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Original article

Bovine colostrum improves intestinal function following formula-induced gut inflammation in preterm pigs☆

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SUMMARY

Background & aims: Only few hours of formula feeding may induce proinflammatory responses and predispose to necrotizing enterocolitis (NEC) in preterm pigs. We hypothesized that bovine colostrum, rich in bioactive factors, would improve intestinal function in preterm pigs following an initial exposure to formula feeding after some days of total parenteral nutrition (TPN).
Methods: After receiving TPN for 2 days, preterm pigs were fed formula (FORM, $n = 14$), bovine colostrum (COLOS, $n = 6$), or formula (6 h) followed by bovine colostrum (FCOLOS, $n = 14$). Intestinal lesions, function, and structure, abundance and location of bacteria, and inflammation markers were investigated.
Results: NEC severity and interleukins (IL)-1β and -8 protein concentrations were lower, while villus height, galactose absorption, and brush-border enzyme activities were increased in the distal small intestine in COLOS and FCOLOS pigs, relative to FORM pigs. Intestinal gene expression of serum amyloid A, IL-1β, -6 and -8, and bacterial abundance, correlated positively with NEC severity of the distal small intestine.
Conclusions: Bovine colostrum restores intestinal function after initial formula-induced inflammation in preterm pigs. Further studies are required to test if bovine colostrum may also benefit preterm infants during the challenging transition from total parenteral nutrition to enteral nutrition, when human milk is unavailable.

1. Introduction

Necrotizing enterocolitis (NEC) is a serious gastrointestinal disease that mainly affects preterm infants. Preterm birth, abnormal bacterial colonization, and enteral feeding especially with milk formula, are factors that predispose to NEC.[1–3] Before the initiation of enteral nutrition, preterm neonates may require a period of total parenteral nutrition (TPN), and NEC occurs very rarely in the absence of enteral food in infants[4] and pigs.[5] Although TPN may be a life-saving therapy for many preterm infants, TPN has in neonatal pigs been shown also to reduce the digestive- and absorptive function,[6] increase intestinal permeability,[7] and cause a degree of mucosal atrophy in the developing intestine.[8] When milk formula feeding is initiated after a few days of TPN in preterm pigs, this may be

Non-standard abbreviations: NEC, necrotizing enterocolitis; TPN, total parenteral nutrition; FORM, treatment group receiving infant formula; FCOLOS, treatment group receiving infant formula followed by colostrum; COLOS, treatment group receiving colostrum; FISH, fluorescence *in situ* hybridization; *TLR4*, toll-like receptor 4; *TNF*, tumor necrosis factor alpha; *DEFB4A*, defensin, beta 4A; SAA, serum amyloid A; *B2M*, beta-2-microglobulin; *ACTB*, beta-actin; *HPRT1*, hypoxanthine phosphoribosyltransferase 1; IL, interleukin; CFU, colony forming units; OA, organic acids.

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associated with a more difficult and less tolerant transition to enteral nutrition.[5] An abrupt transition to milk formula reduces villus height, mucosa percentage, digestive enzyme activities and nutrient absorption, and increases bacterial adherence, mucosal atrophy and inflammatory cytokine levels within just 8 h of feeding in preterm pigs.[5,9,10] In contrast, preterm pigs fed porcine or bovine colostrum are less affected by NEC.[5,10–12] This may be due to the high content of bioactive compounds such as growth factors, antioxidants, antimicrobial and immune-modulatory factors in colostrum, which may support intestinal maturation, balance and priming of the immune system and establishment of a beneficial gut microbiota.[13,14] It is known, that colostrum given as minimal enteral nutrition may prevent the inflammatory cascade leading to NEC lesions in preterm pigs,[12] while human milk may reduce NEC in preterm infants compared with milk formula.[1–3] However, the ability of colostrum to regenerate an already compromised gut exposed to a short period of formula feeding is unknown. Knowledge about the possible therapeutic effect of colostrum on an intestine initially fed formula is important, because lack of mother's milk following preterm birth leads to variable periods of formula feeding. Thus, there is a need to know to which extent mother's own milk, or a possible substitute bioactive product like bovine colostrum, may help to suppress the pro-inflammatory state of the immature intestine resulting from a few days of TPN followed by a period of formula feeding.

We hypothesized that the dysfunction, induced by the combination of TPN with an abrupt transition to milk formula, is reduced by feeding bovine colostrum just after an initial formula feeding period. We used a preterm pig model of NEC[5,10] to investigate the effects of bovine colostrum on intestinal structure, digestive and absorptive functions, microbiota, and plasma and tissue proteins and tissue mRNA levels of inflammatory markers. A group of preterm pigs was fed formula followed by colostrum and was compared with a group of preterm pigs fed only formula (negative control) after the TPN period. Values from these groups were also compared with a third group of preterm pigs fed only colostrum which according to our previous studies protects against NEC.[5,9,10]

2. Materials and methods

2.1. Animals and their treatment

Thirty-four preterm pigs were delivered from four sows by caesarean section (Large White × Danish Landrace × Duroc, Askelygaard Farm, Roskilde, Denmark) at 105–107 d gestation (90–92% gestation). The procedures for caesarean section, passive immunization (maternal serum given three times: 4, 5, and 7 ml/kg body weight during the first 24 h after birth) and nursing of the preterm pigs followed a standard protocol.[5,15] During the first 48 h, pigs were given TPN through a vascular catheter (advancing from 4 to 6 ml/kg/h). The TPN solution was based on Nutriflex Lipid Plus (Braun, Melsungen, Germany) and adjusted in nutrient composition to meet the requirements of pigs.[5] After the TPN period, the pigs were stratified according to birth weight into three total enteral nutrition groups fed either milk formula (FORM, $n = 14$), 6 h of milk formula followed by bovine colostrum (FCOLOS, $n = 14$), or bovine colostrum (COLOS, $n = 6$) until euthanasia. The COLOS group was included as a reference group confirming the protective effects of exclusive feeding with bovine colostrum shown in a previous study.[5] The feeding dose for all groups was 15 ml/kg body weight/3 h. The milk formula contained 80 g Pepdite, 70 g Maxipro, and 75 g Liquigen/l of water,[5] resulting in a dry matter content of 18.8% (all products kindly donated by Nutricia, Allerød, Denmark). Bovine colostrum was obtained from the first milking after parturition (kindly donated by Biofiber-Damino, Gesten, Denmark), sterilized by gamma-irradiation (1 × 10 kGy; Sterigenics, Espergærde, Denmark) and stored at −20 °C. Before use, the colostrum was diluted in tap water to a dry matter content of 14.2%. Both products were warmed to body temperature in a water bath. All animal protocols and procedures were approved by the Danish National Committee on Animal Experimentation (Licence: 2004/561-910).

2.2. Clinical evaluation and tissue collection

Pigs were monitored closely for clinical symptoms of NEC such as abdominal distension, lethargy, cyanosis and bloody diarrhea. Pigs suffering from NEC before the end of the study protocol were immediately euthanized and the tissue collected according to earlier protocols.[5,11] Pigs not showing clinical signs of NEC were euthanized at day 2 of enteral feeding and the tissue collected. All pigs were given a NEC severity score ranging from 1 (no or minimal focal hyperemic gastroenterocolitis) to 6 (severe extensive hemorrhagic and necrotic gastroenterocolitis) in the proximal, middle and distal small intestine, stomach and colon as previously described.[5] Pigs with a severity score of 3 or more in any gastrointestinal region were considered suffering from NEC. A mean NEC severity score was calculated as the mean of the NEC severity score across the intestinal regions. To determine small intestinal enzyme activities, gene expression, and cytokine concentrations, full thickness tissue samples from the small intestinal regions were immediately snap-frozen in liquid nitrogen and stored at −80 °C. A 10 cm segment of each small intestinal region was used to measure intestinal circumference and to determine the proportion represented by mucosa according to Bjornvad et al.[5] Samples from the distal small intestine were collected and fixed in 4% neutral buffered paraformaldehyde for 24 h and transferred to 70% ethanol before preparation for fluorescence *in situ* hybridization (FISH) and evaluation of gut morphology.

Blood collected at euthanasia was used for later determination of inflammatory factors (see below). All blood samples were collected in EDTA- or heparin coated tubes, placed on ice, centrifuged for 10 min at 4 °C and 2500 *g* and the plasma was stored at −20 °C until further analyses.

2.3. Gut morphology and intestinal function

Distal small intestinal villus height and crypt depths were evaluated on scanning pictures obtained from FISH analysis using the morphometric software SoftWoRx Explorer version 1.2.0 (Applied Precision, Issaquah, WA, USA). One representative cross-section was selected from each pig and 10 representative villi and crypts were measured. Intestinal function was evaluated by measuring enzyme activities of dipeptidylpeptidase IV, aminopeptidase N, aminopeptidase A, lactase and maltase according to Sangild et al.[16] Finally, trehalase activity was measured as described for lactase and maltase using 0.6 M D-(+)-trehalose dihydrate (EC 202-739-6, Sigma–Aldrich, Brøndby, Denmark) as a substrate. *In vivo* plasma galactose concentrations were measured according to a previous study[17] before enteral nutrition (0 h) and at 6 h and 30 h after enteral food introduction by collection of a blood sample 20 min after an oral bolus (15 ml/kg, 5% galactose) via an oro-gastric feeding tube. The results from each treatment group at 6 h and 30 h were compared with the baseline level at 0 h. The urinary ratio of lactulose to mannitol, to estimate gut permeability, was determined by giving an oral bolus of 5% lactulose and 2% mannitol (15 ml/kg) 4–6 h prior to euthanasia as previously described.[5]

2.4. Gene expression analyses of inflammatory factors

The expression of proinflammatory markers *IL1B*, *IL6*, *IL8*, toll-like receptor 4 (*TLR4*), tumor necrosis factor alpha (*TNF*), anti-inflammatory marker *IL10*, defensin beta 4A (*DEFB4A*), acute