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DIETARY MODULATION OF BRAIN DEVELOPMENT AND THE IMMUNE SYSTEM IN PRETERM PIGS

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Background and aims: Preterm infants are born with an immature gut, immune system and brain, predisposing them to short- and long-term complications. Optimizing the early diet may help to improve organ maturation. Using preterm pigs as models for immature infants, we hypothesized that a milk diet with a specific combination of pre- and probiotics, and a specific amino acid (PPA) would improve gut, brain and immune maturation relative to control (CON) pigs.
Methods : Preterm pigs (n=40, 90% gestation) were fed increasing volumes of bovine milk with or without PPA for 23 days
Results: The PPA diet tended to increase diarrhea prevalence but weight gain and body composition were similar to values in CON. Cognitive performance was improved in the PPA group (P<0.01) while motor activity and exploratory interest were unaffected. Using ex vivo MRI diffusion imaging, the orientation dispersion index in brain cortical gray matter was higher, and fractional anisotropy (FA) values lower in PPA pigs. Conversely, in associative white matter fiber bundles radial diffusivity was lower and FA values higher (all P<0.05 relative to CON), consistent with enhanced microstructural maturation. Blood leukocyte number and neutrophil phagocytic capacity were higher in PPA pigs (P<0.05), but CRP levels were similar. Relative gut weight, mucosal structure and digestive enzyme activities were similar between groups
Conclusions: A milk diet supplemented with this specific combination of PPA may enhance cognitive performance and support gray and white matter maturation and systemic immunity in preterm neonates, without affecting the gut parameters measured

EARLY ENTERAL FEEDING REDUCES SEPSIS RESPONSE AND NEUROINFLAMMATION IN A PIG MODEL OF NEONATAL BLOODSTREAM INFECTION

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Introduction: Preterm birth and infections account for the majority of early childhood morbidity. Neonatal sepsis remains a major clinical and societal burden due to associated neurodevelopmental disturbances. Milk contains several immunologically active compounds and may support infection resistance. Using a novel preterm piglet model of bloodstream infection, we hypothesized that early enteral milk feeding supports resistance against sepsis and neuroinflammation.
Methods: Forty cesarean born preterm piglets were administered saline (CON) or Staphylococcus epidermidis (SE, 109 CFU/kg) systemically within 6 hours of birth and given total parenteral nutrition (CON-TPN, n=11; SE-TPN, n=15) or enteral nutrition (SE-ENT, n=14, 9 ml/kg/3h cow's colostrum). After 24 hours, we recorded bacteriology, hematology, hemodynamics, thromboelastographies (TEGs), and acid-base balance. Furthermore, in vivo blood-cerebrospinal fluid (CSF) barrier permeability, CSF leukocyte count, microglia numbers and brain innate immune gene expression were assessed.

Results: ENT-SE pigs had fewer bacteria than TPN-SE in blood and CSF (both $p<0.05$), and physical activity levels, blood pressure, blood lactate and acidity were similar to CON-TPN, while TPN-SE pigs showed decreased physical activity, blood pressure and blood pH, and increased lactate (all $p<0.05$). Lymphopenia, thrombocytopenia and procoagulant TEG profiles were observed in both SE-infected groups. In vivo blood-CSF barrier permeability and CSF leukocytes were lower in ENT-SE than TPN-SE pigs (both $p<0.05$). Moreover, the ENT treatment downregulated several immune-related genes in the brain, while microglia numbers were increased in both SE-infected groups.
Conclusions: Using a newly established clinically relevant animal model of neonatal bloodstream

FECAL MICROBIOTA TRANSPLANTATION PROTECTS AGAINST NECROTIZING ENTEROCOLITIS BUT INCREASES OVERALL MORTALITY IN PRETERM NEWBORN PIGS

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Introduction: Necrotizing enterocolitis (NEC) is a severe gastrointestinal complication afflicting preterm infants, and inappropriate gut microbial colonization is a predisposing factor. Fecal microbiota transplantation (FMT) improves gut homeostasis in other gut diseases but has never been tested in preterm infants. Using preterm pigs as models, we hypothesized that FMT protects against NEC in preterm neonates.
Methods: 83 piglets were born by cesarean section at 90% gestation, fed increasing amounts of formula for 5 days, and received either saline (CON, n=42) or fecal material from healthy pigs for 2 days after birth (FMT, n=41, oral and rectal administration, 3*109 CFU/day). To further document the effect of administration route, a subsequent pilot study with only rectal FMT administration was conducted.
Results: Mortality was highest in FMT pigs (46 versus 19%), but NEC incidence was reduced (21 versus 56%, both $p<0.01$). FMT pigs had higher stomach pH and organic acid levels, higher intestinal villus heights, crypt depths, bacterial abundance and goblet cell density, and lower IL6 gene expression. No differences were detected for digestive enzyme activities, nutrient absorption and intestinal permeability. FMT showed 3-fold higher total blood leukocyte counts and more culture-positive organs. Rectal FMT administration was not associated with increased mortality, but tended to reduce NEC incidence and intestinal permeability (both $p=0.08$).
Conclusions: FMT was associated with higher mortality, possibly due to bacterial sepsis following oral treatment, but surviving pigs were more NEC-resistant. Dose, timing and administration route need to be further investigated before FMT is tested in preterm infants.

GASTRIC RESIDUALS PREDICT SYMPTOMS OF NECROTIZING ENTEROCOLITIS IN PRETERM PIGS

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Background and aims: Evaluation of gastric residual volume (GRV) and color is commonly used to adjust enteral feeding volume in preterm neonates. It remains controversial however, if high GRV and/or green tone (bile stain) are biomarkers of necrotizing enterocolitis (NEC) development. Unjustified use of GRV may limit enteral feeding, reduce infant growth and prolong hospitalization. Using preterms pigs as models, we hypothesized that GRV, coupled with bile acid and acidity of gastric residuals, would correlate with progression of NEC lesions.
Methods: Gastric residuals were collected from 5 d-old preterm pigs (n=231) and the association between NEC lesions (both mild and more severe) with GRV, gastric pH and gastric bile acid levels was investigated.

Results: Animals diagnosed with NEC lesions on day 5 (n=98) showed 50% higher GRV after the last meal (29±2 vs. 20±1 ml, P<0.001), increased gastric acidity (pH 3.44±0.07 vs. 3.69±0.08, P<0.05) and increased gastric bile acid levels (145±11 vs. 113±9, P<0.05).
Conclusions: Elevated volume, acidity and bile acid levels of gastric residuals are strong predictors of NEC lesions in preterm pigs. Further studies are required to show how biomarkers related to gastric residuals may help to predict region-specific NEC progression in both piglets and infants.

IMPAIRED POSTNATAL SYSTEMIC IMMUNITY IN PRETERM NEONATES

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Background: Preterm infants and pigs are highly sensitive to infections during the first weeks of life. Limited knowledge is available about the postnatal development of systemic immunity. Using pigs as models, we hypothesized that blood leukocyte functions would be immature following preterm birth but undergo maturation mainly during the first weeks after birth.
Methods: Preterm and near-term pigs (90 and 98% gestation) were reared for 3-4 weeks. We characterized blood leukocyte subsets, their capacity of phagocytosis and formation of neutrophil extracellular traps (NETs), and TLR-mediated immune response.
Results: Compared with near-term, term and adult pigs, newborn preterm pigs had low blood leukocytes, poor neutrophil phagocytic capacity, and limited cytokine responses to TLR1/2/5/7/9, and NOD1/2 agonists. Relative to near-term, responses in preterm pigs remained immature during the first postnatal week, but matured during the following 2-3 weeks, as evidenced by increasing leukocyte numbers (7-fold), frequency of NK cells, neutrophil capacity of phagocytosis and NET formation, and TLR2-mediated IL-6 production. NET formation and phagocytosis against Gram-positive bacteria remained lower in preterm vs. near-term pigs at 2-3 weeks after birth.
Conclusion: Innate immunity and leucocyte responses are very immature during the first week of life after preterm birth. This may explain the high susceptibility to infection in both preterm pigs and infants. The delayed immunity development in preterm pigs during the first month of life may be used to model how medical, antimicrobial and diet interventions may support innate immunity in preterm infants.

CIRCULATING NEUTROPHIL EXTRACELLULAR TRAP COMPONENTS ARE ASSOCIATED WITH SEPSIS AND NECROTIZING ENTEROCOLITIS IN NEWBORN, IMMATURE INFANTS, PIGS AND MICE

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Background: Preterm infants are highly susceptible to late-onset sepsis (LOS) and necrotizing enterocolitis (NEC) but specific biomarkers for diagnosis and effective treatment are lacking. Neutrophil extracellular traps (NETs) are related to sepsis in adults but not investigated in infant conditions. We hypothesized that elevated NET levels (including cell-free DNA, cfDNA) are associated with LOS and NEC development in preterm infants, potentially providing new diagnostic markers.
Methods: Circulating cfDNA and NET protein levels were compared between control and LOS/NEC preterm infants (<32 weeks of gestation, n=54) and preterm pigs (90% gestation, n=114). Finally, cfDNA and splenic

bacterial load were analyzed after inducing sepsis in neonatal mice (*S.epidermidis*, n=21). **Results**: Preterm infants before/at the onset of LOS/NEC (n=27) had elevated circulating cfDNA levels. Pigs developing NEC with septic symptoms (n=15) had elevated intestinal IL-8, IL-6 and IL-1β levels in the small intestine. Further, these pigs showed increased circulating levels of cfDNA, six NET proteins and four acute-phase proteins, while neutrophil phagocytosis and nine negative inflammatory regulators were reduced, together with changes in blood coagulation proteins. Finally, septic mice showed high cfDNA levels together with bacteria in the spleen.
Conclusion: This is the first study to document that circulating NETs are involved in neonatal LOS and NEC. cfDNA and NET proteins may provide new potential diagnostic markers for these diseases.

GUT, IMMUNITY AND BRAIN DEVELOPMENT IS IMMATURE IN PRETERM PIGS AND RESPONSIVE TO DIFFERENT MILK DIETS

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Background and aims: Preterm birth is associated with compromised development of gut function, immunity and cognition and changed cerebellar gene expression level, and early nutrition may affect these endpoints. We hypothesized that an enriched milk diet influences development of the gut, brain and immunity following preterm birth.
Methods: Caesarian-delivered preterm pigs (90% gestation) were fed a base milk diet for 19 days with or without supplementation (PRETERM-CON and PRETERM-SUP, n = 20 and 19, respectively). The preterm pigs were compared with a reference group of pigs born at term (TERM, n = 14). Gut functions, blood cell immunity and cerebellar gene expression of 39 selected neurodevelopmental or intervention related genes were investigated. Further, cognition was tested in a spatial T-maze.
Results: Relative to term pigs, a lower proportion of preterm pigs reached the learning criteria (≥ 80% correct choices/day, $P < 0.01$) but the proportion was higher in PRETERM-SUP versus PRETERM-CON ($P < 0.05$). Preterm pigs showed lower blood neutrophil counts and poorer neutrophil phagocytic capacity at birth and on day 19 ($P < 0.05$), relative to term pigs. Twenty genes showed differential regulation between PRETERM-CON and TERM pigs ($P < 0.05$), while there was no effect of the dietary interventions on cerebellar gene expression. Among preterm pigs, the leukocyte phagocytic capacity was highest in PRETERM-SUP ($P < 0.05$), while there were no differences in intestinal brush-border enzymes between groups.
Conclusions: Cognition and systemic immunity are affected negatively by preterm birth but may be improved by an enriched milk diet.

THE IMMATURE GUT IS HIGHLY RESPONSIVE TO ENTERAL DIETS BEFORE BIRTH

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Background and aims: Enteral diets have marked maturational effects on the gut in preterm neonates, including gut hormone release and brush border enzyme activities. We hypothesized that feeding-stimulated gut maturation in preterm neonates is independent of birth and that such responses may also occur after enteral feeding in utero.

Methods: Eight pregnant sows were anaesthetized at 99-102 d gestation (85% gestation) and the fetal esophagus was ligated and catheterized in 2-3 fetuses in each litter. These fetuses were infused with saline (n=6) amniotic fluid (AF, n=9), porcine milk (PM, n=7) or porcine colostrum (PC, n=9) into the fetal stomach four times daily (10 mL/kg) for 6-8 d before tissue collection.
Results: Plasma gastrin was elevated (~2-fold) for 2-3 h after each infusion of AF, PM or PC and gastrin levels were higher in the stomach lumen (all $P<0.05$). Following 6-8 d of infusion, intestinal lactase, maltase and aminopeptidase activities were higher in infused fetuses, relative to controls, particularly for the colostrum diet. There were limited effects on pancreatic and gastric proteases.
Conclusions: Ingestion of amniotic fluid, milk or colostrum can stimulate gut maturation (endocrinology, enterocyte digestive functions) already before birth. This underlines the importance of enteral food stimulation for preterm neonates and suggests that the postnatal responses are at least partly independent of the birth transition.

CEREBROSPINAL FLUID FROM PRETERM PIGS WITH NECROTIZING ENTEROCOLITIS HAS ALTERED CYTOKINE PROFILE AND PROMOTES HIPPOCAMPAL NEURITOGENESIS

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Introduction: Necrotizing enterocolitis (NEC) in preterm infants is associated with neurodevelopmental delay and cerebral palsy. We hypothesized that intestinal NEC lesions affect inflammatory cytokines in cerebrospinal fluid (CSF) which in turn may affect neurite differentiation.
Methods: Variable degrees of NEC lesions developed spontaneously in piglets reared for 9 days after preterm birth. CSF samples were collected and cytokine profile was evaluated by multiplex cytokine array ELISA. Hippocampal structures were dissected and cytokine mRNAs were measured by qPCR. The ability of piglet CSF to promote neurite outgrowth was quantified by stereology using primary rat hippocampal neurons in vitro.
Results: Relative to healthy controls, pigs with NEC lesions in either intestine or colon showed altered concentrations for 7 of 32 cytokines in CSF and higher neurite outgrowth in the *in vitro* neuronal differentiation model. The qPCR analyses showed higher erythropoietin mRNA in the hippocampus of NEC pigs.
Conclusion: Intestinal NEC lesions affect CSF cytokine profile in preterm pigs and this may affect the differentiation of neurons in the brain. Neuronal plasticity in immature brain may explain that NEC lesions, via changes in CSF cytokine levels, may affect neurodevelopment in preterm neonates.

DIFFERENTIAL EFFECTS OF HUMAN MILK FORTIFIERS BASED ON EITHER BOVINE MILK OR COLOSTRUM IN PRETERM PIGS

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Background: In preterm infants, nutrient fortification of human milk is required to improve postnatal growth, but there are concerns that currently used bovine milk-based nutrient fortifiers may induce intestinal dysfunction. Mildly processed bovine colostrum (BC), containing high amounts of protein and

bioactive components, is shown to stimulate growth and gut health in preterm pigs. Using pigs as models, we hypothesized that a commercial bovine milk-based fortifier (CF) would be inferior to BC in supporting intestinal function and immunity.
Methods: Preterm piglets received enteral nutrition consisting of pasteurized human donor milk (HM, n=16), HM fortified with CF (HM+CF, n=16), HM fortified with BC (HM+BC, n=19) for 9 days before tissue collection.
Results: Relative to the other groups, HM+CF pigs had higher diarrhea severity and lower physical activity when reaching full enteral feeding after 6 days (120 mL/kg/d). Addition of CF (but not BC) decreased intestinal digestive enzyme activities, hexose uptake, villus height/crypt depth, relative to HM. HM+BC pigs had higher body weight gain than HM pigs, with intermediate values for HM+CF pigs, which also showed lower cerebellum weight. Blood urea and creatine kinase levels were markedly higher in HM+CF than in HM+BC pigs, and phagocytic capacity of neutrophils and leucocytes was decreased by adding CF.
Conclusion: Addition of CF to human donor milk may induce adverse effects on growth, intestinal maturation and immunity in preterm pigs. This may be partly related to excessive catabolism of highly processed CF protein. BC may be an alternative to CF as a human milk fortifier for preterm infants.