analyzed across diets and in response to increasing feeding time. The total number of bands increased from 55 T-RFs before feeding to ~170 T-RFs at 34 h. T-RFs with increasing density over feeding time included T-RFs 217 and 594 bp, identified as Enterococcus spp. and Streptococcus spp. T-RF 583 bp increased while T-RF 348 bp decreased significantly over time (P<0.05), but neither of these bands could be identified as specific bacteria using the present pig microbiota libraries. The same four T-RFs changed significantly with time also when pigs diagnosed with NEC were excluded from the analyses (all P<0.05). Diet type (colostrum or formula) did not affect the response to increasing feeding time, except for T-RF 233 bp, which increased with time specifically in the formula group (P=0.002). This T-RF has been identified as Clostridium perfringens and increased density has repeatedly been associated with clinical NEC outbreak in studies on preterm pigs.

**Microarray of intestinal immune genes**

Fourteen differentially expressed genes (7 up, 7 down) were detected when TPN pigs were compared with FORM pigs at 8 h and sixteen differentially expressed genes (8 up, 8 down) were detected when TPN pigs were compared with COLOS pigs at 8 h (Table 2). After 8 h of formula feeding, we detected differential regulation of several key genes coding for proteins involved in innate immune defense: interleukin 18 (IL-18), mannose binding lectin (MBL), surfactant, pulmonary-associated protein D (SFTPD), transferrin (TF), and plasminogen activator inhibitor 1 (PAI-1) were all found to be up-regulated as well as tight junction formation (claudin 1 [CLDN1]) and apoptosis (tumor necrosis factor (TNF) related apoptosis related ligand (TRAIL). Early responses to formula feeding also showed down-regulation in genes related to cell signaling
interleukin 12 receptor beta 2, integrin alpha V, and integrin beta 6), immune regulation (nuclear factor kappa-B, subunit 1) and anti-apoptotic activity (basal cell lymphoma-extra large) (Table 1).

Early response genes to colostrum feeding were more diverse in function (Table 1). Anti-inflammatory (annexin A11) and hypoxia-inducible factor, which is regulated in response to hypoxia, and integrin alpha V, which is involved in cell signaling were among those identified as up-regulated. Others include angiopoietin (angiogenesis), hypoxanthine phosphoribosyltransferase (purine synthesis) and malate dehydrogenase (glucoeneogenesis). In contrast to early response genes after formula feeding, genes related to innate immunity (MBL and SFTPD), tight junction formation (CLDN1) and apoptosis (TRAIL) were down-regulated after 8 h of colostrum feeding.

Quantitative real time PCR for inflammatory genes, and IL-18 ELISA

The genes encoding the proinflammatory cytokines IL-1α, IL-6, and TNF-α, as well as the Toll like receptor (TLR)-4, were significantly up-regulated in FORM compared with both COLOS and TPN pigs and independent of duration of enteral feeding (P<0.05, Figure 5). PBD1 and MYD88 were significantly down-regulated in FORM pigs at 34 h after enteral feeding (P<0.05) (data not shown). IL-1α, IL-6, TLR-2 and TLR-4 were all significantly up-regulated (1.5-2.5 fold) in NEC diseased pigs across the different feeding groups (n=16) compared with healthy pigs (n=36, P<0.05). Analyzed across all the six different diet and time groups (n=51), intestinal TLR-4 expression was markedly upregulated (29-fold, P<0.001), relative to the mean value in a separate group of preterm, caesarean-delivered pigs at birth, before any feeding (n=6).

After 8 h of feeding, IL-18 protein levels in FORM and COLOS pigs were similar to those in TPN pigs (80-110 pg/ml) but by 17 h mean levels increased significantly in FORM pigs (240±24 pg/ml, P<0.001 relative to 8 h) but less in COLOS pigs (150±29 pg/mL, P>0.05). Correspondingly, IL-18 levels increased significantly from 8 to 17 hours of feeding in the pigs.
diagnosed with NEC (from 68±34 to 272±37 pg/ml, P<0.001) while levels remained the same in healthy pigs across diets (155±31 pg/ml).

Discussion

Despite many years of research, the pathogenesis of NEC remains unclear. Mounting evidence suggests a multi-factorial etiology, including the presence of an inappropriate bacterial colonization, intestinal ischemia, reperfusion injury with activation of pro-inflammatory cellular cascades, coupled with intestinal mucosal immaturity and dysfunction (25). Identifying and understanding the early events in the etiology in NEC is crucial in prevention and improved therapy for this devastating disease. In this study we used a previously validated preterm pig model of NEC (3, 23) to characterize the temporal and diet-dependent development of NEC by analyzing intestinal tissues 8, 17, and 34 h after an abrupt transition to full enteral feeding following two days of total parenteral nutrition. In our model, a more gradual parenteral to enteral transition reduces, but does not prevent NEC development, at least not using formula (5). With our standard volumes of enteral feed (15 mL/kg/3 h), we now show that formula-induced mucosal inflammation and histopathological lesions occur as early as 8 h after the transition to enteral feeding, although clinical NEC symptoms may not develop until much later. Formula feeding is sufficient to initiate the cascade, without further stressors such as bacteria, hypoxia or hypothermia, often used to induce NEC in other animal models (6; 11; 35). The immediate effects of formula feeding included changes in histopathological indices, inflammatory gene expressions, brush border enzyme activities and short chain fatty acid levels, while gross parameters of intestinal growth (weight, length, villus, crypt), and bacterial density and composition, were not affected until later, and were similar between formula- and colostrum-fed pigs.
The early lesion progression revealed excessive accumulation of erythrocytes and stasis of the villous capillaries, accompanied by degeneration of the overlaying epithelium, suggesting that mucosal ischemia is an early event in NEC etiology. The early stasis and extravagation of erythrocytes in some preterm pigs fed formula for 8 hours involved the whole lamina propria and caused complete loss of the villous epithelium and subsequently, the less ischemia-sensitive crypt epithelium. Severe histological tissue damage may thus develop hours before the clinical signs of NEC are evident (e.g. abdominal distension, lethargy, blood stools), together with later increased inflammatory cell reactions and signs of epithelial regeneration.

Bacterial colonization immediately after birth is a prerequisite for NEC (6) and the typical NEC–related intestinal dysfunctions are absent under germ-free conditions (23; 27; 35). In preterm pigs, *Clostridium perfringens* has repeatedly been associated with advanced NEC (3; 23; 28) but the results in this study suggest that overgrowth of clostridia is a relatively late phenomenon in NEC etiology. After 8 h of enteral feeding, there was no apparent association between the density of colonic or distal intestinal bacteria, and the degree of intestinal lesions. Host response factors, rather than the composition of the gut microbiota, may be the main determinant of NEC. Regardless, the general presence of bacteria is required for NEC and the high abundance, and low diversity, of bacteria in the retained colonic meconium plug of preterm TPN pigs is noteworthy. In the preterm intestine, intestinal motility is greatly reduced and the lack of stimulation by enteral feeds may further promote intestinal stasis, leading to proliferation of relatively few bacterial populations before full enteral feeding can be gradually introduced. Postnatal TPN, or TPN with too little enteral food stimulation, may facilitate a low-diversity, high-density bacterial colonization that will predispose to disease when advancing feeding volumes are introduced. Because this advancing enteral feeding may also reduce digestive functions (e.g. formula-reduced brush border enzyme activities and nutrient uptake), nutrients will easily be subject to fermentation by the resident
bacteria resulting in a very rapid production of short chain fatty acids, acting as an additional irritant
to an immature epithelium. Hence, the concentration of short chain fatty acids in the stomach
contents of pigs fed formula for just 8 h after TPN in the present study was similar to the levels
observed in pigs fed formula for much longer (5). The above, together with an underdeveloped
innate immune defense (4), may explain our consistent observation that a short period of TPN after
birth increases the feeding-induced NEC sensitivity in preterm pigs (3).

Feeding with maternal milk has long been advocated to prevent NEC but it remains unclear
whether mother’s milk act via provision of gut functional and immunoprotective factors, or whether
it directly promotes a less pathogenic gut microbiota composition short and/or long term (8; 19).
Consistent with our earlier studies, we failed to find a close relationship between diet and the
luminal gut microbiota at different times following feeding. Formula may be devoid of the factors
in mother’s milk that protect against detrimental microbe-epithelial interactions, breakdown of
barrier functions (e.g. claudins) and subsequent uncontrolled immune responses involving
interleukins. Colostrum may have reduced the response to bacteria in this study by lowering TLR-4
extression, relative to formula, but the effect was marginal relative to the 29-fold increase in TLR-4
epression over the 2 d TPN period, in the complete absence of enteral food. This marked postnatal
bacterial receptor upregulation occurs for a number of TLRs (TLR-1, -2, -4, -5, -9) in both preterm
and term pigs fed colostrum (Liangqiang, Sangild & Bering, unpublished observations). More
likely therefore, mothers’s milk act to inhibit bacterial adhesion, rather than TLR expression, and
this effect could be particularly important in the hours following the first introduction of enteral
food. This hypothesis is confirmed by the observation that epithelial adhesion of both Gram-
negative and Gram-positive bacteria, and subsequent IL-1β secretion, were effectively inhibited by
the first boluses of colostrum in preterm pigs (33).
In the current study, we identified early response genes to formula feeding as being primarily involved in innate immune defense and apoptosis compared with early response genes to colostrum feeding, which were more diverse in function, primarily related to tissue development and metabolism. This was confirmed by uploading the microarray into Ingenuity’s (Ingenuity Pathway analysis software (http://ingenuity.com) that generates a series of networks and assign biological functions to the uploaded genes. Innate immune response genes (IL-1α, IL-6, IL-18, TNF-α, TLR-4, MBL, SFTPD, and TF) and mucosal integrity genes (CLDN1) were up-regulated after only 8 h of formula feeding compared with colostrum feeding. These same inflammatory mediators may induce an acute, but highly specific, reduction in certain enterocyte functions, such as lactase and DPPIV activities that in this study occurred in the absence of any gross mucosal atrophy. In colostrum-fed pigs, innate immune defense (MBL and SFTPD) and mucosal integrity (CLDN1) genes were down-regulated. These acute changes following the initiation of enteral feeding highlight the importance of providing an optimal amount and composition of enteral nutrition to preterm infants. Early feeding-induced proinflammatory responses may be difficult to revert with later clinical interventions such as antibiotics, withdrawal of enteral food and anti-inflammatory drugs. Conversely, some of the early proinflammatory signals could be targets for future therapeutic interventions to assist enteral food introduction. These targets include TLR-4, IL-1, IL-6, IL-18 and TNF-α.

IL-18 is a cytokine with potent interferon (IFN)-γ inducing activities and plays an important role in the innate immune response. The IL-18 receptor system and its signal transduction pathway are analogous to the IL-1 receptor system. IL-18, among other functions, promotes IFN-γ and TNF-α production by Th1 cells. Of the many cytokines that play important roles in inflammation, IL-18 has been implicated in inflammatory diseases of the small intestine (20; 22; 30). An IL-18 −/− mouse model showed a significant decrease in NEC incidence after 3 d of formula feeding, confirming IL-
18 as having a crucial role in the pathogenesis of experimental NEC (13). IL-18 protein level was significantly elevated after 17 h of formula feeding whereas the mRNA coding for IL-18 was increased after just 8 h of feeding. This may be due to a delayed activation from inactive to pro-active IL-18 protein, to post-transcriptional regulation or to differences in mRNA and protein turnover rates (9; 36).

Nitric oxide (NO), an important messenger produced by a family of synthases (NO synthase, NOS), is a key factor in intestinal responses to injury. NO production is important in protecting the intestine from ischemic damage through vasodilation and increased blood flow (17). There is evidence indicating that NO suppresses IL-18 processing by inhibiting caspase-1 activity (16). In this study we showed that endothelial NOS (eNOS) mRNA was down-regulated as an early response to formula feeding, consistent with the earlier indication that low mucosal eNOS was related to NEC lesions in preterm pigs (32). Likely an early feeding- and ischemia-induced decrease in NO levels results in activation of caspase-1 and further secretion of IL-18. Hence, early neutralization of IL-18 may be a potential preventive therapy for NEC, together with other early markers of intestinal lesions such as TNF-\(\alpha\) (10; 12), IL-6 and the gram-negative bacterial receptor, TLR-4. Conversely, other factors that have been related to NEC lesions in earlier studies, such as IFN-\(\gamma\) (6; 21; 28), may be involved relatively late in NEC pathogenesis and thus fail to play a key role in early NEC development.

**Perspectives**

We have shown that feeding infant formula, instead of mother’s milk, as the initial enteral diet to TPN-fed preterm neonates very rapidly results in pronounced histopathological, functional and inflammatory responses in the small intestine. These adverse effects may be difficult to reverse with later dietary or medical interventions. More studies are required to better identify how the
many possible diet regimens during the important parenteral to enteral transition phase best serve to provide adequate nutrients coupled with good intestinal health and maturation to the preterm infant. We emphasize the need to optimize the nature and amount of the first enteral food, especially when mother’s milk is not available. In preterm pigs, the only feeding regimen that consistently prevents against NEC lesions is a gradual transition from parenteral nutrition to enteral colostrum feeding within the first week, starting from few hours after birth (5). As the clinical presentation of NEC is diverse and rapid, it is important to understand the early pre-clinical events for NEC as a tool to identify therapies that could support the challenging transition from parenteral to enteral nutrition.
References


31. Thymann T, Møller HK, Støl B, Støy AC, Buddington RK, Bering SB, Jensen BB, Olutoye OO, Siggers RH, Mølbak L, Sangils PT and Brurrin DG. Carbohydrate


**FIGURE LEGENDS**

**Figure 1.** Dimensions of the small intestine after colostrum and formula feeding for 0-34 h following a 2 d period of total parenteral nutrition (means±SEM, n=5-13). The bar graphs show wet weight relative to body weight (A), intestinal length (B), distal intestinal villus height (C) and crypt depth (D). Bars not sharing the same letter differ significantly (P<0.05).

**Figure 2.** Representative H&E-stained cross sections of the distal small intestine in preterm pigs before enteral feeding (A), after 8 h colostrum feeding (B), and after formula feeding for 8 h (C-G) or 34 h (H). The unfed mucosa (A) had long villi and a low density of bacterial colonies was present along the mucosal surface (AFISH, red fluorescence hybridisation using the domain bacterial probe EUB338). After feeding colostrum for 8 h, the long villi were lined with enterocytes distended by immunoglobulin-containing cytoplasmatic vacuoles (Barrows) while empty vacuoles were present after formula feeding (C arrows). This material was washed out during tissue preparation. Moderate NEC lesions in pigs fed formula for 8 h (D) were associated with capillary stasis of villi (arrow heads) and necrosis of stroma cells (star), without notable circulatory disturbance between crypts, and a moderate density of bacterial colonies without signs of intraepithelial invasion (DFISH, red fluorescence hybridisation using the domain bacterial probe EUB338). At higher magnification (E) epithelial degeneration was evident with pyknotic nuclei (arrows) compared to the unaffected enterocytes (arrow heads) and separation from the basement membrane, and adjacent capillaries and stromal cells were undergoing necrosis (star). Severe NEC lesions in pigs fed formula for 8 h were associated with total loss and necrosis of the villous epithelium, without or with breakdown of the crypt epithelium (F and G, respectively, see arrows). Cellular inflammatory reactions (star) were...
generally absent except in some pigs fed formula for 34 h with atrophic villi covered by leukocytic debris (H, arrows). Magnifications were x10, except AFISH and DFISH and E (all x 40).

**Figure 3.** Brush border enzyme activities in the small intestine after colostrum and formula feeding for 0-34 h following a 2 d period of total parenteral nutrition (means±SEM, n=5-13). The bar graphs show lactase (A), maltase (B), sucrase (C), ApN (D), ApA (E) and DPPIV (F) activities where one enzyme unit represents one mmol of substrate released per min per g of intestine. Bars not sharing the same letter differ significantly (P<0.05).

**Figure 4.** Microbiology of colon contents as indicated by the mean intensity values of the dominating terminal restriction fragment length polymorphism (T-RFLP) bands after various times of enteral feeding (8-34 h). The graph shows pooled values across the colostrum and formula diets (n=14-20). * Significant change in intensity with increasing feeding time (P<0.05, 8-34 h, linear regression analysis).

**Figure 5.** Gene expression changes in the distal small intestine after colostrum and formula feeding for 0-34 h following a 2 d period of total parenteral nutrition (means±SEM, n=5-13). The bar graphs show changes in IL-1α (A), IL-6 (B), TLR-4 (C) and TNF-α (D) expression in the distal small intestine of enterally-fed animals, relative to TPN pigs (set to 1). Fold changes were analyzed across time points for each diet and bars not sharing the same letter differ significantly (P<0.05).
Table 1. Intestinal genes showing significant transcriptional response (↑ up-regulated, ↓ down-regulated) to 8 h of enteral feeding (formula or colostrum) after a 2 day period of total parenteral nutrition (TPN, control)

<table>
<thead>
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<th>Gene</th>
<th>Description of genes</th>
<th>Function</th>
<th>P-value</th>
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<tr>
<td><strong>FORMULA FEEDING</strong></td>
<td></td>
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<tr>
<td>MBL2</td>
<td>mannose-binding lectin</td>
<td>innate immunity</td>
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<td>IL-18</td>
<td>interleukin 18</td>
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<td>0.02↑</td>
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<tr>
<td>SFTP2D</td>
<td>surfactant, pulmonary-associated protein D</td>
<td>innate immunity</td>
<td>0.03↑</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor 1</td>
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<td>0.03↑</td>
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<td>TRAIL</td>
<td>TNF related apoptosis induced ligand</td>
<td>apoptosis</td>
<td>0.03↑</td>
</tr>
<tr>
<td>CLDN1</td>
<td>claudin 1</td>
<td>tight junctions</td>
<td>0.03↑</td>
</tr>
<tr>
<td>TF</td>
<td>transferrin</td>
<td>innate immunity</td>
<td>0.05↑</td>
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<td>IL12RB2</td>
<td>IL-12 receptor beta 2</td>
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</tr>
<tr>
<td>BCLXL</td>
<td>basal cell lymphoma-extra large</td>
<td>anti-apoptosis</td>
<td>0.02↓</td>
</tr>
<tr>
<td>ITGB6</td>
<td>integrin beta 6</td>
<td>cell signaling</td>
<td>0.03↓</td>
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<tr>
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<td>transferase activity</td>
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<td>NFKB1</td>
<td>nuclear factor kappa-B, subunit 1</td>
<td>immune regulation</td>
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<tr>
<td>NOS3</td>
<td>nitric oxide synthase 3 (endothelial)</td>
<td>vascular function</td>
<td>0.05↓</td>
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**COLOSTRUM FEEDING**

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<th>P-value</th>
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<td>tissue hypoxia</td>
<td>0.02↑</td>
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<tr>
<td>ANGPT1</td>
<td>angiopoietin 1</td>
<td>angiogenesis</td>
<td>0.02↑</td>
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<td>ITGAV</td>
<td>integrin alpha V</td>
<td>cell signaling</td>
<td>0.04↑</td>
</tr>
<tr>
<td>PLAT</td>
<td>plasminogen activator, tissue</td>
<td>fibrinolysis</td>
<td>0.04↑</td>
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<td>myxovirus (influenza virus) resistance 1</td>
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<td>0.00↓</td>
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<td>claudin 1</td>
<td>tight junctions</td>
<td>0.00↓</td>
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<td>mannose-binding lectin</td>
<td>innate immunity</td>
<td>0.01↓</td>
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<tr>
<td>TRAIL</td>
<td>TNF related apoptosis induced ligand</td>
<td>apoptosis</td>
<td>0.01↓</td>
</tr>
<tr>
<td>SLC5A1</td>
<td>solute carrier family 5</td>
<td>glucose transport</td>
<td>0.01↓</td>
</tr>
<tr>
<td>SFTPD</td>
<td>surfactant, pulmonary-assoc. protein D</td>
<td>innate immunity</td>
<td>0.02↓</td>
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<tr>
<td>B3GALT3</td>
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<tr>
<td>NANS</td>
<td>N-acetylneuraminic acid synthase</td>
<td>carbohydr. synthesis</td>
<td>0.05↓</td>
</tr>
</tbody>
</table>
Figure 1

A: Weight

B: Length

C: Villus

D: Crypt

Length of enteral feeding (h)

Intestinal weight (g/kg)

Intestinal length (cm)

Villus height (μm)

Crypt depth (μm)
Figure 3

A: Lactase

B: Maltase

C: DPP IV

D: ApA

Bars indicate significant differences at the 0.05 level.
Figure 4

- 8 h
- 17 h
- 34 h

Absolute intensity vs. T-RF (bp)
Figure 5

A: IL-1α

B: IL-6

C: TLR-4

D: TNF-α

Length of enteral feeding (h)