Enteral feeding induces diet-dependent mucosal dysfunction, bacterial proliferation, and necrotizing enterocolitis in preterm pigs on parenteral nutrition

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Departments of 1Human Nutrition, 2Large Animals and 3Anatomy and Cell Biology, Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark; 4Department of Animal Health, Welfare and Nutrition, Faculty of Agricultural Sciences, University of Aarhus, Tjele, Denmark; 5Department of Surgery, Maastricht University, Maastricht, The Netherlands; 6National Veterinary Institute, Technical University of Denmark, Copenhagen, Denmark; and 7U. S. Department of Agriculture/ Agricultural Research Service Children’s Nutrition Research Center, Houston, Texas

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Bjornvad CR, Thymann T, Deutz NE, Burrin DG, Jensen SK, Jensen BB, Mølbak L, Boye M, Larsson LI, Schmidt M, Michaelsen KF, Sangild PT. Enteral feeding induces diet-dependent mucosal dysfunction, bacterial proliferation, and necrotizing enterocolitis in preterm pigs on parenteral nutrition. Am J Physiol Gastrointest Liver Physiol 295: G1092–G1103, 2008. First published September 25, 2008; doi:10.1152/ajpgi.00414.2007.—Preterm neonates have an immature gut and metabolism and may benefit from total parenteral nutrition (TPN) before enteral food is introduced. Conversely, delayed enteral feeding may inhibit gut maturation and sensitize to necrotizing enterocolitis (NEC). Intestinal mass and NEC lesions were first recorded in preterm pigs fed enterally (porcine colostrum, bovine colostrum, or formula for 20–40 h), with or without a preceding 2- to 3-day TPN period (n = 435). Mucosal mass increased during TPN and further after enteral feeding to reach an intestinal mass similar to that in enterally fed pigs without TPN (+60–80% relative to birth). NEC developed only after enteral feeding but more often after a preceding TPN period for both sow’s colostrum (26 vs. 5%) and formula (62 vs. 39%, both P < 0.001, n = 43–170). Further studies in 3-day-old TPN pigs fed enterally showed that formula feeding decreased villus height and nutrient digestive capacity and increased luminal lactic acid and NEC lesions, compared with colostrum (bovine or porcine, P < 0.05). Mucosal microbial diversity increased with enteral feeding, and Clostridium perfringens density was related to NEC severity. Formula feeding decreased plasma arginine, citrulline, ornithine, and tissue antioxidants, whereas tissue nitric oxide synthetase and gut permeability increased, relative to colostrum (all P < 0.05). In conclusion, enteral feeding is associ-ated with gut dysfunction, microbial imbalance, and NEC in preterm pigs, especially in pigs fed formula after TPN. Conversely, colostrum milk diets improve gut maturation and NEC resistance in preterm pigs subjected to a few days of TPN after birth.

neonate; colostrum; formula; intestine; bacterial colonization

PREMATURITY AND ENTERAL FEEDING predispose to development of necrotizing enterocolitis (NEC) in both infants and animal models (5, 32, 39). The incidence of NEC is increased with a rapid transition to full enteral feeding, particularly formula feeding, but can be reduced with colostrum and minimal enteral feeding (4, 32). A period of total parenteral nutrition (TPN) is often required before enteral feeding is tolerated in preterm infants (19). TPN has been advocated to improve neonatal hemodynamic and metabolic stability, prevent NEC development (7), and accelerate body growth (19). Studies in term neonatal piglets have shown that TPN induces mucosal atrophy (35), reduces digestive and absorptive function (8), increases intestinal permeability (24), and enhances the growth of mucolytic bacteria, particularly clostridia (15). Nevertheless, the loss of intestinal function during TPN may be less pronounced in preterm compared with term piglets (36, 38). It is not known whether a period of TPN prior to enteral feeding positively or negatively affects the feeding-associated gut trophic response and NEC incidence in preterm neonates (34).

The gastrointestinal responses to different diets fed just after birth have been investigated in both term infants and pigs. In newborn term pigs, cow colostrum and infant formula differ from sow colostrum feeding regarding some intestinal functions (enzyme activities, nutrient absorption) but both formula and colostrum diets are well tolerated and induce normal intestinal mucosal growth with absence of intestinal atrophy or inflammation (20). In contrast, our recent studies on preterm pigs showed that formula feeding immediately after birth rapidly induced mucosal atrophy, dysfunction, and NEC development in a significant proportion of animals (40–57% incidence) (5, 39). Such detrimental responses were absent in both fetal piglets fed the same formula in utero, newborn germ-free formula-fed preterm pigs, and newborn preterm piglets fed sow’s colostrum just after birth (5, 39). This indicates that dietary factors, bacterial colonization, and certain birth-related events all play important roles in NEC development. That the intestinal microflora may play a key role in NEC development is further suspected by the preventive effects of probiotics and antibiotics (9, 27) and increased density of certain pathogens (e.g., Clostridia) in previous NEC studies (9, 14).

We hypothesized that the potential beneficial metabolic effects of TPN, prior to the start of enteral feeding, could help preterm neonates to resist feeding-induced mucosal dysfunction and inflammation. To investigate whether the previously reported protective effect of colostrum depends on provision of...

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species-specific colostrum, we also compared sow and cow colostrum feeding. First, we performed a large screening study of NEC incidence and intestinal growth in preterm pigs after feeding sow’s colostrum, cow’s colostrum, or formula for 20–40 h, and with or without a prior 2- to 3-day TPN period. Spontaneous NEC tends to be an epidemic disease with variable incidence also in preterm piglets, and we needed a large sample size to be able to conclude whether a few days of TPN would affect NEC incidence relative to the alternative, enteral feeding just after birth. On the basis of the first study, we investigated in more detail the gut responses to enteral nutrition after 3 days of TPN. Intestinal structure, digestive and absorptive functions, mucosal bacterial community, and nutrient fermentation were analyzed and related to enteral diet and NEC incidences. Finally, concentrations of vitamin E (α-tocopherol), selected plasma amino acids, and tissue inducible nitric oxide synthetase (iNOS) activity were recorded because of their potential roles in the diet-induced inflammatory responses.

MATERIALS AND METHODS

Experiment 1: NEC Incidence and Gut Growth With and Without TPN

Animals. The incidence of NEC and the gut trophic response to enteral feeding were studied in groups of piglets fed enterally immediately after birth or after 2–3 days of TPN administration. Hence, 435 preterm piglets from 47 pregnant sows were delivered by caesarean section (105–108 days gestation) and allocated to eight treatment groups. Four groups received no TPN: one group of newborn preterm section (105–108 days gestation) and allocated to eight treatment preterm piglets from 47 pregnant sows were delivered by caesarean enteral feeding were studied in groups of piglets fed enterally imme-

Porcine colostrum was collected manually from different sows (Large White × Landrace, Research Station Sjælland II, Denmark) within 6 h of completed farrowing. Bovine colostrum was collected from one dairy cow (Holstein Friesian, Gjordslev gods, Denmark) from the first milking after parturition. The formula was made of three commercially available products used for feeding infants (per liter of water: 80 g Pedipette 2-0, 70 g Maxipro, and 75 ml Liquigen-MCT, all products kindly donated by SHS International, Liverpool, UK). The macronutrient content per liter of formula was as follows: energy, 4,140 kJ; protein (mainly whey protein concentrate), 64 g; carbohydrate (mainly glucose), 45 g; fat, 61 g (saturated 44 g; monounsatu-
rated 10 g; polyunsaturated 4 g); sodium, 0.30 g; potassium, 0.64 g; calcium, 0.59 g; and phosphorus, 0.42 g (5, 20, 39). The macronutrient content of the formula was designed to match the composition of sow’s milk during midlactation (not sow’s colostrum) (25). Sow’s and cow’s colostrum have higher protein concentrations (145 g protein/l) due to high immunoglobulin contents. In newborn animals, immunoglobulins are transported by pinocytosis to the circulation as intact molecules and do not as such contribute to the plasma amino acid pool. Colostrum and formula were stored at −20°C until used.

The TPN solution was infused continuously for 2–3 days via the arterial catheter by use of automatic infusion pumps (Infusomat Secura, Braun). The nutritional goal was to provide the pigs with sufficient energy and protein to allow for a slightly positive energy balance. The infusion rate was 4 ml kg⁻¹ h⁻¹ in the first 24 h postpartum, gradually increasing to 6–8 ml kg⁻¹ h⁻¹ during the subsequent 1–2 days. This way, the pigs received an average of 450 kJ kg⁻¹ day⁻¹, 6.5 g amino acids kg⁻¹ day⁻¹, and a fluid intake of 160 ml kg⁻¹ per day during the TPN period. In contrast to humans, transplacental transport of immunoglobulins does not occur in pigs, which makes neonatal pigs dependent on colostral immunoglobulins for passive immunization (40). Therefore all the pigs were immunized with maternal serum (5 ml/kg at 6, 12, and 18 h and 9 ml/kg at 24 h postpartum) that was injected via the arterial catheter. The serum was separated aseptically from maternal blood (4,000 g, 4°C, 10 min) that was collected from a maternal uterine vein at the time of surgery.

Tissue collection and clinical NEC evaluation. The piglets were evaluated for clinical symptoms of intestinal disease every 3 h (feeding intolerance, stool consistency and color, abdominal distension, and respiratory distress). If severe respiratory distress or abdominal distension was observed, euthanasia and tissue collection were instituted immediately. In each litter of pigs, individuals started to show NEC symptoms from −20 h after the start of enteral feeding. All pigs in each litter were killed from 20–40 h after the start of enteral feeding to maintain the time frame of enteral food exposure and NEC observation similar among groups. All pigs were euthanized for tissue collection (pentobarbital sodium, 200 mg/kg, intra-arterially through the umbilical catheter). The entire gastrointestinal tract (GIT) was removed and macroscopically evaluated for pathological changes indicative of inflammation or necrosis, and digital pictures were taken of the entire GIT. The empty small intestinal weight was determined, after which a 10-cm segment was taken from each intestinal region (proximal, middle, distal) and slit along its length, and the mucosal layer was gently scraped off with a slide. For each segment, the proportion of mucosa and muscularis were determined on a dry matter basis.

The following clinical NEC scoring system was applied to charac-
terize the extent to which damage as indicated by macroscopic evidence of inflammation, edema, hemorrhage, necrosis, and pneu-
motasis intestinatis occurred in the stomach, small intestine, and/or colon: 1, no or minimal focal hyperemic gastroenterocolitis; 2, mild focal gastroenterocolitis; 3, moderate locally extensive gastroenterocolitis; 4, severe locally extensive hemorrhagic gastroenterocolitis; 5, severe locally extensive hemorrhagic and necrotic gastroenterocolitis;
When a pig was given a NEC score of 3 or more for one of the gastrointestinal sections, this animal was considered positive for NEC in the clinical NEC incidence calculation. In experiment 2, this macroscopic score was combined with a microscopic histopathological scoring (see below).

Experiment 2: Gut Responses to Enteral Nutrition in Preterm TPN Pigs

Animals. Forty preterm piglets were obtained from four litters delivered by cesarean section at 107–108 days of gestation (Duroc × Yorkshire × Danish Landrace, Research Station Sjælland II, Denmark, term = 115 ± 2 days) and reared in incubators as described above (38, 39). During 3 days of continuous TPN, the pigs from each litter were randomly divided into four groups. The first group served as controls to illustrate gastrointestinal morphology and function following a 3-day TPN period and were killed for tissue collection immediately after completion of the TPN period (TPN, n = 7). The other three groups were switched to full enteral nutrition and fed different milk diets (15 mL·kg⁻¹·h⁻¹) in the following 32–42 h as described previously (5, 39) and for experiment 1 above: sow’s colostrum (TPN-Sow, n = 9), cow’s colostrum (TPN-Cow, n = 8) and formula (TPN-Form, n = 12). Throughout the experiment, rectal temperature and oxygen saturation for the piglets were monitored, and during the enteral feeding period potential clinical signs of NEC (feeding intolerance, abdominal distension, hemorrhagic diarrhea, and respiratory distress) were recorded every 3 h. To minimize variation resulting from age differences, and to maintain the tissue collection protocol identical to that used in previous studies on preterm pigs (5, 39), euthanasia and tissue collection for remaining pigs were initiated at 32–38 h after the start of enteral feeding when one or more piglets in a litter had already been euthanized because of clinical signs of NEC.

Blood sampling, lactose digestion, and intestinal permeability studies. Every 24 h during the TPN period and every 6 h during the enteral feeding period, arterial blood samples (1.5 mL) were collected into heparinized syringes. Blood glucose and urea measurements (Reflotron, Roche, Mannheim, Germany) and blood-gas analyses were performed immediately after blood sampling (Rapidlab, 348 pH/blood-gas analyzer, Bayer, East Walpole, MA). Remaining blood was transferred to ice-chilled EDTA-containing tubes and centrifuged (4,000 g, 4°C, 8 min), and plasma was stored at −20°C until biochemical analyses. For determination of amino acid concentrations, plasma was transferred to tubes containing sulfosalicylic acid and snap frozen in liquid nitrogen.

To estimate in vivo lactose digestive capacity, piglets were fasted for 6 h at the end of the 1- to 2-day enteral feeding period and subsequently given a bolus (15 mL/kg) of a 10% lactose solution (Sigma, St. Louis, MO) through the orogastric tube. Blood samples were retrieved into heparinized syringes through the umbilical artery catheter at time t = 0, 10, 20, 30, 40, and 70 min after the bolus was given. All samples were centrifuged within 10 min (6,000 g, 4°C, 2 min), and plasma was isolated and frozen at −20°C for later analysis of galactose concentration as a reflection of lactose digestion and absorption as described previously (41). This test was only carried out for the TPN-Sow and TPN-Form groups. To measure in vivo gut permeability, the piglets were given a bolus of 0.5 g lactulose/kg and 0.3 g mannitol/kg (Sigma) at 3–5 h before tissue collection. Concentrations of mannitol and lactulose in urine collected by cystocentesis at tissue collection were analyzed as described previously (6).

Tissue collection with combined clinical and histopathological NEC evaluation. Organs were weighed and samples were taken from the entire gut for later analyses, as described previously (38). In addition to the clinical NEC evaluation, as described for experiment 1, a histopathological evaluation was performed on paraformaldehyde-fixed intestinal samples. The samples were embedded in paraffin, sectioned (3 µm), and mounted on slides to be stained with hematoxylin and eosin. Mean villus height (µm), villus width (µm), and crypt depth (µm) were measured in 15 representative vertically well-oriented villus crypt columns by use of a light microscope (Orthoplan, Leitz, Germany) and NIH Image J software (version 1.22, National Institutes of Health, Bethesda, MD). Villus morphology data were collected only from sections without gross necrotic lesions. In all sections, disruption of villus architecture, separation of submucosal layers, blood congestion, and transeptal necrosis were noted as previously described (5). These histopathological observations were used in combination with the macroscopic evaluation done at autopsy (clinical NEC score) to determine the final NEC score.

Cell proliferation. To assess regenerative ability, proliferating cells were identified in the crypts by immunohistochemistry. Antibodies directed against proliferating cell nuclear antigen (PCNA) were used by following the manufacturer’s instructions (Dakocytomation, Glostrup, Denmark). Briefly, all tissue sections were deparaffinized and processed for antigen retrieval by heat treatment with microwaves (3 × 5 min, 0.01 M citrate). Sections were then washed with 0.05 M PBS (pH 7.4) and blocked in 10% goat serum for 30 min at room temperature. Sections were incubated with mouse anti-PCNA antibody (PC10, DAKO M879, Dakocytomation) for 20 h at 4°C. Sections were then washed and incubated for 1 h with Alexa 488 goat anti mouse IgG-FITC-conjugated secondary antibody (A-11029, Molecular Probes, Invitrogen, Taasstrup, Denmark) preabsorbed for 24 h with swine serum. Finally, a nucelic counterstain was performed using Hoechst 0.1 µg/ml for 1 min. All incubations were carried out in a humidified chamber to minimize evaporation of antibody solution. After being washed all sections were mounted with Fluorescent Mounting Medium (DAKO). Total nuclei and PCNA positive cells were counted in 15 well-oriented crypts for each section.

Gut tissue and digesta analyses. Pancreatic trypsin and chymotrypsin activities and intestinal brush-border enzyme activities were measured for three segments of the small intestine and the proximal colon as described previously (38–40). Colonic tissue was included because digestive enzymes are temporarily present here during the early neonatal period. Measurement of intestinal iNOS activity was quantified on the basis of conversion of L-[¹⁴C]arginine to L-[¹⁴C]citrulline in intestinal tissue extracts as described previously (35, 46). The activity was related to DNA concentration in the tissue which was analyzed spectrophotometrically. The concentration of short-chain fatty acid (SCFA) in digesta samples was measured as described previously using gas chromatography (21).

TNF-α mRNA levels were measured as a marker of local tissue inflammation. Total RNA was extracted from individually frozen samples (50–100 mg) of proximal and distal small intestine by using TRIzol reagent (Invitrogen, Taasstrup, Denmark) as described previously (13). The primers used to detect porcine TNF-α (pTNF-α) mRNA were pTNF-α forward (5'-GGTGATCCCTACTAATCCTCCC-3') and pTNF-α reverse (5'-TAGACCTGCCCAGATTGCAG-3'). β-Actin primer sets were included as housekeeping control genes as already described (5).

Microbial community analyses. Terminal restriction fragment length polymorphism (T-RFLP) analysis was done using distal small intestinal tissue and colon luminal content, respectively. DNA from the samples was extracted and purified as described previously (42). Four replicate 50-µl PCR mixtures were made from each sample using the universal eubacterial primer pairs S-D-Bact-0099-f-S-20 (5'-AGAGTTTGATCMTGGCTCAG-3') and the T-RFs with a relative abundance higher than 0.05% were measured as described previously using gas chromatography (21).

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2% were selected for further analysis. Potential bacterial species were identified from a swine-specific clone library (29) incorporated in TORAST (Dresden University of Technology, Dresden, Germany). The potential clones were aligned with the WEB program BLAST (http://www.ncbi.nlm.nih.gov) to find the closest cultivable isolate.

**Bacterial fluorescent in situ hybridization.** The location and identity of bacteria adhering to the mucosa or present in the mucosa (e.g., invasive pathogens) was investigated by using specific bacterial oligonucleotide probes and fluorescent in situ hybridization on formaldehyde-fixed sections of distal small intestine (23). All bacteria not firmly attached to mucosal or food residue surfaces were excluded (in contrast to the T-RFLP analyses of frozen sections). Fixed tissue samples were analyzed from all pigs in all treatment groups. In addition, distal intestinal samples were analyzed from newborn preterm pigs ($n = 5$) obtained just after birth by caesarean section. Tissue sections (3 μm) were deparaffinized in xylene, transferred to ethanol, and circumscribed with a hydrophobic PAP-pen (Daido Sangyo, Tokyo, Japan) prior to hybridization at a temperature of 49°C. For hybridization, a general probe targeting all domain bacteria was used (EUB338, systematic name: S-D-bact-0338-a-A-18, sequence: GCT-GCCTCCCGTAGGAGT) (1) and a specific probe targeting Clostridium perfringens (systematic name: S-S-Cl.perf.-185-a-A-18, sequence: TGGTTGAAATGATGATGCC, MWG-Biotech, Ebersberg, Germany). The latter probe was tested with pure cultures of known bacteria and found to be specific for the target species. The probes were 5’-labeled with either FITC (green color) or isothiocyanate derivative Cy3 (red color). An ArrayWoRx microarray scanner (Applied Precision, Issaquah, WA) was used for scanning in situ hybridization of the entire intestinal cross sections. Since the resolution of the scanner was 5.06 quah, WA) was used for scanning in situ hybridization of the entire intestinal weight and NEC incidence were analyzed using Yates-corrected $\chi^2$ values. In experiment 2, the data were analyzed by a two-way ANOVA using the MIXED procedure of SAS (SAS/STAT version 8.1, SAS Institute, Cary, NC). Diet (TPN, TPN-Sow, TPN-Cow, and TPN-Form) and intestinal region (proximal, middle, and distal) were considered as fixed effects whereas pig and litter were considered as random effects. Sample sizes for all analyses were $n = 7$ (TPN), $n = 9$ (TPN-Sow), $n = 8$ (TPN-Cow), and $n = 12$ (TPN-Form), unless otherwise indicated. The values in TPN-Sow and TPN-Cow were often similar and are then referred to as pooled mean values for colostrum-fed pigs ($n = 17$). Furthermore, for some intestinal dimensions there were no differences between enteral diets and they were pooled for comparison of TPN vs. enteral feeding ($n = 29$). The results are given as the means ± SE and differences between two means were tested by the least significant difference test. The NEC scoring system is comprised of nonparametric data, and therefore the median NEC score was calculated for each group and statistically evaluated by the Mann-Whitney test for statistical evaluation of plasma amino acid and galactose concentrations, repeated-measures ANOVA analyses were performed and post hoc comparisons were carried out by use of the Bonferroni test. $P = 0.05$ was used as the critical level of significance for all statistical evaluations.

**RESULTS**

**Experiment 1: NEC Incidence and Gut Growth With and Without TPN**

A significantly higher NEC incidence was observed in piglets fed formula immediately after birth, relative to those fed sow’s or cow’s colostrum ($P < 0.05$, Fig. 1). The same diet effect was present for pigs fed enterally after 2–3 days of TPN but the NEC incidences for both the TPN-Sow (26%) and the TPN-Form (62%) groups were significantly higher than for the corresponding Sow and Form groups (5 and 39%, respectively). Across all pigs fed cow’s colostrum ($n = 46$), only two (4%) developed NEC. Consequently, the overall NEC incidence (across diets) was significantly higher for pigs fed enterally after TPN (46%, $n = 179$) than for pigs fed enterally just after birth (30%, $n = 227$, $P < 0.001$).

Enteral feeding induced a marked increase in intestinal weight in all groups of preterm pigs, with the highest values observed in preterm pigs fed sow’s colostrum just after birth (+82% increase, Fig. 1). Total intestinal mass did not change after 2–3 days of TPN administration, despite an increased

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**Statistical Analysis**

In experiment 1, differences between individual diet groups for intestinal weight and NEC incidence were analyzed using Yates-corrected $\chi^2$ values. In experiment 2, the data were analyzed by a two-way ANOVA using the MIXED procedure of SAS (SAS/STAT version 8.1, SAS Institute, Cary, NC). Diet (TPN, TPN-Sow, TPN-Cow, and TPN-Form) and intestinal region (proximal, middle, and distal) were considered as fixed effects whereas pig and litter were included as random effects. Sample sizes for all analyses were $n = 7$ (TPN), $n = 9$ (TPN-Sow), $n = 8$ (TPN-Cow), and $n = 12$ (TPN-Form), unless otherwise indicated. The values in TPN-Sow and TPN-Cow were often similar and are then referred to as pooled mean values for colostrum-fed pigs ($n = 17$). Furthermore, for some intestinal dimensions there were no differences between enteral diets and they were pooled for comparison of TPN vs. enteral feeding ($n = 29$). The results are given as the means ± SE and differences between two means were tested by the least significant difference test. The NEC scoring system is comprised of nonparametric data, and therefore the median NEC score was calculated for each group and statistically evaluated by the Mann-Whitney test for statistical evaluation of plasma amino acid and galactose concentrations, repeated-measures ANOVA analyses were performed and post hoc comparisons were carried out by use of the Bonferroni test. $P = 0.05$ was used as the critical level of significance for all statistical evaluations.
mucosal mass (+33%, $P < 0.001$). Nevertheless, total intestinal mass increased when enteral food was introduced (+35–60%, Fig. 1) to reach a mass similar to that in pigs fed enterally immediately after birth. Among the three enteral diet groups, feeding-induced increase in intestinal mass was similar when expressed as mucosal dry mass (+25–50%) in pigs exposed to TPN, whereas for newborn pigs without prior TPN the increase in mucosa was +100–150%.

**Experiment 2: Gut Responses to Enteral Nutrition in Preterm TPN Pigs**

There was no significant difference in body weight at birth among the three feeding groups (average 1.222 ± 52 g), following 3 days of TPN (average 1.305 ± 57 g) or enteral feeding (average 1.337 ± 59 g, Table 1). Mean rectal temperature in the pigs immediately after delivery was 36.7 ± 0.1°C. Despite the fact that the piglets were kept in heated incubators, the rectal temperature dropped to an average of 34.2 ± 0.2°C within half an hour and subsequently slowly increased to 36.5 ± 0.3°C at 12 h postpartum. The rectal temperature after half an hour in heated incubators was negatively correlated to birth weight ($P < 0.01$).

During the TPN period, the mean partial pressure of CO$_2$ decreased from 63.9 ± 5.2 mmHg shortly after delivery to 53.2 ± 2.2 mmHg at 24 h and to 44.2 ± 2.6 mmHg at 72 h postpartum ($P < 0.05$). Mean PO$_2$ increased from 47.6 ± 8.4 mmHg at delivery to 113 ± 9.2 mmHg at 24 h ($P < 0.01$) and remained at that level the subsequent 2 days. Nevertheless, because of a decreasing concentration of hemoglobin from 8.7 ± 0.3 at delivery to 5.9 ± 0.3 g/100 ml 72 h later ($P < 0.05$), the oxygen concentration in blood decreased from 10.3 ± 0.8 at delivery to 7.5 ± 0.4 ml/100 ml at 72 h ($P < 0.05$). Mean pH increased from 7.34 ± 0.02 at delivery to 7.40 ± 0.03 during the first 24 h and remained at that level for the subsequent days. At delivery, blood glucose was 2.69 ± 0.17 mmol/l and decreased during placement of catheters to 1.06 mmol/l just prior to TPN administration and remained low (3.5 ± 0.1 mmol/l) during the TPN period.

During the 3 days of TPN administration, no defecation was observed although the meconium plug was passed in a few pigs and no pigs developed NEC. Initially, the shift to enteral feeding was well tolerated for all pigs. Formula-fed pigs started to develop diarrhea at 12–24 h, whereas colostrum-fed pigs passed normal stools after -24 h. At the time of tissue collection, 32–38 h following institution of enteral feeding, clinical symptoms of NEC were observed in six of twelve TPN-Form pigs (50%), in one of nine (11%) TPN-Sow pigs, and in none of the TPN-Cow pigs. However, by later macroscopic and microscopic evaluation of the intestine, even more pigs could be identified as having the pathological changes characteristic of NEC. Hence, four of nine (44%) TPN-Sow and three of eight (38%) TPN-Cow pigs had NEC-like inflammatory and necrotic lesions in part of their GIT, whereas 10 of 12 (83%) TPN-Form pigs had severe local or extensive signs of NEC. Formula-fed pigs with NEC had a higher median NEC score (5, range 3–6, $n = 10$) than colostrum-fed NEC pigs (3, range 3–5, $n = 7$, $P < 0.05$). Histologically, villus destruction was most severe in the TPN-Form group whereas separation of the submucosal layers was the most prominent finding in the seven colostrum-fed pigs with signs of NEC. Lesions were primarily located to the distal small intestine and colon but the stomach was affected in several pigs. Three TPN-Form pigs had extensive NEC involving the entire GIT. In the pigs with necrotic stomachs, there was a marked increase in plasma pepsinogen (9.8 ± 1.7 ng/ml, $n = 9$) compared with pigs with clinically normal stomachs (3.8 ± 2.9 ng/ml, $n = 20$, $P < 0.001$).

**Gut Mass, Morphology, and Permeability**

Compared with intestinal dimensions after 3 days of TPN, enteral feeding increased intestinal length (368 ± 12 vs. 335 ± 12 cm, $P < 0.05$), intestinal weight (Table 1), proportion of mucosa (68 ± 2 vs. 63 ± 2%, $P < 0.05$), and villus width (83 ± 1 vs. 75 ± 2 μm, $P < 0.05$), with no differences among the three enteral feeding groups. Villus height in the distal intestine was higher in the two colostrum groups compared with TPN and TPN-Form (Fig. 2), although the values were similar in the proximal part among all four groups (808 ± 32 μm). There was no difference in crypt depth between the enteral feeding groups (Fig. 2). Compared with TPN pigs, the number of PCNA-positive cells was unaltered with formula feeding whereas TPN-Cow increased PCNA positive cell counts (Fig. 2). Following an oral lactulose-mannitol bolus, the urinary lactulose-to-mannitol ratio was significantly lower in the TPN-Cow group compared with TPN, TPN-Cow, and TPN-Form (Fig. 2, $P < 0.05$). The high ratio for the TPN-Cow group reflected a low absorption of mannitol relative to the other groups. Formula feeding increased the relative weight (g/kg body wt) of the stomach by 50%, compared with TPN, and the relative colon weight by 30%, compared with the TPN and TPN-Sow groups (Table 1). Pancreatic relative weight was decreased for TPN-Cow (1.60 ± 0.05 g/kg body wt) compared with TPN-Sow (1.96 ± 0.11 g/kg body wt). The relative weights (g/kg body wt) of the adrenal glands were increased for the TPN-Cow (0.20 ± 0.02) and TPN-Form groups (0.21 ± 0.02) vs. TPN (0.14 ± 0.02) and TPN-Sow (0.17 ± 0.01) groups ($P < 0.05$). The relative weight of the kidneys was

Table 1. Body weight, relative gut weights, and NEC incidence in preterm pigs

<table>
<thead>
<tr>
<th></th>
<th>TPN (n = 7)</th>
<th>TPN-Sow (n = 9)</th>
<th>TPN-Cow (n = 8)</th>
<th>TPN-Form (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body, kg</td>
<td>1.42 ± 0.13</td>
<td>1.32 ± 0.12</td>
<td>1.16 ± 0.15</td>
<td>1.38 ± 0.13</td>
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<tr>
<td>Small intestine, g/kg body wt</td>
<td>23.3 ± 1.6a</td>
<td>32.0 ± 2.2b</td>
<td>33.7 ± 1.8b</td>
<td>34.8 ± 2.0b</td>
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<tr>
<td>Stomach, g/kg</td>
<td>5.12 ± 0.16a</td>
<td>6.64 ± 0.95ab</td>
<td>6.29 ± 0.60ab</td>
<td>7.67 ± 0.92b</td>
</tr>
<tr>
<td>Colon, g/kg</td>
<td>6.41 ± 0.57a</td>
<td>6.17 ± 0.35a</td>
<td>7.58 ± 0.66ab</td>
<td>8.17 ± 0.81b</td>
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<tr>
<td>Pigs with NEC</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>10</td>
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Values are means ± SE. Measurements were taken following 3 days of total parenteral nutrition (TPN) alone or with an additional 2 days of enteral feeding with either sow’s colostrum (TPN-Sow), cow’s colostrum (TPN-Cow), or formula (TPN-Form). NEC, necrotizing enterocolitis. a,bMeans not sharing the same superscript letter differ significantly ($P < 0.05$).
increased for TPN-Cow pigs (9.5 ± 0.6) relative to the other three groups (8.3 ± 0.2, \( P < 0.05 \)). No treatment effects were noted for relative weights (g/kg body wt) of the liver (26.4 ± 0.7), heart (7.2 ± 0.2), or lungs (19.1 ± 0.7).

Enzyme Activities in the Small Intestine, Colon, and Pancreas and In Vivo Lactose Digestion

Compared with TPN pigs, only TPN-Form feeding increased iNOS activity (Fig. 3, \( P < 0.01 \)), although there was a trend for higher levels in TPN-Cow (Fig. 3, \( P < 0.1 \)). Furthermore, the TNF-\( \alpha \) mRNA levels were significantly elevated in TPN-Form pigs compared with colostrum-fed pigs (16.2 ± 2.2 vs. 9.6 ± 1.7 in relative abundance, \( P < 0.05 \)).

Across all brush-border enzymes and intestinal regions, the two colostrum groups had similar mean hydrolytic activities, and these were generally higher than the TPN and TPN-Form groups (Fig. 4). In all three regions of the small intestine, TPN-Form was associated with decreased lactase activity, compared with the other three groups (Fig. 4). In the colon, the tissue, ratio of PCNA-positive cells in intestinal crypts, and urinary lactulose-to-mannitol ratio were observed.

Microbial Community Analyses and SCFA Concentrations

The intestinal microflora was characterized by a very low diversity in both distal small intestinal tissue and colon content, compared with T-RFLP profiles from suckling and older pigs (29, 42). In the tissue of the distal small intestine, only seven different T-RF sizes were detected, of which the bands of 232 and 217 bp made the major relative abundance (Fig. 5). The two T-RFs found in the pig intestinal clone library were different *Clostridium* species (97% similarity), respectively (29). The diversity in the colon luminal content was low, consisting of eleven detected bands, of which the same two T-RFs, *Clostridium* species (232 bp) and *Enterococcus faecium* (99% similarity), and *Enterococcus faecalis* (99% similarity), respectively (29). The diversity in both distal small intestinal tissue and colon content, accounted for most of the total band intensity. The fewest T-RFs were found in TPN pigs, where only bands of 209, 217, and 232 bp were found in the colon content and only one T-RF was identified in the small intestinal tissue at 209 bp (closest known bacteria, *Lactobacillus vitulinus*, similarity 91.8%). Enteral feeding increased the numbers of T-RFs and specifically the band intensity at 232 bp, representing the *Clostridium* species. No major differences among the diets were observed.

To further investigate associations between microbial community and animal health, the percent relative abundance of the average T-RF’s intensities were plotted against clinical NEC-score, representing either no or moderate inflammation (grade 1–3) or typical NEC lesions (grade 4–6). The *Clostridium* species accounted in average for ~70% of the total microbiota in the distal small intestinal tissue for the pigs with the NEC.
lesions (4–6) whereas in the healthy pigs the relative abundances of E. faecium and Clostridium species were more similar (Fig. 5, \(P < 0.05\)). In the colon content the relative abundance of the T-RFs between the two groups of pigs were more similar.

Except for lactic acid, SCFA concentrations were generally below detection limits. In formula-fed pigs, lactic acid concentrations were increased by 60–100% in the distal small intestine (12.4 ± 2.8 mmol/kg) and colon contents (20.7 ± 4.2 mmol/kg), compared with TPN (colon, 8.0 ± 1.6 mmol/kg, \(P < 0.01\)) and colostrum groups (distal intestine, 6.7 ± 0.8 mmol/kg and colon, 11.6 ± 3.0 mmol/kg, both \(P < 0.05\)).

**Fluorescent In Situ Hybridization**

On the basis of the T-RFLP findings, the fluorescent in situ hybridization aimed to investigate the general bacterial and the C. perfringens populations associated with the mucosa of the distal small intestine. Consistent with the low number of bacterial species found in the 3-day-old TPN group by T-RFLP analysis, the corresponding in situ hybridization sections with the general bacterial probe (red color) showed a complete lack of mucosa-adhering microbes (Fig. 6B), similar to the picture shown in the 0-day-old newborn preterm pigs (Fig. 6A). Confirming an earlier study (36), 3-day-old preterm TPN pigs did not show TPN-induced mucosal atrophy, relative to newborn preterm pigs (Fig. 6, A and B). Following enteral feeding, the number of bacteria detected in and around the mucosa in clinically healthy pigs increased in the groups fed sow’s colostrum (Fig. 6C) and cow’s colostrum (Fig. 6E) whereas they were virtually absent in tissues from healthy pigs fed the (sterile) formula (Fig. 6G). All pigs diagnosed with severe NEC had a high number of bacteria adhering to the mucosa, regardless of treatment, sow colostrum (Fig. 6D), cow colostrum (Fig. 6F), or formula (Fig. 6H). Mucosa-associated C. perfringens was detected in half of the fed pigs but their mucosa-associated presence was not consistently related to diet or NEC-like lesions. They remained at the surface of the epithelium in healthy pigs (Fig. 6I) whereas they invaded the mucosal tissues of pigs with NEC (Fig. 6J).

**Plasma Amino Acid and \(\alpha\)-Tocopherol Levels**

Average plasma amino acid levels were calculated for samples taken during both the TPN period (every 24 h) and the enteral phase (every 6 h). The results showed that the concentration of arginine decreased with the introduction of enteral feeding, but most for the formula group (TPN, 275 ± 20 vs. colostrum, 140 ± 9, and formula, 58 ± 6 μg/ml, \(P < 0.01\)). For citrulline the mean value increased for both colostrum groups (TPN, 78 ± 3, and formula, 69 ± 6, vs. colostrum, 125 ± 9 μg/ml, \(P < 0.05\)). A similar trend was present for ornithine with the highest values detected in the two colostrum groups (TPN, 69 ± 9 vs. colostrum, 180 ± 14, and formula, 94 ± 9 μg/ml, \(P < 0.05\)). With the introduction of enteral nutrition, the antioxidant status, as illustrated by \(\alpha\)-tocopherol levels, increased with colostrum feeding in both tissue (Fig. 3) and plasma (2.96 ± 0.32 vs. TPN, 1.14 ± 0.15 μg/ml), but not with formula feeding (plasma, 1.31 ± 0.18 μg/ml and Fig. 3).

**DISCUSSION**

Our previous studies showed that the preterm piglet is a clinically relevant animal model of NEC and is characterized by hallmark clinical and histological signs of NEC observed in human infants. Moreover, we showed that the NEC incidence is significantly higher in formula compared with colostrum-fed piglets and occurred exclusively in preterm and not term piglets. Our previous studies tested the effect of enteral nutrition given immediately at birth, yet in clinical practice many preterm infants receive TPN prior to enteral food introduction. Thus one of the aims of the present study was to test whether an initial period of TPN administration prior to enteral feeding would affect NEC incidence and the gut trophic response. Secondly, we wished to study the responses to three different diets following TPN. Using a large sample material to account for the variable NEC incidence, we show that 2–3 days of TPN, prior to enteral feeding, results in an elevated NEC incidence in both formula- and sow colostrum-fed pigs. This occurred despite the fact that TPN improved blood chemistry values and body temperatures and increased mucosal growth. We conclude that an initial TPN period does not improve NEC resistance in preterm pigs but may actually sensitize the intestine to inflammation.

Despite the fact that NEC was present in both formula- and colostrum-fed pigs in experiment 2, the clinical, structural, and...
biochemical indices suggested that the intestine remained healthier in the colostrum groups. The partly protective effects of colostrum could result from the combined actions of colos-tral nutrients and bioactive components (e.g., immune-modu-lating compounds, antioxidants, and growth factors). These protective effects appear to be species independent, because cow’s and sow’s colostrum had similar effects on NEC incidence and the majority of intestinal functional indexes. Clinical symptoms of NEC were not observed during TPN administra-tion and generally occurred in individuals after 20–35 h of enteral feeding. Our ongoing studies have shown that few pigs, if any, develop NEC lesions during the following 2–3 days (our unpublished results). This highlights the causative role of enteral feeding introduction in this animal model rather than the possible negative effects of hemodynamic instability, hypoxia and hypothermia occurring in the first 24 h after birth. Compared with earlier studies (5, 39), the crypt depth and iNOS activity in sow colostrum-fed pigs were increased to levels only observed in formula-fed preterm piglets previously. Additionally, even colostrum-fed pigs were frequently as-sociated with NEC lesions and bacteria were present in high numbers not only along the surface of the epithelium but also invading the mucosal barrier. This supports the conten-tion that TPN, or the lack of enteral feeding, acts as a “first hit,” increasing the inflammatory response to a secondary insult (28, 34). Compared with colostrum-fed pigs, formula-fed pigs had a less preserved villus architecture and more severe NEC lesions with or without pneumatosis intes-tinalis, and in some cases the lesions and edema (leading to higher wet weights) included the stomach and colon. Stom-acch inflammation and necrosis resulted in increased leakage of pepsinogen into the systemic circulation, which may provide a diagnostic marker of NEC development in this region. Across the groups, enteral feeding also lead to a

Fig. 4. Brush border enzyme [lactase, maltase, dipeptidyl peptidase IV (DPPIV), and aminopeptidase N (ApN)] specific activity in piglets following 3 days of TPN (n = 7) alone or with an additional 2 days of enteral feeding with either sow’s co-lostrum (n = 9), cow’s colostrum (n = 8), or formula (n = 12) (means ± SE). Means not sharing the same letter differ significantly (P < 0.05).

Fig. 5. Relative abundance values for CfoI terminal restriction fragments (T-RFs) in samples of distal small intestinal tissue relative to the NEC score [1–3 = healthy or moderately inflamed (n = 13); 3–6 = NEC (n = 13)]. Data are average values ± SE for the 2 groups of pigs. T-RF 217 bp represent Enterococcus faecium (similarity 99%) whereas T-RF 232 bp represent different Clostridium species, including C. perfringens (similarity 97–99%) (*P < 0.05).
Fig. 6. Representative pictures of bacterial in situ hybridization carried out on formaldehyde-fixed whole mount cross sections from the distal intestine of preterm pigs at birth (A), following 3 days of TPN (B) or with an additional 2 days of enteral feeding with sow’s colostrum (C and D), cow’s colostrum (E and F), or formula (G and H). Hybridization with a general bacterial probe (red color, A–H) showed that no microcolonies of bacteria were attached to tissues from newborn or 3-day-old TPN pigs (A and B). Yellow staining represents blood residuals in vessels or congested and hyperemic tissue. Clinically healthy pigs fed sow’s or cow’s colostrum (C and E) had a variable density of adhered bacteria (white arrows) whereas no microcolonies were detected in healthy formula-fed pigs (G). NEC lesions were associated with intense bacterial overgrowth, pneumatosis intestinalis, mucosal atrophy, or complete loss of villi (D, a pig fed sow’s colostrum), mucosal atrophy, and overgrowth of bacteria attached to both mucosal surfaces and luminal food remnants (F and H, pigs fed cow’s colostrum and formula, respectively). When colonies of rod-formed \textit{C. perfringens} were present in healthy pigs (green color, I, a pig fed sow’s colostrum), they remained at the surface of the villus epithelium, whereas they completely invaded the mucosal epithelium of pigs showing necrotic NEC lesions in the presence of villus-associated \textit{C. perfringens} (arrows in J, a pig fed sow’s colostrum).
degree of adrenocortical activation, as indicated by elevated adrenal gland weights.

Additional studies are required to clarify whether and why TPN may sensitize the preterm intestine to development of NEC following introduction of enteral food. It cannot be excluded that the nature of the TPN solution itself plays a role. The lipid emulsion in the TPN solution used in this experiment was based on soybean oil, containing a high content of n-6 polyunsaturated fatty acids. Soybean oil intravenous emulsions have been suggested to exhibit immunosuppressive effects resulting in impairment of chemotaxis, phagocytosis, antigen-presenting cell function, and cytokine production in vitro (26, 37), although the evidence is less clear from in vivo studies (44). Soybean-based intravenous emulsions have also been shown to cause increased pulmonary vasoconstriction in piglets (3), possibly through thromboxane production. General or intestinal ischemia-reperfusion is suggested to play a major role in the development of NEC, and thus increased vasoconstriction may add to the susceptibility to NEC in a preterm neonate with compromised hemodynamics (11, 12).

Despite a decreased villus height in clinically healthy formula-fed pigs, there was no increase in crypt depth or number of proliferating cells in the crypts relative to colostrum-fed pigs. This could reflect a further decreased regenerative ability associated with a subclinical inflammatory response that was evident from increased tissue TNF-α mRNA and iNOS activity in pigs that appeared clinically healthy at the time of tissue collection. The increase in tissue iNOS in formula-fed pigs could explain the lower plasma levels of arginine since this is the substrate for nitric oxide synthesis. The product of the nitric oxide synthase reaction, citrulline, and also ornithine were decreased in formula-fed pigs. Alternatively, decreased plasma arginine and citrulline levels may reflect decreased endogenous intestinal synthesis due to prematurity or gastrointestinal injury (47). Antioxidant capacity, indicated by vitamin E (α-tocopherol) concentrations, in formula-fed piglets was markedly decreased both in tissue and plasma, and increased iNOS activity may further deplete arginine and antioxidant stores. The villus atrophy and inflammation was coupled with loss of mucosal integrity and compromised tight junction function on the basis of increased mucosal permeability estimated via the urinary lactulose-to-mannitol ratio in formula- vs. colostrum-fed pigs. Collectively, the results indicate that formula feeding was associated with more mucosal inflammation and greater loss of mucosal integrity.

Formula feeding decreased the specific activity of most brush-border enzymes relative to colostrum feeding, in both the small intestine and the colon. The decreased lactase activity was associated with impaired intestinal lactose digestive capacity, and/or lowered hexose absorptive capacity as indicated by lower plasma galactose following an oral lactose bolus in formula- vs. colostrum-fed piglets. This, together with the lowered maltase and peptidase activities in formula pigs, may have caused a general reduction in both carbohydrate and protein digestion, rapidly leading to diarrhea in the formula group. As a result of decreased digestive capacity, undigested nutrients may facilitate bacterial overgrowth and fermentation, as indicated by increased luminal lactic acid concentrations. SCFA may play a direct role in NEC development (10, 16) since acetic acid has been shown to induce colitis in rats (33) and prolonged exposure to excessive amounts of SCFA causes mucosal injury (31). Furthermore, a lowered pH may promote proliferation of potentially pathogenic bacteria, such as C. perfringens (9).

On the basis of the molecular fingerprint (T-RFLP) of the gastrointestinal microflora in this study, TPN immediately after delivery by caesarean section led to a microbial community of only few species after 3 days. The small intestinal tissue mainly harbored a single strain, whereas the colon luminal content was dominated by only three strains (including a group mainly consisting of Clostridia). In the absence of luminal nutrients, we did not see an upregulation of mucolytic bacteria like Clostridia as reported previously (8, 15), possibly because our pigs were completely deprived of enteral feeding before TPN. However, when enteral nutrition was introduced after TPN, there appeared to be a major growth in Clostridia numbers, especially at the tissue level. The presence and intensity of the Clostridium band seemed not to be associated with the type of enteral diet, but more with the degree of NEC lesions across enteral diets. It is noteworthy that the increase in Clostridia in the mucosa of the distal small intestine was not present to the same extent in colon contents, suggesting that the more transient bacteria in fecal samples may not always be an accurate reflection of the flora resident at the intestinal mucosa, including Clostridia. Although luminal nutrient fermentation and toxin production by Clostridia species may play a significant role, the lack of mucosa-associated C. perfringens in several NEC pigs (both formula and colostrum) suggest that clostridial mucosal colonization is not a major determinant of NEC. We have also been unable to detect mucosa-associated C. butyricum, C. difficile, and C. paraputrificum (data not shown) known to be key pathogens in a quail model of NEC (45). The correlation between NEC lesions and Clostridia species by T-RFLP analyses may thus suggest a role mainly for bacteria present in the lumen or in the mucous layer that are preserved for the T-RFLP analyses but absent at in situ hybridization on formaldehyde-fixed tissues. It is currently unknown whether the identified differences in microbial community profiles are the result of NEC or play a role in the pathogenesis of NEC. Regardless, it seems that the etiology of NEC contains a strong microbial component and further studies with pre- and probiotics, potentially using germ-free and gnotobiotic animals, would be helpful to clarify the nature of the interactions.

Perspectives

The present NEC model is clinically relevant and can be used for further investigations of the factors that predispose the preterm neonate to this serious intestinal disease. The spontaneous nature of the model avoids the need to introduce hypoxia, hypothermia, pathogens, or nonphysiological diets, to study NEC development (11, 16–18). In contrast to preterm infants, a large proportion of preterm pigs develop NEC already 1–2 days after the introduction of enteral feeding. Although the incidence of spontaneous NEC in preterm pigs can be quite variable, like in infants, the mechanisms may be similar between the species and allow for prospective controlled studies in a relatively short time frame. Consistent with observations in infants, NEC is rarely seen during an initial TPN period, which may help such preterm neonates to reach a degree of metabolic and hemodynamic stability. Regardless,
introduction of enteral feeding after the TPN period is associated with a high NEC incidence. Feeding either porcine or bovine colostrum may provide some mucosal protection and higher digestive capacity, relative to formula, although all three enteral diets may be associated with intestinal atrophy, malabsorption, and inappropriate bacterial colonization. Our study suggests that minimal enteral feeding could be important in association with parenteral nutrition immediately after birth. Using the preterm pig, we may in the future help to define the nature and amount of enteral nutrients that provide maximal NEC protection and optimal gut and metabolic adaptation in the preterm neonate.

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