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Gut and immune effects of bioactive milk factors in preterm pigs exposed to prenatal inflammation

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Running title: Bioactive milk diets in preterm neonates born with prenatal inflammation

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Abstract

Prenatal inflammation may predispose to preterm birth and postnatal inflammatory disorders, such as necrotizing enterocolitis (NEC). Bioactive milk ingredients may help to support gut maturation in such neonates, but mother’s milk is often insufficient after preterm birth. We hypothesized that supplementation with bioactive ingredients from bovine milk (osteopontin, OPN; caseinoglycomacropeptide, CGMP; colostrum, COL) supports gut, immunity and NEC resistance in neonates born preterm after gram-negative infection before birth. Using preterm pigs as a model for preterm infants, fetal pigs were given intra-amniotic injections of lipopolysaccharide (LPS, 1 mg/fetus) and delivered three days later (90% gestation). For five days, groups of LPS-exposed pigs were fed formula (FOR), bovine colostrum (COL), or formula enriched with OPN or CGMP. LPS induced intra-amniotic inflammation, postnatal systemic inflammation but limited effects on postnatal gut parameters and NEC. Relative to FOR, COL feeding to LPS-exposed pigs showed less diarrhea, NEC severity, reduced gut IL-1β and IL-8 levels, greater gut goblet cell density and digestive enzyme activities, and blood helper T cell fraction. CGMP improved neonatal arousal, gut lactase activities and reduced LPS-induced IL-8 secretion in intestinal epithelial cells (IECs) in vitro. Finally, OPN tended to reduce diarrhea and stimulated IEC proliferation in vitro. No effects on villus morphology, circulating cytokines or colonic microbiota were observed among groups. In conclusion, bioactive milk ingredients exerted only modest effects on gut and systemic immune parameters in preterm pigs exposed to prenatal inflammation. Short-term, prenatal exposure to inflammation may render the gut less sensitive to immune-modulatory milk effects.

New and noteworthy: Prenatal inflammation is a risk factor for preterm birth and postnatal complications including infections. However from clinical studies, it is difficult to separate the effects of only prenatal inflammation from preterm birth. Using caesarean-delivered preterm pigs with prenatal inflammation, we documented some beneficial gut effects of bioactive milk diets, relative to formula, but prenatal inflammation appeared to decrease the sensitivity of enteral feeding. Special treatments and diets may be required for this neonatal population.
Introduction

Preterm births (<37 weeks of gestation) represents 15 million infants every year (10% of all pregnancies), and is the most important cause of neonatal mortality (5, 6). Prenatal infection and inflammation, including chorioamnionitis (CA), inflammation of the fetal membranes, is a main predisposing factor for preterm birth (40-70% cases) (14, 16, 25). The incidence of CA is inversely related to gestational age at birth, and many CA cases are related to infection with the low-virulent bacteria, Ureaplasma (15, 59). CA may be associated with increased risks of neonatal early-onset sepsis (EOS) (2, 24), necrotizing enterocolitis (NEC) (1, 3), and neurodevelopmental disorders (58), but it remains elusive if these effects are direct or indirect. In addition, it is unclear how the first milk diets (mother’s own milk, standard formula, enriched formula) (11) may modify the gut and systemic responses to prenatal inflammation. For preterm infants with CA, sub-optimal neonatal feeding may induce a second inflammatory insult to further increase the risk of infection and NEC (53). Our recent study on formula-fed preterm pigs has shown that CA induced by 3 days of intra-amniotic (IA) exposure to LPS exerted strong fetal gut responses and postnatal systemic inflammation but did not increase NEC sensitivity on day 5 (33). Thus, CA may predispose to EOS in preterm infants while the effects on NEC are more unclear. Sheep studies have shown CA-induced fetal inflammatory responses in the gut, lung and brain at birth (26, 50), but neither of these earlier studies allowed postnatal recovery and recording of inflammation and NEC sensitivity after enteral feeding. Possibly, an optimized milk diet for preterm neonates may not only protect the immature gut against NEC, but also reverse negative impacts of CA on the postnatal systemic immunity and inflammation in multiple organ systems.

In preterm pigs delivered by caesarean section after unaffected pregnancies, feeding bovine colostrum (COL) reduces NEC incidence, improves gut maturation, and down-regulates expression of intestinal genes related to inflammation, relative to infant formula (20, 37, 48, 49). Initial preterm infant studies also show that COL decreases diarrhea (51) and the time to full enteral feeding (22). Similar to
mother’s milk, COL contains multiple bioactive ingredients including anti-bacterial, anti-inflammatory proteins and peptides (immunoglobulins, lactoferrin, transforming growth factor β, casein, osteopontin (OPN)) (10). Bioactive components in COL may provide a better option or supplement than conventional infant formula for preterm infants, and may help to reverse gut inflammation induced by prenatal inflammation in infants born after CA. Among the milk bioactive components, OPN is an important protein with higher levels in COL than in bovine mature milk and human milk (29). It exerts anti-inflammatory properties (38) and anti-bacterial activities as well as protects the epithelial barriers via interactions with macrophages and neutrophils (23, 54, 60). OPN-enriched formula fed to newborn infants shows systemic immune-modulatory effects with lower plasma levels of TNF-α and a higher frequency of blood T cells than regular infant formula (29, 55). Caseinoglycomacropeptide (CGMP) is another promising milk peptide derived from kappa-casein with multiple immune-modulatory effects. For instance, CGMP decreases inflammatory gene expressions in porcine intestinal epithelial cells in vitro (18), downregulates the secretion of pro-inflammatory cytokines IFN-γ and TNF-α on rat splenocytes (41). CGMP is also considered a prebiotic component, via its decoy functions against pathogenic E.coli adhesion to the gut mucosa (8, 17), and via its ability to promote the growth of Bifidobacteria and Lactococcus spp. (7, 42).

Based on the already reported immune-modulatory effects of COL, OPN and CGMP, and the effects of prenatal inflammation on postnatal gut and systemic inflammation (33), we hypothesized that feeding COL, or formula (FOR) enriched with OPN or CGMP, would be superior to FOR feeding alone in protecting against NEC and systemic inflammation in preterm neonates born after prenatal inflammation. Preterm pigs were given an IA dose of LPS, as previously described (33), and were fed one of the above four diets for five days. The study primary outcome was NEC incidence and secondary outcomes included gut functions (absorption, enzyme activities, permeability), gut inflammation (inflammatory cytokines), and systemic immune endpoints (hematology and neutrophil functions).
Material and Method

Preterm pig experiments

All animal procedures were approved by the Danish National Committee of Animal Experimentation licence number 2014–15–0201–00418, which is in line with directive 2010/63/EU from the European Parliament. The schematic overview of the animal experimental design was depicted in Fig. 1. Eight pregnant sows (Large White x Danish Landrace x Duroc) were operated by laparotomy at d 103 of gestation (term at 117±2 days of gestation), and each fetus received either an IA dose of 1 mg LPS (LPS, n = 141, from *E.coli* 055:B5, Sigma Aldrich, Copenhagen, Denmark) or control (CON, n = 47, saline or no injection) in an area close to the mouth, as previously described (33). Post-surgical monitoring of the sows included frequent clinical evaluation and temperature measurement until the time of planned delivery to record any potential signs abortion or infection. Preterm pigs were then delivered by caesarean section at d 106 (89-92% gestation age). In experiment 1, to confirm the postnatal gut and systemic effects of prenatal LPS, a proportion of the surviving pigs at birth with and without IA LPS injection (25 CON and 26 LPS pigs) were fed formula until euthanasia on postnatal d 5. In experiment 2, to test the effects of bioactive milk components in pigs born with prenatal inflammation, 44 caesarean delivered piglets with IA LPS injection from five of the above eight pregnant sows were block-randomized, according to birth weight and gender, into four groups fed with four different diets: formula (FOR, n=11), bovine colostrum (COL, n=10), casein-glycomacropeptide-enriched formula (CGMP, n=11) and osteopontin-enriched formula (OPN, n=12). Pigs that died before birth or during the first 24 h after birth were excluded from the data analyses.

After birth, each piglet was transferred to a pre-heated individual incubator (37-38°C) with supplemental oxygen (0.5-2 L/min, for the first 24 h). If necessary, resuscitation was performed by physical stimulation and positive airway ventilation until stable respiration. Each pig was then inserted a vascular catheter (4F, Portex, Kent, UK) via the umbilical artery for blood sampling and parenteral nutrition (PN) and an orogastric catheter (6F, Portex, Kent, UK) for enteral nutrition (EN), as described previously (21). During
the first 24 h, piglets also received maternal plasma (16 mL/kg) via the umbilical catheter for provision of passive immunity. All piglets were nourished by PN (Kabiven, 3210 kJ/L, modified to meet piglet nutrient requirement (44, 47), gradually decreased amount, 96-48 mL/kg/d), and by EN (gradually increased amount of 24-120 mL/kg/d) until postnatal d 5, when they were euthanized for tissue collection. Four enteral diets were designed isoenergetically (Table 1). Infant formula consisted of whey protein concentrate Lacprodan 80D (Arla Food Ingredients, Viby, Denmark), Seravit-SHS, Liquidgen MCT-SHS, Calogen LCT and Fantomalt (Nutricia, Birkerød, Denmark). For enriched formulas, the levels of supplemented CGMP and OPN were 30 g/L and 2.2 g/L, respectively. Bovine colostrum powder (Colodan, Biofiber Damino, Gesten, Denmark) was a commercial product previously used in clinical trials of preterm infants (22). Clinical conditions and faecal characteristics were evaluated twice daily as previously described (33). The time from birth until first signs of eyelid opening, standing and walking were recorded. At euthanasia, organs were weighed and tissues were snap-frozen and stored at -80°C or fixed in paraformaldehyde 4% for later analyses. Five regions of the gastrointestinal tract including stomach, proximal, middle and distal small intestines and colon were evaluated for macroscopic NEC-like lesions by a scoring system from 1 to 6, as previously described (21). A pig with a score of at least 3 in any the five gastrointestinal regions was defined as NEC.

Hematology and systemic immune analyses

At birth, amniotic fluid was collected for manual leukocyte counting to evaluate the levels of intra-amniotic inflammation, as previously described (33). Arterial blood (d 1, 3 and 5) was collected for hematology by an automatic cell counter (Advia 2120i Hematology System Siemens, Germany) and systemic immune analyses including blood T cell phenotyping and blood neutrophil phagocytosis function. For blood T cell characterization, blood erythrocytes were lysed (1 × BD FACS Lysing solution, BD Biosciences, Lyngby, Denmark) and the remaining leukocytes were permeabilized (Fixation/Permeabilization Concentrate, eBioscience, ThermoFisher, Roskilde, Denmark) for 30 min at 4°C in the dark and washed with permeabilization buffer (eBioscience). The cells were incubated 15 min in the
dark at 4°C with porcine serum (Thermofisher) for Fc receptor blocking, and then stained with a mixture of
PerCP-Cy5.5 conjugated anti-pig CD3 antibody (IgG2a isotype, BD Biosciences, Lyngby, Denmark), FITC-
conjugated anti-pig CD4 antibody (IgG2b isotype, BioRad, Copenhagen, Denmark), PE-conjugated mouse
anti-pig CD8 antibody (IgG2a isotype, Biorad) and APC-conjugated anti-mouse/rat Foxp3 antibody (IgG2a
isotype, eBioscience). Samples were then analyzed by BD Accuri C6 flow cytometer (BD Biosciences). PerCP-
Cy5.5-conjugated mouse IgG2a negative control antibody (BD Bioscience), APC-conjugated rat IgG2a
negative control antibody (eBioscience), PE-conjugated mouse IgG2a negative control antibody and FITC-
conjugated mouse IgG2b negative control antibody were used as isotype controls. T cell subsets were
analyzed including helper T cells (Th, CD3+CD4+CD8− lymphocytes), cytotoxic T cells (Tc, CD3+CD4−CD8+
lymphocytes) and regulatory T cells (Treg, CD3+CD4+Foxp3+ lymphocytes). Blood neutrophil phagocytosis
function was analyzed by flow cytometry using the pHrodo Red E.coli (560/585 nm) Bioparticles
Phagocytosis Kit for Flow cytometry (Thermofisher) as previously described (32). Phagocytic rate was
assessed as percentage of neutrophils exerting phagocytosis (pHrodo+ neutrophils), and phagocytic capacity
was assessed as the fluorescent intensity of pHrodo+ in the pHrodo+ neutrophil population.

Serum biochemistry, tissue cytokines and brush border enzyme activities

At euthanasia, serum samples were collected for biochemical analyses by Advia 1800 Chemistry
System (Siemens, Germany). Distal small intestinal tissues were homogenized and analyzed for IL-1β, IL-8,
IL-10 and I-FABP by ELISA (R&D Systems, Abingdon, UK). In addition, the small intestinal homogenates
(proximal, middle and distal regions) were also analyzed for brush border enzyme activities including
peptidases (aminopeptidase N (ApN), aminopeptidase A (ApA) and dipeptidylpeptidase IV (DPPIV)) and
disaccharidases (sucrase, maltase and lactase) by spectrophotometry, as previously described (45, 46).

Gut histology and goblet cell density

Fixed tissues from the small intestinal regions (proximal, middle and distal) were embedded in
paraffin, sectioned and stained with hematoxylin and eosin for measurement of villus height and crypt
depth by ImageJ (version 1.50i, NIH, USA). Mucin-containing goblet cells in the distal small intestine and colon were stained with Alcian blue and Periodic acid-Shiff (AB-PAS) and quantified, as previously described (28).

**Gut microbiota composition**

Colon contents collected at euthanasia were used to extract total cellular DNA using PowerSoil DNA Isolation Kit (MoBio Laboratories, CA, US) (27, 40). The bacterial V3 region of 16S rRNA was chosen for NextSeq paired-end 150bp amplicon analysis using primer compatible with Nextera Index Kit (Illumina, CA USA): NXt_388_F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACWCCTAGGGWGGCAGCAG-3′ and NXt_518_R: 5′-GTCTCGTGGGCTCGGAGATGTGTTATAAGAGACAGATTACCAGCCGCTGCTGG-3′ (Integrated DNA Technologies; Leuven, Belgium).

**In vitro cell culture, proliferation and cytokine secretion**

IPEC-J2, an intestinal epithelial cell line (IEC), isolated from jejunum of a neonatal, unsuckled piglet (ACC 701, DSMZ, Braunschweig, Germany) was cultured in Advanced Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F-12) supplemented with heat inactivated 5% fetal bovine serum, 1% GlutaMAX and 0.2% penicillin-streptomycin (all from Gibco, New York, USA), at 37 °C and 5% CO2. All experiments were performed in 3-4 independent replicates at cell passages 5-15.

For proliferation assay, the cells were seeded at 5×10^4 cells/mL in a 96-well plate, cultured for 24 h, and then serum starved for another 24 h. Thereafter, the cells were stimulated with bovine OPN (Lacprodan OPN-10, Arla Foods Ingredients, Viby, Denmark) or CGMP (Lacprodan CGMP-20, Arla Foods Ingredients) at various doses of 0.01-1 g/L (sterile filtered at 0.22 um) for 24 h, and then incubated with the CellTiter 96 AQsens One Solution Reagent (Promega, Nacka, Sweden) for 4 h at 37 °C and 5% CO2 before measurement of the absorbance at 490 nm. Cell proliferation was then quantified relative to controls (set as 100 %), which was cultured in the same serum-free medium.
For cytokine secretion assays, the cells were seeded at $1 \times 10^5$ cells/ml in a 24-well plate, cultured until reaching 70-80% confluence, and serum starved for 24h. The cells were then stimulated with 0.05 g/L CGMP or OPN with or without LPS (1 µg/mL, 0127:B8; InvivoGen, Toulouse, France) for 24 h. Supernatants were collected for analysis of IL-8 (specific porcine ELISA DuoSet kits, R&D Systems, Abingdon, UK).

Statistical analysis

All statistical analyses were performed using R, version 3.4.3. In vitro data including cell proliferation and secreted cytokines were analysed by linear models with protein concentration and passage as fixed factors (proliferation) or with treatment (with or without CGMP/OPN treatment) and cell passage as fixed factors (cytokines), using lmer function. Incidences of diarrhea and NEC and first stand were compared using Fisher exact tests. NEC severity score was analysed by non-parametric Mann-Whitney test. All other continuous data from the animal experiment (except gut microbiota) were analysed by linear mixed models with diet as a fixed factor, and litter as a random factor using lmer function. The post hoc Dunnet tests were used to compare interventions with the negative control (FOR). Data transformation (log or sqrt) was performed if necessary. Residuals and fitted values were used to evaluate normal distribution and variance homogeneity. Data are shown as mean ± SEM, and P values < 0.05 were regarded as statistically significant and P values < 0.1 were considered as tendencies of being significant.

For microbiome analysis, the raw sequencing reads were merged and trimmed, and chimeric reads were removed, resulting in zero-radius Operational Taxonomic Units (zOTUs) with UNOISE implemented in Vsearch (12, 13, 43). The green genes (13.8) 16s rRNA gene database was used as reference for annotation. Qiime pipeline (v1.9.1) (9) together with R packages vegan, ggpubr and ggplot2 were used for data analysis. All the samples were rarefied to the minimum sampling depth (32719 counts) for alpha diversity index calculation. The raw OTU table was normalized with cumulative sum scaling (39) to calculate unweighted and weighted Unifrac distance, and adonis from R package (vegan)(36) was performed to test differences.
among treatments after removing the litter effect. Specific taxa comparisons were analysed by ANCOM with FDR correction (30). P values < 0.05 were considered as statistically significant.
Results

Effects of fetal LPS exposure on postnatal gut outcomes and systemic inflammation in preterm pigs

In experiment 1 testing fetal and postnatal effects of IA LPS, the incidence of fetal death was higher in LPS vs. CON pigs (45/141 vs. 4/47, P < 0.01). Total leukocyte levels in the amniotic fluid were highly elevated in LPS vs. CON pigs (P < 0.001, Fig. 2A), indicating LPS-induced IA inflammation. LPS pigs took longer time to stand for the first time, approximately 49 h in LPS vs. 31 h in CON pigs (P < 0.05, Fig. 2B). At euthanasia on day 5, circulating parameters showed responses to LPS with a tendency to increased IL-1β and glucose levels (P = 0.09 and 0.07, respectively), increased iron level (P < 0.01) and decreased levels of albumin and alanine aminotransferase activities (ALT), relative to CON pigs (P < 0.05 and 0.01, respectively, Fig. 2C-G). In contrast, gut parameters on day 5 showed no clear LPS effect on the measured inflammatory endpoints or NEC incidence (35-48%, Fig. 2H). Lactase activity across three regions of the small intestine did not differ between LPS and CON pigs (Fig. 2I), but it showed tendency to be lower in the middle small intestine of LPS vs. CON pigs (P = 0.07).

Clinical evaluations and physical activity in LPS-exposed preterm pigs

There was a tendency for more CGMP pigs to be on their feet within the first two days, relative to the FOR group (P = 0.06, Fig. 3A). During the study, diarrhea occurrence was dominant in FOR pigs (91%), but lower in the remaining groups (40% in COL pigs, 4/10, P < 0.05; 55% in CGMP pigs, 6/11, P = 0.15; 58% in OPN pigs, 7/12, P = 0.15, Fig. 3B). NEC incidence in the whole gastrointestinal tract was not different among the groups but the NEC severity score tended to be lower in COL vs. FOR pigs (P = 0.08, Fig. 3C-D).

Intestinal morphology, brush border enzyme and nutrient absorption in LPS-exposed preterm pigs

Gut morphology data showed similar villus height (Fig. 4A) and crypt depth (Fig. 4B) across all the three regions of the small intestine. FOR pigs had the lowest lactase activities across three intestinal regions, relative to the other three groups (Fig. 4C). Comparing lactase activity in each specific small intestinal
region, COL pigs had 1.5 to 2-fold higher level than FOR pigs in proximal, middle and distal regions (P < 0.01, P < 0.001 and P = 0.07, respectively), and CGMP pigs had higher levels than FOR pigs only in middle region (P < 0.01, Fig. 4C). Plasma galactose levels following administration of a bolus of galactose solution, used as a hexose absorption marker, showed numerically higher values in CGMP vs. FOR pigs (Fig. 4D).

**Gut cytokines, proteins and goblet cell density in LPS-exposed preterm pigs**

The pro-inflammatory cytokines IL-1β and IL-8 and the anti-inflammatory cytokine IL-10 were evaluated in the distal small intestinal tissues. IL-1β levels tended to be lower in COL vs. FOR pigs (P = 0.06, Fig. 5A), and IL-8 levels in COL and CGMP were lower than in FOR pigs (P < 0.001 and P = 0.09, respectively, Fig. 5B). No differences in IL-10 levels were observed (Fig. 5C). I-FABP, a gut maturation marker, showed a tendency to be elevated in the distal small intestine of COL pigs (but not OPN nor CGMP pigs), relative to FOR pigs (P = 0.06, Fig. 5D). Both colon and distal small intestinal mucin-containing goblet cell densities were significantly higher in COL pigs than FOR pigs (P < 0.05, Fig. 5E-F) with intermediate values in OPN and CGMP pigs.

**Blood biochemistry and systemic immunity in LPS-exposed preterm pigs**

Comparing serum biochemical parameters at euthanasia on day 5, COL pigs had higher levels of cholesterol, phosphate, urea nitrogen, Mg and K than FOR pigs (all P < 0.05, Table 2). CGMP-enriched formula-fed pigs had 2.4-fold higher levels of urea nitrogen (P < 0.001), and tended to have increased serum levels of P (P = 0.07) and Na (P = 0.08), relative to FOR pigs (Table 2). Systemic parameters on d 5 modulated by LPS including albumin, ALT (Table 2), glucose and IL-1β (Fig. 6A-B) were not different among the feeding groups while serum Fe decreased in COL pigs (relative to FOR pigs) to the basal levels found in pigs without LPS exposure (P < 0.05, Fig. 6C). Total blood leukocyte, neutrophil, lymphocyte and monocyte counts on d 5 were not different among the groups (Table 2).

On day 3, COL pigs had higher frequencies of blood helper T cells (CD3⁺CD4⁺CD8⁻ lymphocytes) than FOR pigs (P < 0.05), whereas expression levels of CD4 on blood helper T cells tended to be lower in COL and
OPN pigs than in FOR pigs (P = 0.07 and 0.06, respectively, Fig. 6D-E). In vitro blood neutrophil phagocytic capacity against E. coli on day 5 showed tendency to be lower in COL vs. FOR pigs (P = 0.07) while OPN and CGMP pigs showed similar levels to that in FOR pigs (Fig. 6F).

Colon microbiota composition in LPS-exposed preterm pigs

High quality reads were merged and aligned to 1098 zOTUs, then classified into 55 bacterial groups at genus level (28 identified genera and 27 unambiguously identified at upper levels). The numbers of observed OTU (alpha diversity) were similar among the groups (Fig. 7A). Enterococcus, Streptococcus, Klebsiella, Clostridium and spp. and members of Enterobacteriaceae, Clostridiaceae were dominant groups in the colon content without significant differences in abundance of any bacterial groups among the four treatments (Fig. 7B). Using PCoA analysis for both unweighted and weighted Unifract distance matrix, no difference was detected among the four groups (Fig. 7C-D). No specific bacterial groups were significantly affected by COL, OPN or CGMP intervention, relative to FOR.

In vitro cell studies

We used porcine IPEC-J2 cell line to test the in vitro effects of CGMP and OPN on IEC proliferation and related cytokine secretion. Among tested protein/peptide concentrations (0, 0.01, 0.1 and 1 g/L), CGMP did not stimulate IEC proliferation. In contrast, OPN increased cell proliferation in a dose-dependent manner with the highest proliferation of approximately 150% at 1 g/L (Fig. 8A).

For the cytokine secretion assay, cell stimulation with CGMP or OPN alone slightly down-regulated the levels of IL-8, relative to control cells cultured in the same serum-free medium (P = 0.06 and P < 0.05, respectively; Fig. 8B). LPS stimulation led to a 1.7-fold increase in IL-8 levels (P < 0.001; Fig. 8B). Co-stimulation with LPS and CGMP, but not LPS and OPN, decreased IL-8 secretion, relative to LPS stimulation alone (P < 0.01; Fig. 8B).
Clinical studies indicate that prenatal inflammation may have a variety of effects on the preterm neonate and its organs, depending on the nature, timing and length of fetal inflammatory exposure (52). Studies in fetal lambs also support this conclusion (26, 34, 57), but these studies did not allow rearing of preterm lambs exposed to prenatal inflammation, to investigate clinical effects after birth and postnatal feeding, such as NEC. In our previous preterm pig study (33), we induced prenatal inflammation by an intra-amniotic LPS injection three days before preterm birth and found that IA LPS led to strong fetal gut immune responses and increased postnatal diarrhea and symptoms of systemic inflammation when preterm pigs were fed infant formula. However, the postnatal NEC outcomes were not affected by IA LPS. In this study, we first confirmed the most important effects of IA LPS in formula-fed pigs on postnatal day 5, including increased systemic inflammation and decreased gut enzyme activities in LPS vs. CON pigs, with no impact on NEC incidence. Then we aimed to dampen these detrimental effects of IA LPS by feeding bioactive milk diets. We found that colostrum feeding reduced diarrhea and NEC severity, lowered levels of gut inflammatory cytokines, and increased gut enzyme activities and goblet cell density. Enrichment of formula with CGMP and OPN exerted some beneficial effects (reduced diarrhea and gut cytokines, increased gut lactase activity) and the results were supported by proliferative and anti-inflammatory properties of CGMP and OPN in vitro. However, we conclude that in the presence of prenatal inflammation, the in vivo effects of these interventions were modest, relative to our previous studies on preterm pigs not subjected to prenatal inflammation (31, 48). Possibly, exposure to inflammation before birth makes the preterm newborn neonate less sensitive to the immune-modulatory effects of bioactive milk diets after birth.

For those preterm infants with insufficient or delayed intake of mother’s own milk, intact bovine colostrum may be a better alternative than processed infant formulas as it contains high levels of multiple bioactive components, including IgG, lactoferrin and OPN (10). In recent years, multiple preterm pig studies have showed positive effects of bovine colostrum to improve gut function and maturation, as well as to
decrease gut inflammatory responses (20, 48). In addition, bovine colostrum also reduced the time to full
enteral feeding and increased protein intake in preliminary studies in preterm infants, relative to infant
formula (22). This preliminary infant study did not allow us to evaluate if the postnatal effects of colostrum
feeding may depend on the inflammatory status at birth and previous animal studies with colostrum have
not addressed this research question. Now we demonstrate that in preterm pigs exposed to IA
inflammation, bovine colostrum feeding reduces or has tendency to reduce diarrhea, NEC severity and gut
inflammatory cytokine levels, reflecting an anti-inflammatory activity. For some of the parameters such as
NEC severity and gut IL1-β, the tendency may have become significant if the study sample size was greater.
Nevertheless, the effects of colostrum in this current study were much less pronounced than a previous
study using similar products and feeding regimes but without prenatal inflammation (e.g. clear colostrum
protective effects on NEC lesions in all regions, gut nutrient absorption, permeability, mucosal morphology
and tissue cytokines) (48). This suggests that adverse inflammatory conditions before birth may lead to a
less responsive gut status to immune-modulatory diets after birth. An immune-modulatory diet, like
colostrum, may be most effective in a state where the gut has not already been challenged with pro-
inflammatory stimuli before birth.

In addition to gut effects, some systemic immune parameters were also modulated by colostrum
feeding, including the increased percentage of blood T-helper cells, as well as decreased blood neutrophil
phagocytosis function, relative to formula feeding. Increased percentage of blood T-helper cells were
associated with the immune maturation over the first two weeks of life in preterm pigs (40), suggesting
systemic immune maturational effects of bovine colostrum in the current study. In contrast, decreased
neutrophil phagocytic capacity may relate to the numerical increase in blood neutrophil counts in COL vs.
FOR pigs, as newly recruited neutrophils from the bone marrow to the circulation may have a relatively less
mature function. It remains to be elucidated however, if COL pigs have an improved systemic immune
competence and infection resistance, relative to FOR pigs. Further, colostrum feeding in LPS-exposed pigs
reversed levels of several parameters including serum iron and gut lactase activity to levels found in
formula-fed pigs without prenatal LPS challenge. These effects may be derived from both anti-inflammatory and nutritional properties of colostrum (relative to formula). The different nutritional composition between colostrum and formula may also explain differences in serum levels of BUN, P, Mg and K.

CGMP and OPN have been reported to exert immune-modulatory functions both in vivo and in vitro (18, 29, 41, 55). In the current study, we showed proliferative effects of OPN and anti-inflammatory functions of CGMP in IECs in vitro, coupled with some moderate improvements of gut functions in preterm pigs (e.g. less diarrhea and inflammatory cytokines and higher lactase activity). For CGMP, a previous in vitro study using an IEC cell line demonstrated that CGMP decreased inflammatory gene expressions including IL1B, IL8 and TNFA (18). On the other hand, the in vivo beneficial trends of OPN in this study was very modest (only weak tendency of reduced diarrhea), in contrast with a previous study showing significant effects of OPN to reduce NEC severity and gut inflammation in preterm pigs without prenatal inflammation (31). Strong innate immune modulations in vitro and modest in vivo effects support our speculation that effects of bioactive milk proteins after birth is less pronounced if the newborn has already been exposed to prenatal inflammation. Similar to colostrum, CGMP- and OPN- supplemented formula diets may be more effective to prevent feeding-induced inflammatory reactions than to act as a therapy when gut inflammation is already present due to prenatal inflammatory stimuli.

Interestingly, none of the diet interventions led to changes in the gut microbiota composition or diversity, relative to infant formula. Both CGMP and OPN have previously been reported to modulate microbiota composition and play a role in maintaining gut bacterial homeostasis (19, 35). In preterm pigs without prenatal inflammation, colostrum feeding also reduced the abundance of Enterococcus spp. (56), a bacterial genus that is often associated with increased gut inflammation (4). As indicated in a previous pig study (33), fetal LPS-induced gut inflammation may alter bacterial gut colonization during early life, thereby affecting the host-microbiota interactions and responses to nutrition. Similar abundance of Enterococcus
spp. in all four groups in this study also supports that the effects of colostrum, CGMP and OPN supplementation were relatively mild.

In conclusion, milk diets containing bioactive components such as bovine colostrum, OPN- or CGMP-enriched formulas exerted modest protective effects on the immature gut in preterm pigs exposed to prenatal inflammation. Possibly, prenatal inflammation inhibits the protective effects of milk bioactive diets. Potentially, the postnatal effects of prenatal inflammation are highly dependent on the nature, timing and length of the inflammatory insult. Thus, preterm neonates exposed to prenatal inflammation may require careful evaluation and highly individualized clinical care and diets. Further investigations are required to define the optimal nutritional regimen for preterm infants subjected to prenatal inflammation, especially when mother’s own milk is lacking or absent.

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References


Table 1. Nutritional compositions in the four diets used in preterm pigs

<table>
<thead>
<tr>
<th></th>
<th>Formula</th>
<th>CGMP-Formula</th>
<th>OPN-Formula</th>
<th>Colostrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g/L)</td>
<td>40.6</td>
<td>70.6</td>
<td>42.8</td>
<td>67.6</td>
</tr>
<tr>
<td>Carbohydrate (g/L)</td>
<td>45.0</td>
<td>34.9</td>
<td>45</td>
<td>26.8</td>
</tr>
<tr>
<td>Lipid (g/L)</td>
<td>41.3</td>
<td>31.5</td>
<td>41.3</td>
<td>36.8</td>
</tr>
<tr>
<td>Energy (kJ/L)</td>
<td>3017.0</td>
<td>3016.9</td>
<td>3054.7</td>
<td>3004.7</td>
</tr>
</tbody>
</table>
Table 2. Blood biochemistry and hematology on d 5 in preterm pigs born with prenatal inflammation

<table>
<thead>
<tr>
<th></th>
<th>FOR (n=11)</th>
<th>COL (n=10)</th>
<th>CGMP (n=11)</th>
<th>OPN (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total protein (g/L)</strong></td>
<td>30.0±0.8</td>
<td>31.8±1.2</td>
<td>31.4±1.6</td>
<td>29.9±0.9</td>
</tr>
<tr>
<td><strong>Albumin (g/L)</strong></td>
<td>11.1±0.4</td>
<td>11.9±0.6</td>
<td>12.2±0.7</td>
<td>11.1±0.4</td>
</tr>
<tr>
<td><strong>ALT (U/L)</strong></td>
<td>18.6±0.6</td>
<td>20.4±0.7</td>
<td>23.7±3.6</td>
<td>18.3±0.9</td>
</tr>
<tr>
<td><strong>Cholesterol (mmol/L)</strong></td>
<td>2.4±0.1</td>
<td>3.0±0.2*</td>
<td>2.4±0.2</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td><strong>Iron (µmol/L)</strong></td>
<td>6.2±0.8</td>
<td>4.5±1.4*</td>
<td>3.6±0.4</td>
<td>4.0±0.7</td>
</tr>
<tr>
<td><strong>Phosphate (mmol/L)</strong></td>
<td>1.5±0.1</td>
<td>1.9±0.1**</td>
<td>1.7±0.1*</td>
<td>1.6±0.1</td>
</tr>
<tr>
<td><strong>Urea nitrogen (mmol/L)</strong></td>
<td>3.5±0.6</td>
<td>11.4±0.7***</td>
<td>8.4±0.9***</td>
<td>4.2±0.8</td>
</tr>
<tr>
<td><strong>Mg++ (mmol/L)</strong></td>
<td>0.80±0.03</td>
<td>1.05±0.05***</td>
<td>0.84±0.04</td>
<td>0.84±0.03</td>
</tr>
<tr>
<td><strong>Na+ (mmol/L)</strong></td>
<td>148.0±0.7</td>
<td>147.1±1.1</td>
<td>152.2±2.8*</td>
<td>149.3±1.9</td>
</tr>
<tr>
<td><strong>K+ (mmol/L)</strong></td>
<td>3.4±0.1</td>
<td>4.2±0.1***</td>
<td>3.6±0.1</td>
<td>3.6±0.2</td>
</tr>
<tr>
<td><strong>Total leukocytes</strong></td>
<td>4.3±0.5</td>
<td>6.8±1.3</td>
<td>4.2±0.6</td>
<td>3.5±0.5</td>
</tr>
<tr>
<td><strong>Neutrophils</strong></td>
<td>2.7±0.5</td>
<td>4.2±1.3</td>
<td>2.4±0.4</td>
<td>2.0±0.4</td>
</tr>
<tr>
<td><strong>Lymphocytes</strong></td>
<td>1.5±0.1</td>
<td>2.3±0.3</td>
<td>1.7±0.2</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td><strong>Monocytes</strong></td>
<td>0.10±0.02</td>
<td>0.10±0.02</td>
<td>0.07±0.01</td>
<td>0.10±0.02</td>
</tr>
</tbody>
</table>

* P < 0.05; ** P < 0.01; *** P < 0.001, relative to FOR. # P = 0.07-0.08, relative to FOR. Values (mean ± SEM) in three diet treatment groups were compared with values in FOR pigs (control). ALT:Alanine aminotransferase.
Figure legends

**Fig. 1.** Schematic overview of the animal experimental design. Fetal pigs from eight litters received either LPS injection or saline/no injection (CON) at day 103 of gestation (3 days before delivery by caesarean section). In experiment 1, a proportion of LPS and CON pigs were reared until postnatal day 5 to characterize effects of IA LPS on postnatal outcomes. In experiment 2, IA LPS-exposed pigs were randomized into 4 groups fed with 4 different diets until postnatal day 5. Exp, experiment; IA, intra-amniotic; LPS, lipopolysaccharide.

**Fig. 2.** Effects of intra-amniotic (IA) LPS on gut and systemic parameters in formula-fed preterm pigs at postnatal day 5. (A-B) Amniotic fluid leukocyte levels and time from birth to first stand. (C-G) Circulating levels of IL-1β, glucose, iron, albumin, and alanine aminotransferase (ALT). (H-I) NEC incidence and small intestinal lactase activity. n = 32-36 in each group for (A) and 25-26 in each group for (B-I). Values are mean ± SEM. *, P < 0.05. CON and LPS: control and LPS-exposed pigs.

**Fig. 3.** Physical activities and clinical evaluations in IA LPS-exposed preterm pigs following five days of postnatal feeding with various bioactive diets. (A) Number of pigs with first stand after the first 2 days after birth, (B) diarrhea incidence during study, (C) NEC incidence, and (D) average NEC severity score across the gastrointestinal tract. All treatment groups were used to compare to FOR group (control). n = 10-12 in each treatment group. Values are shown by mean ± SEM. *, P < 0.05. IA: intra-amniotic.

**Fig. 4.** Gut morphology, brush border enzymatic activities and nutrient absorption in IA LPS-exposed preterm pigs following five days of postnatal feeding with various bioactive diets. (A-C) Villus height, crypt depth and lactase activities across the three small intestinal regions. (D) Plasma galactose concentration following a galactose test. All treatment groups were compared with FOR group (control). n = 10-12 in each group for (A-C) and n = 8-10 in each group for (D). Values are mean ± SEM. *, ** and *** P < 0.05, 0.01 and 0.001. IA: intra-amniotic. Prox: proximal, Mid: middle, Dist: distal intestine.
Fig. 5. Gut inflammatory cytokines and mediators and goblet cell density in IA LPS-exposed preterm pigs following five days of postnatal feeding with various bioactive diets. (A-D) Distal small intestinal IL-1β, IL-8, IL-10 and I-FABP, respectively. (E-F) Goblet cell density in distal small intestine and colon. All treatment groups compared with FOR group (control). n = 10-12 in each group. Values are mean ± SEM. * and *** P < 0.05 and 0.001. IA: intra-amniotic.

Fig. 6. Systemic immune parameters in IA LPS-exposed preterm pigs following five days of postnatal feeding with various bioactive diets. (A-C) Serum/plasma levels of glucose, IL-1β and iron at euthanasia on d 5, respectively. (D-E) Percentage of helper T cells and fluorescent intensity of CD4 on d 3. (F) Neutrophil phagocytic capacity on d 5. All treatment groups were compared with FOR group (control). n = 8-12 in each group for (A-C) and n = 5-10 in each group for (D-F). Values are mean ± SEM. *, p<0.05. IA: intra-amniotic; MFI: median fluorescent intensity.

Fig. 7. Colonic microbiota composition in IA LPS-exposed preterm pigs following five days of postnatal feeding with various bioactive diets. (A) Number of operational taxonomic units (OTUs). (B) Relative abundance of specific genera, with species with an relative abundance below 1% being grouped to “others”. (C-D) Principle coordinates analysis (PCoA) plots based on unweighted and weighted Unifrac distance matrix. n = 6-10 in each group.

Fig. 8. In vitro effects of OPN and CGMP on intestinal epithelial cells (IECs). (A) In vitro IEC proliferation stimulated by different concentrations of CGMP and OPN (0, 0.01, 0.1 and 1 g/L) for 24 h. (B) IL-8 secretion in IECs following cell stimulation with CGMP or OPN with or without LPS presence. n = 3-4 in each treatment group. Values (mean ± SEM) not sharing the same letters are significantly different (P < 0.05). * and **, P < 0.05 and 0.01, respectively. IEC: intestinal epithelial cell.
### Prenatal period

- **d -3**
  - Saline/No injection
  - n = 47

- **d 1**
  - LPS
  - n = 141

### Postnatal period

- **Postnatal period (d 1)**
  - Exp. 1
    - CON
    - n = 25
    - LPS
    - n = 26

- **Exp. 2**
  - LPS
  - FOR (n = 11)
  - COL (n = 10)
  - CGMP (n = 11)
  - OPN (n = 12)

- **d 5 (Postnatal day)**
  - 106d
  - 110d (Gestational age)
A  

In vitro IEC proliferation

Relative proliferation (%)

0 0.01 0.1 1

CGMP

OPN

(g/L)

B  

IL-8 secretion

Fold change vs. Control

Control  CGMP  LPS  LPS+CGMP

Control  OPN  LPS  LPS+OPN

P = 0.06

**  ***
(Postnatal day) d -3  d 1  d 1  d 5

Saline/No injection
n = 47  n = 43

LPS
n = 141  n = 96

Exp. 1
CON
LPS
n = 25  n = 26

LPS induces:
- IA inflammation
- Fetal gut and postnatal systemic inflammation

Exp. 2
LPS
FOR (n = 11)
COL (n = 10)
CGMP (n = 11)
OPN (n = 12)

Moderate gut protective effects in COL, CGMP and OPN pigs born with prenatal inflammation

(Gestational age) 103d  106d  106d  110d