Preterm birth makes the immature intestine sensitive to feeding-induced intestinal atrophy

Charlotte Reinhard Bjornvad,1 Mette Schmidt,2 Yvette Miata Petersen,1 Søren Krogh Jensen,4 Hanne Offenberg,3 Jan Elnif,1 and Per Torp Sangild1

Divisions of 1Animal Nutrition, 2Reproduction, and 3Anatomy, Royal Veterinary and Agricultural University, Frederiksberg, Denmark; and 4Danish Institute of Agricultural Sciences, Tjele, Denmark

Submitted 15 November 2004; accepted in final form 8 June 2005

Preterm birth makes the immature intestine sensitive to feeding-induced intestinal atrophy. Am J Physiol Regul Integr Comp Physiol 289: R1212–R1222, 2005. First published June 16, 2005; doi:10.1152/ajpregu.00776.2004.—Preterm birth and formula feeding predispose to small intestinal dysfunction, which may lead to necrotizing enterocolitis (NEC). In piglets, we tested whether the physiological and environmental transitions occurring at birth affect the response of the immature intestine to enteral feeding. Pig fetuses (106 days gestation, term = 115 days) were prepared with esophageal feeding tubes and fed either sow’s colostrum (n = 8) or infant formula (n = 7) in utero. After 24 h of oral feeding, the pig fetuses were delivered by cesarean section and their gastrointestinal morphology and function were compared with those of preterm newborn (NB) littermates that were not fed (n = 8) or fed colostrum (n = 7) or formula (n = 13) for 24 h after birth. Before birth, both colostrum and formula feeding resulted in marked increases in intestinal mass, brush-border enzyme activities, and plasma glucagon-like peptide 2 concentrations, to levels similar to those in NB colostrum-fed piglets. In contrast, NB formula-fed piglets showed reduced intestinal growth, decreased brush-border enzyme activities, and intestinal lesions, reflecting NEC. NB formula-fed pigs also showed impaired enterocyte endocytotic function and decreased antioxidative capacity, whereas brush-border enzyme mRNA levels were unaltered, relative to NB colostrum-fed pigs. Our results indicate that the feeding-induced growth and enzyme maturation of the immature intestine are not birth dependent. However, with a suboptimal diet (milk formula), factors related to preterm birth (e.g., microbial colonization and metabolic and endocrine changes) make the immature intestine sensitive to atrophy and development of NEC.

IN ALL MAMMALS, THERE IS A FUNDAMENTAL TRANSITION IN THE FEEDING-INDUCED INTESTINAL ATROPHY
study, we hypothesized that preterm birth and the associated physiological and environmental changes alter the response of the immature GIT to enteral food introduction in a diet-dependent manner.

The first aim of this study was to investigate the ability of the immature GIT to respond to enteral nutrition fed in utero. Second, we wished to examine whether birth was required for formula feeding to exert a detrimental effect on the immature small intestine. Hence, key parameters of small intestinal structure and function were evaluated in immature pigs fed formula or colostrum, either before or after preterm delivery by cesarean section. Plasma levels of glucagon-like peptide 2 (GLP-2) were measured because this intestinotrophic peptide is responsive to the first oral feed and stimulates intestinal blood flow (3, 31). In addition, because macromolecule endocytosis is a fundamental feature of enterocytes in the perinatal period of piglets, the ability of the small intestine to absorb albumins was recorded (18, 36). Finally, vitamin E levels were determined because this key antioxidant may reduce intestinal damage and necrosis by decreasing oxidative stress (28).

MATERIALS AND METHODS

Surgical Preparation of Sows, Fetuses, and Newborn Piglets

A total of 16 fetuses (F) and 32 newborn (NB) pigs were obtained from five sows (105–106 days of gestation, term is at 115 ± 2 days). All were bolus fed with either sow’s colostrum or formula every 3 h for 24 h, except eight NB piglets that acted as unfed controls and were killed immediately after birth. The National Committee on Animal Experimentation, Denmark, approved the experiments.

Fetal piglets. The pregnant sows (Large White × Landrace) were sedated (1.6 g/kg im azaperone; Janssen, Beerse, Belgium), and a superficial ear vein was catheterized. General anesthesia was induced (5–10 mg/kg iv thiopental sodium; Abbott Laboratories, North Chicago, IL) and maintained with isoflurane (0.5–1 l/min in oxygen; Abbott Laboratories). After intubation, the sow was covered with heating blankets to prevent anesthesia-induced hypothermia, and care was taken to maintain aseptic conditions around a ventral fascial incision. After exposure of the uterus, a maximal number of four fetuses per sow were chosen randomly for surgery. A small incision (5 cm) was made in a relatively nonvascular area of the uterus, and the allantoic and amniotic membranes were then carefully anchored onto the uterine wall incision. The head of a fetus was exteriorized and covered with a sterile wet heating pad to prevent dehydration and cooling. A midline incision was made in the ventral neck, and surrounding tissue was gently separated to expose the fetal esophagus. A silastic catheter (vinyl tube, inner diameter of 0.86 mm, outer diameter of 1.52 mm; Dural Plastics and Engineering, Auburn, Australia) was passed down the esophagus, such that the tip reached the stomach. The esophagus was ligated, and amniotic fluid swallowed by the fetus was returned to the amniotic cavity by a small catheter inserted cranially into the esophagus (36). After catheterization of each fetus, the fetal skin incision, fetal membranes, and uterine incision were sutured separately (39). Antibiotics (2 mg/kg iv enrofloxacin, Baytril; Bayer, Leverkusen, Germany) were administered to the amniotic cavity of the fetus. The ventral fascial incision of the sow was sutured, and finally all catheters were exteriorized on the back of the sow and kept in a small plastic pouch. The duration of the surgery was ~3 h. After the surgery, all of the sows in the study were given antibiotics (20 ml/day im streptomycin; Boehringer Ingelheim, Copenhagen, Denmark) and progesterone (50 mg/day im; University Pharmacy, Royal Veterinary, and Agricultural University, Copenhagen, Denmark) to prevent preterm delivery.

The catheterized fetuses were fed 15 ml every 3 h; two fetuses from the same sow were fed colostrum (F-colostrum group, n = 8) and two were fed formula (F-formula group, n = 8). At the first feeding (0 h), a macromolecule marker, BSA, was included in the milk diets (20 mg/ml, A-4503; Sigma, St. Louis, MO); similarly, at 18 h, human serum albumin (HSA) was included as a marker (20 mg/ml, A-1653; Sigma). Twenty-four hours later, operated and unoperated piglets were delivered by cesarean section under general anesthesia as described previously (33). Of the 16 catheterized fetuses, 15 were alive at the time of delivery. Arterial blood samples were collected in ice-chilled EDTA-containing tubes from the catheterized fetuses and from two unfed control piglets from each sow (NB-unfed group, n = 8). A lethal dose of anesthetic (200 mg/kg pentobarbital sodium; University Pharmacy, Royal Veterinary and Agricultural University, Copenhagen, Denmark) was then administered, and tissue samples were collected. The blood samples were centrifuged (4,000 g, 8 min, 4°C), and plasma was stored at −20°C until biochemical analyses.

Neonatal piglets. The remaining piglets from each sow were each prepared with an orogastric feeding tube (infant feeding tube 6F; Pharmaplast, Roskilde, Denmark) for enteral feeding. To prevent the pigs from chewing the feeding tube, it was passed through the cheek and secured to the skin with sutures. Furthermore a vascular catheter (infant feeding tube 4F; Portex, Kent, UK) was inserted into the dorsal aorta via the transected umbilical cord and sutured to the skin (33). The newborn pigs were reared individually in infant incubators (Air-Shields, Hatboro, PA), which were maintained at 35°C and 80–100% moisture. Throughout the experiment, rectal temperature and arterial oxygen saturation were monitored. Blood samples were collected every 6 h from the piglets, and blood-gas analyses were performed immediately after blood sampling (Rapidlab, 348 pH/blood-gas analyzer; Bayer, East Walpole, MA). Each pig was fed either sow’s colostrum (NB-colostrum group, n = 7) or formula (NB-formula group, n = 13) (15 ml/kg every 3 h) via the orogastric tube until blood sampling and tissue collection 24 h later.

Collection and Preparation of Colostrum and Formula

Porcine colostrum was collected manually from a number of sows (Large White × Landrace; Research Station Sjælland III) within 6 h of completed farrowing. The formula was made of three commercial products used for feeding infants 0–2 yr of age (per liter of water: 80 g Pedipet 2-0, 70 g Maxipro, and 75 ml Liquigen-MCT; all products kindly donated by SHS International, Liverpool, UK). The contents of macronutrients according to the producer were as follows (per liter of solution): energy, 4,140 kJ; protein (mainly whey protein concentrate), 67 g; carbohydrate (mainly glucose), 45 g; fat, 61 g (of which 44 g was saturated, 10 g was monounsaturated, and 4 g was polyunsaturated); sodium, 0.30 g; potassium, 0.64 g; calcium, 0.59 g; and phosphorous, 0.42 g. The energy and protein contents of the formula were designed to match the composition of sow’s milk during lactation (21). Colostrum and formula were stored at −20°C. While frozen, colostrum and formula were sterilized by gamma irradiation (2 × 5.0 kGy; LR Plast, Glostrup, Denmark), and sterility was confirmed by microbiological culturing (Labovet, Glostrup, Denmark).

Tissue Collection

For all pigs, the GIT was macroscopically evaluated for pathological changes, indicative of inflammation or necrosis, and digital pictures were taken of the GIT. The small intestine from the pyloric sphincter to the ileocecal junction was rapidly excised by cutting along the mesenteric border, and its length was measured in a relaxed state. The small intestine was carefully emptied of its contents and then placed on an ice-cold metal plate and divided into three equally long segments, designated proximal, middle, and distal small intestine. The three segments were weighed, and, from the middle of each segment, tissue samples of 2–3 cm were obtained. Only samples of tissue that did not appear necrotic (dark red, brown, or gray in color) were collected for biochemical analyses. These tissue samples were either snap frozen in liquid nitrogen and kept at −80°C for later
determination of enzyme activity and mRNA levels or they were fixed in 4% paraformaldehyde for 48 h and then transferred to 70% ethanol to be stored at 4°C for later histological preparation. Furthermore, a 10-cm segment was taken from each intestinal region to measure intestinal dimensions. For this segment, the proportion of mucosa was determined on a dry matter basis after drying the mucosa and the muscularis layers at 50°C for 72 h. Stomach, colon, and other internal organs (heart, lungs, liver, spleen, pancreas, adrenals, and kidneys) were excised and weighed.

**Clinical NEC Evaluation**

The groups of NB pigs were evaluated for symptoms of intestinal disease every 3 h (stool consistency and color, abdominal distension, pain at palpation, and respiratory distress). At signs of severe pain, euthanasia and tissue collection were performed immediately. At tissue collection, the GIT was visually evaluated for typical signs of NEC. The following clinical scoring system was applied to characterize the extent to which damage, as indicated by gut distension, edema, hemorrhage, necrosis, and/or pneumatosis intestinalis, occurred in one or more regions of the gut (stomach, small intestine, colon): 0 = no damage, 1 = 0–25% affected, 2 = 25–50% affected, 3 = 50–75% affected, and 4 = 75–100% affected. When a pig was given a score of at least 1 for one of the organs, this animal was judged to suffer from NEC.

**Histological Morphology**

The paraformaldehyde-fixed intestinal samples were processed routinely for histology. The samples were embedded in paraffin, sectioned (3 μm), and mounted on slides and 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 using a light microscope (Orthoplan; Leitz) and NIH Image J software (version 1.22c, National Institutes of Health, Bethesda, MD). Villus morphometry data were collected from sections without necrotic lesions. In all sections, necrosis, loss of villi, and disruption of normal mucosal architecture were noted. Histopathological scoring was performed on all sections according to the following criteria: 0 = normal histological appearance, 1 = mild local disruption of villus tips and no changes in the submucosal layer; 2 = local villus disruption and separation of the submucosal layer; 3 = regional villus disruption, blood congestion, and separation of the submucosa and lamina propria; and 4 = extensive complete destruction of villus architecture, blood congestion, and transmural necrosis (Fig. 1). With this histopathological scoring system, piglets with a score of 2 or more were defined as suffering from NEC.

**Plasma Analyses**

Concentrations of BSA and HSA were determined by rocket immunoelectrophoresis as described previously (36), and cortisol concentration in plasma was determined by ELISA (Biomar Diagnostics, Marburg, Germany). α-Tocopherol (vitamin E) in plasma and intestinal tissue was analyzed by normal phase HPLC after alcoholic saponification at 80°C for 30 min and extraction into heptane as described by Jensen et al. (19). Circulating GLP-2 levels were measured by radioimmunoassay using an NH₂-terminal-specific antiserum. This assay specifically detects the full-length, bioactive porcine GLP-2-(1–33) peptide (16).

**Enzyme Activity**

Frozen samples from the proximal, middle, and distal regions of the intestine from each piglet were extracted in 1.0% Triton X-100 (10 ml/g tissue) and homogenized (0°C, 1 × 20 s). Only intestinal samples that appeared healthy or mildly hemorrhagic were analyzed. The homogenates were assayed for disaccharidase (lactate, maltase, and sucrase) and peptidase [aminopeptidase N (ApN), dipeptidylpeptidase IV (DPPIV), and aminopeptidase A (ApA)] activities, as previously described (35). Briefly, sucrose (0.01 M, no 194018; ICN, Aurora, OH) and lactose (0.12 M, no. L-3625; Sigma) dissolved in sodium maleate buffer (50 mM, pH 6.0) were used as substrates for sucrase-isomaltase (EC 3.2.1.48–10) and lactase-phloridzin hydrolase (EC 3.2.1.23–62), respectively. Maltose (0.0112 M, no. L-5885; Sigma) was used to measure maltase activity, which represents the combined activity of sucrase-isomaltase and maltase-glucosamylase (EC 3.2.1.20). ApN (EC 3.4.11.2), DPPIV (EC 3.4.14.5), and ApA (EC 3.4.11.7) activities were measured using three peptidase-specific substrate solutions: 10 mM L-alanine-4-nitroanilide (Merck, Darmstadt, Germany) in 50 mM Tris·HCl, pH 7.3, 15 mM glycyl-L-proline-L-glutamic acid 4-nitroanilide (Innovativeive 1) in 50 mM Tris·HCl, pH 8.0, and 10 mM α-L-glutamic acid 4-nitroanilide (In-
stitute of Protein Chemistry, Hørsholm, Denmark) in 50 mM Tris-HCl, pH 8.0, respectively. Frozen pancreas was homogenized in Tris-HCl buffer (100 mmol/l containing 20 mmol/l CaCl₂, pH 7.9, 2 min, 0°C) and centrifuged (20,000 g, 45 min, 4°C), and the supernatant was used for analysis of amylase activity. Ethylidene-p-nitropheno-nyl D-maltoheptasidase was used as a substrate for amylase, and the liberated glucose was determined spectrophotometrically at 405 nm (577–50P; Sigma). For all enzymes, a hydrolytic rate of 1 μmol substrate released per minute at 37°C was considered to represent one unit of enzyme activity. Enzyme activities were expressed per gram of wet intestine (proximal, middle, and distal) or pancreas.

RNA Extraction and Reverse Transcription

Total RNA was extracted from individually frozen samples (50–100 mg) of distal small intestine (9). RNA was dissolved in 0.1% diethylpyrocarbonate-treated water, and recovery (absorption at 260 nm) and purity (ratio of absorption at 260 nm to 280 nm) were determined with a spectrophotometer (GeneQuant Pro; Amersham Pharmacia Biotech, Cambridge, UK). RNA quality was assessed using RNA 6000 Micro Assay on an Agilent 2100 bioanalyzer (Agilent Technologies, Nærum, Denmark). On the basis of the integrity of the 18S and 28S bands, only samples showing no sign of degradation were chosen for further evaluation. RNA was reverse transcribed into cDNA as described previously (30).

Real-Time Fluorescence PCR

PCR reactions were carried out on a light cycler instrument (Roche Diagnostics, Mannheim, Germany), and products were detected with SYBR Green I, which is included in the FastStart Master SYBR Green I mix (Roche). Reactions were carried out in 20-μl volumes consisting of 1X FastStart Master SYBR Green I mix (which includes Tag DNA polymerase, reaction buffer, dNTPs, and SYBR Green I), 3–5 mM MgCl₂, and 0.5 μM gene-specific primer. The primers used are given in Table 1. The β-actin and the 18S primer sets were included as housekeeping control genes. The specificity of the primers was tested by submitting them into a BLAST analysis. Before intestinal samples were examined, the PCR conditions for each primer pair were optimized by determining the MgCl₂ concentration and annealing temperature at which only the specific product was seen. After the first optimization steps, the PCR products were run on a 2% agarose gel and stained with ethidium bromide to confirm that the PCR product had the expected length. In each reaction, 2 μl of cDNA were amplified. A standard curve was included in every run, and it consisted of serial dilutions (10×) of purified PCR products. With use of the standard curve, copy numbers of the target cDNA in intestinal samples were calculated. The amplification program was as follows: preincubation for Fast Start polymerase activation at 95°C for 10 min, followed by 45 amplification cycles of denaturation at 5 s (20°C/s), annealing at 52–63°C for 10 s (20°C/s), elongation at 72°C for 6–20 s (20°C/S) depending on the primer set, and acquisition of fluorescence at 72°C. At the end of the last cycle, the melting curve was generated by starting the fluorescence acquisition at 65°C and taking measurements every 0.1 s until 95°C was reached.

For each primer set, the relative mRNA abundance was estimated by relating the obtained value to the values obtained for β-actin. This was done to eliminate variations due to differences in RNA quality. Results from the fed NB groups cannot be directly compared with results from the other three treatment groups because these two sets of

Table 1. Oligonucleotide sense and antisense sequences, annealing temperature, MgCl₂ concentration, and elongation time for quantitative RT-PCR analyses

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Annealing Temperature, MgCl₂ Concentration, and Elongation Time</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGA</td>
<td>58°C, 5 mM MgCl₂, 20 s</td>
<td>5’-CAGAACGTGCAAGACTGTC-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-AGAACTGTCGTGGACATCC-3’</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-TGCAGTCGTGGACATCC-3’</td>
<td></td>
</tr>
<tr>
<td>SI</td>
<td>60°C, 4 mM MgCl₂, 20 s</td>
<td>5’-CCCACTTTGCTGTTGA-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-CAGACCTTCCAGGTACATCC-3’</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-CGAGATGTCCAGGTACATCC-3’</td>
<td></td>
</tr>
<tr>
<td>ApA</td>
<td>63°C, 5 mM MgCl₂, 23 s</td>
<td>5’-GTCTTACACCGTCCAGATCC-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-CTGTTAGTGATAGATCC-3’</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-GCAAATCACAGTGCAAACCTCC-3’</td>
<td></td>
</tr>
<tr>
<td>ApN</td>
<td>58°C, 3 mM MgCl₂, 20 s</td>
<td>5’-ACACTACGTCTCATCCACCC-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-GCCACGATGTGACACCTCC-3’</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-CTGTTAGTGATAGATCC-3’</td>
<td></td>
</tr>
<tr>
<td>DPPIV</td>
<td>62°C, 3 mM MgCl₂, 24 s</td>
<td>5’-CCCTTCGGGTGCTGTGGTA-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-TCCTCGGGGCGGTCGTGGTA-3’</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-TGGATTTGACGTGAGCTC-3’</td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>54°C, 5 mM MgCl₂, 18 s</td>
<td>5’-GATACCGCAGCTAGGAAT-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-ATGCTGATATCCTGTCCTG-3’</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-CCGTCCTGCTGCCTGTAG-3’</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>63°C, 4 mM MgCl₂, 15 s</td>
<td>5’-CAAGCCACTATGTGGCGTGG-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-CCCTCAAGCTCACTCTGTC-3’</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-GCTTACTGCACAGGTCGCT-3’</td>
<td></td>
</tr>
<tr>
<td>SGLT-I</td>
<td>58°C, 4 mM MgCl₂, 12 s</td>
<td>5’-CGAAGATGTGTGCTGTCGCC-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-GATGCTCGGGCGAGATGTGG-3’</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-CGAGACAGCGGCTTCCTCC-3’</td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>60°C, 3 mM MgCl₂, 14 s</td>
<td>5’-CGGTCCTGCTGCTGTAG-3’</td>
</tr>
<tr>
<td>Sense</td>
<td>5’-GCTGTAACCTCATACACTCCTCC-3’</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5’-TAGACCTCTGCCGATTCAGC-3’</td>
<td></td>
</tr>
</tbody>
</table>

MGA, maltase-glucosamylase; SI, sucrase-isomaltase; ApA and ApN, aminopeptidase A and N; DPPIV, dipeptidyl peptidase IV; SGLT-1, sodium-glucose coupled transporter 1; eNOS, endothelial nitric oxide synthase.
samples were assayed separately due to a limited sample number capacity of the light cycler instrument.

Statistical Analysis

The data were analyzed by a two-way ANOVA using the MIXED procedure of SAS (SAS Institute, Cary, NC). The fixed variables were diet (unfed, colostrum, formula), birth status (fetal, newborn), and intestinal region (proximal, middle, and distal); pig and litter were included as random variables. Results are given as means ± SE, and differences between two means were tested by the least significant difference test. \( P = 0.05 \) was used as the critical level of significance for all statistical evaluations.

RESULTS

Clinical Evaluation and Blood Chemistry Values

One F-formula piglet died before delivery by cesarean section, probably due to sepsis. There were no signs of intestinal inflammation in this or in any of the other fed piglets at delivery. In the newborn group, 20 of 24 piglets survived the first 12 h. This survival rate (>80%) within the first 12 h after birth is common for newborn preterm piglets (33). Despite rearing in heated incubators (36–37°C), the premature piglets were hypothermic in the beginning of the feeding period (35.6 ± 0.2°C at 6 h postdelivery), with normothermia attained at 12 h (38.0 ± 0.2°C) after birth. Among the seven NB-colostrum piglets, none of these showed clinical signs of gastrointestinal disease during the feeding period. Among the 13 NB-formula piglets, 2 (15%) showed severe clinical signs of NEC, characterized by diarrhea, abdominal distension, fever, and respiratory distress. At necropsy, extensive inflammation and necrosis were found in their stomachs, small intestine, and colon, resulting in NEC scores of 1–2. Despite rearing in heated incubators (36–37°C), the premature piglets point, were severely impaired by formula relative to colostrum feeding. Assuming a similar capacity to take up BSA and HSA (18), the ability to absorb albumin macromolecules decreased significantly from the first feeding (time 0, BSA) to the sixth feeding (time 18, HSA), especially for the formula-fed groups. There were no difference in macromolecule uptake between fetal and newborn piglets (Table 2).

Organ Weights and Intestinal Morphology

Between unfed and fed groups, there were no significant differences in weight at delivery (1.10 ± 0.04 kg). Compared with NB-unfed, colostrum feeding, both before and after birth, increased the relative length (average 3.2 ± 0.1 vs. 2.4 ± 0.2 m/kg; \( P < 0.02 \)), weight (average 36.5 ± 1.4 vs. 20.5 ± 1.2 g/kg; \( P < 0.0001 \), and mucosal mass of the small intestine (average 25.2 ± 1.0 vs. 11.1 ± 0.5 g/kg; \( P < 0.0001 \)) (Table 3 and Fig. 2). Formula feeding resulted in a lower relative small intestinal weight (31.5 vs. 20.5 mg/kg; \( P < 0.02 \)) and mucosal mass (22.2 vs. 0.9 g/kg; \( P < 0.05 \)) compared with the corresponding colostrum pigs. Plasma GLP-2 increased dramatically in response to feeding (10- to 15-fold), with the highest levels attained in the two formula-fed groups. Plasma α-tocopherol was lowest in the unfed group; both F- and NB-colostrum piglets had higher plasma levels of α-tocopherol compared with formula-fed piglets (Table 2).

Blood Gases, Cortisol, GLP-2, α-Tocopherol, and Albumin Levels

Fed fetuses showed decreased blood pH, Hct, Hb, and oxygen and sodium concentrations compared with NB-unfed piglets, whereas postnatally these parameters increased, except for Hct and Hb, which were similar to NB-unfed results (Table 2). All groups, except the NB-colostrum, tended to have increased plasma cortisol levels, relative to NB-unfed, and the highest and most variable results were found in the NB-formula pigs. This group also showed lowered blood pH compared with the corresponding colostrum pigs. Plasma GLP-2 increased dramatically in response to feeding (10- to 15-fold), with the highest levels attained in the two formula-fed groups. Plasma α-tocopherol was lowest in the unfed group; both F- and NB-colostrum piglets had higher plasma levels of α-tocopherol compared with formula-fed piglets (Table 2).

Table 2. Blood-gas values and cortisol, GLP-2, α-tocopherol, and albumin (BSA, HSA) concentrations in plasma at tissue collection for F and NB piglets, either not fed or fed colostrum or formula for 24 h

<table>
<thead>
<tr>
<th>Variable</th>
<th>NB-Unfed</th>
<th>F-Colostrum</th>
<th>F-Formula</th>
<th>NB-Colostrum</th>
<th>NB-Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidity (pH)</td>
<td>7.38 ± 0.02a</td>
<td>7.32 ± 0.02a</td>
<td>7.29 ± 0.02a</td>
<td>7.47 ± 0.02a</td>
<td>7.38 ± 0.01b</td>
</tr>
<tr>
<td>Hct, %</td>
<td>27.4 ± 1.4b</td>
<td>25.0 ± 0.9a</td>
<td>24.50 ± 1.6a</td>
<td>27.67 ± 1.9b</td>
<td>28.00 ± 1.2b</td>
</tr>
<tr>
<td>Hb, mg/100 ml</td>
<td>9.08 ± 0.42b</td>
<td>7.43 ± 0.52a</td>
<td>6.59 ± 0.94a</td>
<td>9.01 ± 0.55b</td>
<td>9.95 ± 0.23b</td>
</tr>
<tr>
<td>Pco2, Torr</td>
<td>60.5 ± 2.7</td>
<td>62.2 ± 2.1</td>
<td>59.7 ± 1.8</td>
<td>54.5 ± 6.2</td>
<td>56.0 ± 2.0</td>
</tr>
<tr>
<td>Sodium, mM</td>
<td>137.0 ± 0.9b</td>
<td>132.0 ± 0.8a</td>
<td>131.1 ± 1.0a</td>
<td>147.9 ± 0.6b</td>
<td>146.7 ± 0.8c</td>
</tr>
<tr>
<td>Potassium, mM</td>
<td>4.14 ± 0.28b</td>
<td>4.57 ± 0.30b</td>
<td>4.85 ± 1.70b</td>
<td>4.84 ± 0.32c</td>
<td>4.47 ± 0.37b</td>
</tr>
<tr>
<td>Calcium, mM</td>
<td>1.16 ± 0.08b</td>
<td>1.39 ± 0.08a</td>
<td>1.31 ± 0.35b</td>
<td>0.98 ± 0.09a</td>
<td>1.13 ± 0.33a</td>
</tr>
<tr>
<td>Cortisol, ng/ml</td>
<td>65.8 ± 5.1a</td>
<td>89.3 ± 4.8b</td>
<td>98.1 ± 7.4b</td>
<td>76.2 ± 13.7ab</td>
<td>139.4 ± 32.5a</td>
</tr>
<tr>
<td>GLP-2, nmol/ml</td>
<td>6.8 ± 1.7a</td>
<td>59.1 ± 11.2b</td>
<td>103.9 ± 13.1c</td>
<td>68.3 ± 9.1b</td>
<td>80.3 ± 7.6bc</td>
</tr>
<tr>
<td>α-Tocopherol, µg/ml</td>
<td>0.28 ± 0.03a</td>
<td>0.64 ± 0.07b</td>
<td>0.49 ± 0.05b</td>
<td>1.49 ± 0.21d</td>
<td>0.53 ± 0.10a</td>
</tr>
<tr>
<td>BSA, µg/ml</td>
<td>337 ± 47bc</td>
<td>171 ± 29a</td>
<td>415 ± 52b</td>
<td>250 ± 49ab</td>
<td>22 ± 5a</td>
</tr>
<tr>
<td>HSA, µg/ml</td>
<td>241 ± 22b</td>
<td>26 ± 7a</td>
<td>174 ± 73a</td>
<td>22 ± 5a</td>
<td>22 ± 5a</td>
</tr>
</tbody>
</table>

Values are means ± SE. F, fetal; NB, newborn; GLP-2, glucagon-like peptide 2; HSA, human serum albumin. Means not sharing the same superscript letter differ significantly (\( P < 0.05 \).)
and fed fetuses, whereas feeding after birth increased the villus height of the NB-colostrum group (Fig. 2). Villus width increased significantly with feeding, which was most pronounced for the NB-colostrum group. For fetal fed pigs, there was no treatment difference in villus height, villus width, crypt depth (Figs. 2 and 3), or histological NEC score (Fig. 1 and Table 3). After birth, however, formula feeding decreased villus height and villus width and increased crypt depth, compared with colostrum feeding. A total of 38% of NB-formula piglets had a score of 2 or more in the histological NEC evaluation, whereas none of the seven NB-colostrum piglets scored more than 1 (Fig. 1 and Table 3).

There were no differences between unfed and colostrum-fed piglets for the relative weights of heart, stomach, pancreas, adrenals, or colon (Table 3). Compared with colostrum, formula increased the relative stomach weight both before and after birth and increased the relative weight of the liver and colon but only after birth (Table 3). Relative heart, lung, kidney, pancreas, and adrenal mass were not different between colostrum and formula feeding (Table 3).

Tissue Enzyme Activities and \( \alpha \)-Tocopherol Levels

From analyses across intestinal regions for disaccharidases and peptidases, colostrum induced an increase in maltase and ApA activities compared with NB-unfed pigs (Fig. 4). This increase was most pronounced in the postnatal piglets. Colostrum feeding decreased lactase and DPPIV (0.71 ± 0.12 vs. 1.79 ± 0.19 U/g; \( P < 0.01 \)) activities for both fetal and postnatal piglets compared with unfed control pigs. Only after

<table>
<thead>
<tr>
<th>Table 3. Body weight, relative organ weights, and NEC incidence in F and NB piglets, either unfed or fed colostrum or formula for 24 h</th>
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</thead>
<tbody>
<tr>
<td><strong>NB-Unfed</strong></td>
</tr>
<tr>
<td>Body weight, kg</td>
</tr>
<tr>
<td>Small intestine, g/kg body wt</td>
</tr>
<tr>
<td>Stomach, g/kg body wt</td>
</tr>
<tr>
<td>Colon, g/kg body wt</td>
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<tr>
<td>Heart, g/kg body wt</td>
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<tr>
<td>Lungs, g/kg body wt</td>
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<tr>
<td>Liver, g/kg body wt</td>
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<tr>
<td>Spleen, g/kg body wt</td>
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<td>Kidney, g/kg body wt</td>
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<tr>
<td>Adrenals, g/kg body wt</td>
</tr>
<tr>
<td>Pancreas, g/kg body wt</td>
</tr>
<tr>
<td>NEC incidence</td>
</tr>
</tbody>
</table>

Values are means ± SE. NEC incidence is the number of piglets with NEC/total number of piglets. Means not sharing the same superscript letter differ significantly (\( P < 0.05 \)).
birth did colostrum feeding decrease sucrase activity compared with NB-unfed (0.11 ± 0.01 vs. 0.18 ± 0.01 U/g; P < 0.05). There was no difference in ApN activity between colostrum-fed and NB-unfed groups.

F-formula pigs maintained enzyme activities similar to those in F-colostrum pigs, except for maltase (Fig. 4). After birth, however, formula feeding induced lower activity of maltase, lactase, ApN, and ApA compared with colostrum feeding (Fig. 4). Feeding in utero had no effect on pancreatic amylase activity (average was 22.5 ± 1.9 U/g), whereas feeding after birth doubled pancreatic amylase activity (average was 49.3 ± 5.9 U/g; P < 0.05) relative to values at birth. Small intestinal α-tocopherol levels were equal in unfed and formula-fed piglets, but colostrum-fed piglets showed a significantly higher intestinal α-tocopherol level, irrespective of birth (Fig. 5).

**Tissue mRNA Levels**

For the formula-fed piglets there was a wide variation in total RNA quality corresponding to the degree of tissue inflam-
information and destruction (Fig. 6). Only samples of intact total RNA were evaluated for specific mRNAs. ApN, DPPIV, ApA, and sodium-glucose cotransporter 1 (SGLT-1) mRNA levels were all significantly decreased for the fetal fed groups compared with the NB-unfed group (Table 4 and Fig. 7). There were no differences in maltase-glucoamylase, sucrase-isomaltase, TNF-α, or endothelial nitric oxide synthase (eNOS) mRNA levels between F-fed and NB-unfed pigs (Fig. 7). After birth, maltase-glucoamylase mRNA levels significantly increased for the NB-formula pigs compared with the NB-colostrum pigs, whereas, for sucrase-isomaltase, ApN, DPPIV, ApA, and TNF-α expression, there were no differences between the two NB fed groups (Table 4 and Fig. 7). Formula feeding after birth increased SGLT-1 and eNOS mRNA expression compared with colostrum feeding.

DISCUSSION

This study shows that feeding colostrum to the late-gestation fetus results in a marked increase in mucosal weight and in enzymatic changes (increases in maltase and peptidase activities, decrease in lactase activity), comparable with those observed in the preterm neonate born at the same gestational age. Only intestinal ApA and pancreatic amylase appeared to be more responsive to the feeding of colostrum after birth than before birth, and the changes were similar to those in newborn pigs born at term (18). Thus the trophic and maturational effects of the first enteral food are relatively independent of the
physiological changes occurring during parturition and birth. The effects may in part be mediated via release of gut growth factors, such as GLP-2, for which intestinal receptor transcripts are expressed already before birth (31). Surgery and 1 day of feeding in utero induced a slight increase in plasma cortisol, relative to unoperated fetuses. This is unlikely to have contributed to maturational changes in the gut of fed fetuses because more prolonged exposure to elevated fetal cortisol levels is required to induce growth and maturation of the GIT (35, 42). In addition, the fetal surgery in itself did not previously lead to changes in intestinal growth and function comparable with those of enteral feeding (29). Fetal distress in response to surgery and feeding may, however, explain the lighter lungs in fed fetuses at delivery, reflecting a higher degree of lung expansion relative to unfed controls. Because of fetal surgery limitations and because the main goal of our investigations was to compare the effects of two different diets ingested either before or after birth, we did not include sham-operated control fetuses. Thus the same unfed newborn pigs were chosen as a reference group for all fed pigs.

In the fetuses, formula feeding induced intestinal growth and brush-border enzyme responses similar to those for colostrum feeding, except for maltase activity, which was lower in the formula-fed fetuses. It has previously been noted that maltase activity is particularly sensitive to colostrum ingestion in newborn pigs (18). The specific nutrients and bioactive factors present in colostrum may induce a posttranslational modification of the maltase-glucoamylase protein at the brush border, resulting in higher enzyme activity. The stimulation seems to be posttranslational rather than transcriptional because maltase-glucoamylase mRNA levels remained similar between fetal feeding groups and even lower in the newborn pigs fed colostrum relative to pigs fed formula.

Another important finding in this study is that formula feeding reduced mucosal structure and function and induced the development of NEC within 24 h in preterm piglets but not in fetuses. In pigs fed the same formula at term, there is no development of NEC (18) and NEC is also absent in preterm or term pigs fed total parenteral nutrition (33). Although none of the colostrum-fed preterm pigs showed any clinical or histological signs of intestinal disease, 5 of 13 NB-formula pigs had inflammatory and necrotic lesions, with or without the presence of pneumatosis intestinalis, in large parts of the GIT. The pathological lesions appeared very similar to those observed in

<table>
<thead>
<tr>
<th>SI</th>
<th>NB-Unfed</th>
<th>F-Colostrum</th>
<th>F-Formula</th>
<th>NB-Colostrum</th>
<th>NB-Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.51 ± 1.95</td>
<td>3.09 ± 2.56</td>
<td>3.58 ± 2.09</td>
<td>1.32 ± 0.30</td>
<td>1.79 ± 0.27</td>
</tr>
<tr>
<td>ApA</td>
<td>2.84 ± 0.27 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.70 ± 0.27 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.28 ± 0.29 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.83 ± 0.21</td>
<td>0.46 ± 0.21</td>
</tr>
<tr>
<td>ApN</td>
<td>2.43 ± 0.33 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.08 ± 0.33 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.95 ± 0.35 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.74 ± 0.11</td>
<td>0.46 ± 0.10</td>
</tr>
<tr>
<td>DPP IV</td>
<td>1.65 ± 0.12 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.73 ± 0.12 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.64 ± 0.13 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.63 ± 0.07</td>
<td>0.59 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE. Means for NB-unfed, F-colostrum, and F-formula not sharing the same superscript letter differ significantly (P < 0.05).

**Fig. 7.** mRNA levels for maltase-glucoamylase (MGA), sodium-glucose cotransporter 1 (SGLT1), TNF-α, and endothelial nitric oxide synthase (eNOS) in the distal small intestine from F and NB piglets either unfed or fed colostrum or formula for 24 h (means ± SE). Means from the NB-unfed and F groups differ if they do not share the same lower case superscript letter (P < 0.05). Means from the 2 NB fed groups differ if they do not share the same upper case superscript letter (P < 0.05).
human infants with NEC (1, 17, 38). The lesions, starting with villus destruction leading to separation of the mucosal layer and lamina propria followed by transmural necrosis and often including pneumatosis intestinales, are well-known characteristics of infant NEC (1, 38). The finding that pathological changes and intestinal dysfunctions were absent in immature piglets fed formulaf formula in utero suggests that NEC is not solely caused by the formula itself but develops in a complex interplay of predisposing factors, including prematurity, formulaf formula feeding, and the physiological and environmental changes around birth itself. These factors may include neonatal hypothermia, intestinal ischemia, and bacterial colonization, as indicated from previous NEC models artificially using such conditions to induce NEC (4, 5, 7, 11, 12). Presence of bacteria may be required for NEC development, and bacterial toxins could initiate an uncontrolled inflammatory response by an immature intestinal immune system. Necrotic lesions in the stomach were sometimes observed in the piglets developing NEC, and infiltration of inflammatory cells together with edema may explain the fact that the stomachs tended to be heavier in formulaf-fed than in colostrum-fed pigs. In the presence of a low intraluminal gastric acid and gastrointestinal proteolytic activity, the incompletely developed epithelial barrier may be particularly vulnerable to bacterial colonization and pathogenic overgrowth (4, 8, 10).

It is noteworthy that, even without severe intestinal hemorrhage and necrosis at the time of tissue collection, the intestinal morphology and enzyme function of NB-formula pigs was significantly affected. We do not know whether the seemingly healthy NB-formula piglets would have remained clinically healthy or would have developed NEC at a later time point. Regardless, however, the intestinal structural and functional indexes in healthy formulaf-fed pigs showed a state of villus atrophy and reduced digestive capacity compared with preterm formulaf-fed piglets fed colostrum, and this may predispose these piglets to NEC development. Formula feeding also significantly decreased the enterocyte endocytotic capacity, relative to colostrum, as indicated by the lowered BSA and HSA levels in plasma following inclusion of these macromolecules into the oral feeds at the start and end of the 24-h feeding protocol. However, all fetal and preterm newborn pigs already showed relatively low macromolecule absorption, relative to the values obtained for term newborn pigs (18), indicating that a diet-induced decrease in enterocyte function in preterms could be critical for the ability to digest and absorb nutrients.

The intestinal inflammatory responses are likely to involve altered local nitric oxide production, facilitating an acute increase in intestinal blood flow that may lead to oxidative damage and necrosis. Inducible nitric oxide synthase (iNOS) activity is believed to be a major contributor to such inflammatory reactions (14, 20). Consistent with this, our own preliminary investigations have shown that iNOS activity was indeed clearly elevated in the preterm formulaf-fed pigs (20.2 ± 3.1 nmol·min⁻¹·g tissue protein⁻¹) relative to unfed pigs (9.8 ± 1.0 nmol·min⁻¹·g tissue protein⁻¹) or pigs fed colostrum (6.4 ± 0.8 nmol·min⁻¹·g tissue protein⁻¹). Also, the eNOS mRNA level was elevated, as indicated by elevated tissue eNOS mRNA levels. The proinflammatory cytokine TNF-α, which has been shown to be elevated in other models of NEC (6), did not in this study show any difference at the mRNA level.

Vitamin E (α-tocopherol) is a key component in preventing oxidative stress, in part mediated by excessive iNOS production. α-Tocopherol levels were low at birth but increased after enteral feeding. Colostrum induced an especially much higher concentration of α-tocopherol in plasma and tissue compared with formulaf. The levels of α-tocopherol in formulaf and sow’s colostrum were similar (37); however, because the synthetic α-tocopherol included in the formulaf was an equimolar mixture of eight different stereoisomers that were esterified to acetate (to increase stability), the bioavailability and biological activities of the vitamin were reduced compared with the α-tocopherol present in sow colostrum (23). Despite the low antioxidant status in formulaf-fed fetuses, none of these developed NEC-like symptoms, again probably reflecting that the transitions at birth (microbiological, physiological) play a role in formulaf-induced intestinal inflammation.

For all brush-border hydrolases, the mRNA levels were decreased after introducing enteral nutrition in utero, relative to their unfed littermates. This reflects a distinct discrepancy between biological activity and mRNA levels in response to the first enteral food intake. Apparently, enteral feeding enhances enzyme activity, probably by inducing a posttranslational modification, without stimulating enzyme transcription. Apart from maltase-glucosamylase and SGLT-1 mRNA levels that were increased for the NB-formula group, there were no differences between the NB feeding groups despite the observed formulaf-induced decrease in enzyme activity. The detrimental effects of formula feeding on the digestive capacity in the preterm neonate therefore do not result from direct effects on the nuclear genes for the functional proteins present on the enterocyte but seem to result mainly from the mucosal atrophy and breakdown of existing functional proteins on the brush border.

This study has shown that the fetal GIT tolerates enteral feeding and that the trophic response to the first enteral food is similar to that after birth. Formula feeding per se does not seem to cause intestinal malfunction, but birth coupled with immaturity increases the sensitivity of the intestine to NEC-like disease in response to formula feeding. We speculate that this is related to hypothermia, hypoxia, and/or ischemia in response to preterm birth, together with the introduction of a suboptimal intestinal microbial environment and an immature immune response. Colostrum feeding protects against this response, probably by provision of bioactive factors that are able to modulate the immature immune response and promote a more beneficial microbial colonization.

ACKNOWLEDGMENTS

Senior scientist Tim Kåre Jensen and technical assistants Anni Ravn Pedersen and Ulla Loose Andreassen at the Danish Institute for Food and Veterinary Research are thanked for preparation and discussion of histological specimens. Anna Siekierska, Bente Synnetsvedt, Ebba de Neergaard Harrison, and Anne Fris Pedersen are thanked for skilled technical assistance.

GRANTS

The work was supported by a grant from the Danish Research Agency (Program 23-00-0102). This work was also supported by the Danish Agricultural and Veterinary Research Council (Programme 9702803).

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