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Dietary-dependent changes in the enteric nervous system and endothelium of premature piglets ☆

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ABSTRACT

After birth, intestinal morphology and function have to adapt at a high pace. This remodelling is more challenging in low birth weight neonates or in preterms, where it may result in necrotizing enterocolitis (NEC). We hypothesized that in the preterm piglet, feeding induces maladaptations of the enteric nervous system (ENS) and vasculature. Using image analysis, the densities of neurons expressing VIP, glial cells containing GFAP and the endothelium containing eNOS on immunohistochemically stained small intestinal sections of preterm 1) unfed piglets, 2) piglets receiving total parenteral nutrition (TPN) for 2–3 days and 3) piglets fed 2 days sow's colostrum (SOW) or formulated milk (FOR) following TPN were estimated.

After enteral feeding, the ENS and vascular endothelium grew in the same order as the intestine. However, feeding formula increased the density of VIP'ergic myenteric neurons, lowered eNOS in the endothelium and resulted in a reactive gliosis. In conclusion, formula induces destructive changes in the immature small intestine, whereas colostrum prevents their occurrence. These conditions may be among the factors that predispose to NEC.

1. Introduction

The pig industry is confronted with substantial losses during the perinatal period (Su et al., 2007). Abnormal prenatal maturational processes result in low birth weight, which is a predisposing factor for neonatal mortality (Tuchscherer et al., 2000; van der Lende et al., 2001; Milligan et al., 2002). Feeding supplemental milk seems insufficient – when compared to colostrum – in reducing mortality and growth retardation in runts (Wolter et al., 2002).

The enteric nervous system (ENS) ensures intestinal motility and digestion and maintains a high degree of plasticity to accurately meet changes in feeding route and type. Concomitantly the intestinal vasculature needs to respond to postnatal changes with sufficient intestinal blood flow and oxygen delivery.

In order to understand the adaptive processes triggered by colostral and not by replacer milk, this study describes the morphological development of important cells of the enteric nervous system and vasculature in the preterm piglet.

2. Material and methods

2.1. Animals

Delivery, incubation, surgery and assignment to feeding groups of the premature piglets (Large White × Danish Landrace at 93–94% of gestation) were implemented as described earlier (Sangild et al., 2002). The experiments were approved by the National Committee on Animal Experimentation of Denmark and the Ethical Committee on Animal Experimentation of the University of Antwerp, Belgium.

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The piglets were randomly divided into 4 groups. One group of newborn piglets received no nutrition (NB) (n = 5) (mean kill weight: 1324 ± 58 g). All other pigs received total parenteral nutrition (TPN) via a vascular catheter. After 2–3 days of TPN, a group of piglets was sacrificed (TPN) (n = 10) (mean kill weight: 1287 ± 233 g) whereas the rest started enteral nutrition via an orogastric tube (15 mL/kg/3 h for 32 h). One group received sow's colostrum (SOW) (n = 9) (mean kill weight: 1378 ± 181 g). The remaining piglets were given an infant milk formula designed to match sow's colostrum (FOR) (n-10) (mean kill weight: 1158 ± 149 g). The composition of TPN (using Nutriflex Lipid Plus, Braun, Melsungen, Germany) and of the formula (using Peptide 2-0, Maxipro and Liquigen-MCT, SHS International, Liverpool, UK) were modified and designed as described earlier (Sangild et al., 2002). All piglets were euthanized after anesthesia with an intra-cardiac injection of sodium pentobarbital (Nembutal®, Abbott Laboratories, Chicago, USA).

2.2. Immunohistochemistry

Randomly taken tissue samples (2–3 cm) of the proximal 1/3 and distal 1/3 of the small intestine were fixed for 2 h in 4% paraformaldehyde (0.01 mol/L, pH 7.4) and processed for paraffin embedding.

Paraffin sections (5 μm) were immunohistochemically stained to detect ß-III tubulin, vasoactive intestinal peptide (VIP), von Willebrandt factor (vWF), endothelial nitric oxide synthase (eNOS), glial fibrillary acidic protein (GFAP) and hypoxia-inducible factor-1α (HIF-1α) (Table 1). Non-specific staining was blocked by incubation in 3% H_2O_2 and 10% normal serum. Subsequently, primary antibodies, secondary antibodies (according to the manufacturer's recommendations) and staining substrate (3-amino-9-ethylcarbazole; or diaminobenzidine) were applied. All dilutions were made in 0.01% Tris-buffered saline (pH 7.4) with 0.3% Triton-X100.

2.3. Quantitative analyses

The analyses were carried out as described in (Van Ginneken et al., 2002). In short, volume densities of ßIII-tubulin, VIP, GFAP, eNOS and vWF were estimated by using an overlay and counting the number of grid points within the immunoreactive area and the number of grid points within the intestinal wall or immunoreactive reference space. For VIP and GFAP, ßIII-tubulin is considered the reference space whereas vWF-IR is considered reference for eNOS-IR.

The arcsine-transformed volume densities were analyzed using repeated-measures ANOVA. Post-hoc comparisons were performed using an LSD-test. *P*-values less than 0.05 were considered significant.

Table 1
Immunohistochemical protocols.

Antigen retrieval	Primary antibody	Secondary antibody
	Anti-ßIII-tubulin (Promega) 1:200, 37 °C, 90 min	Anti-mouse Envision (Dako)
TRIS-EDTA (pH 9), MW, 10 min	Anti-VIP (Chemicon) 1:500, 37 °C, 90 min	Anti-rabbit Envision (Dako)
Tris (pH 10), PrC, 10 min	Anti-GFAP (Abcam) 1:500, 37 °C, 90 min	Anti-mouse Envision (Dako)
Trypsin 0.05%, 10 min at 37 °C	Anti-vWF (Dako) 1:500, 37 °C, 90 min	Anti-rabbit Envision (Dako)
Tris (pH 10), PrC, 10 min	Anti-eNOS (BD Transduction Lab) 1:500, 37 °C, 90 min	Powervision (Immunologic)
TRIS-EDTA (pH 9), MW, 20 min	Anti-HIF1α (Novus Biologicals) 1:100, 4 °C, 20 h	Anti-mouse Envision (Dako)

MW: microwave; PrC: pressure cooker.

3. Results

Microscopic evaluation of the intestine revealed a higher prevalence of NEC-like lesions (e.g. blood congestion, loss of villi, and transmural necrosis) in the formula-fed piglets when compared with the other groups that were investigated.

The volume density of ßIII-tubulin-immunoreactivity (IR) or vWF-IR did not vary between groups, indicating the ENS and the endothelium grew in the same order as the intestine.

Within the ENS, VIP-IR and GFAP-IR increased in the inner submucous plexus (ISP) and VIP-IR increased in the myenteric plexus (MP) of TPN-fed piglets (VIP(ISP): 22.5 ± 3.1%; VIP(MP): 31.3 ± 5.0%; GFAP(ISP): 41.1 ± 2.5%) compared to NB (VIP(ISP): 11.8 ± 3.1%; VIP(MP): 19.0 ± 0.7%; GFAP(ISP): 31.3 ± 2.1%). Subsequent enteral feeding of either colostrum or formula maintained VIP(ISP) at the level of TPN. Whereas, VIP(MP) and GFAP(ISP) decreased after subsequent colostrum feeding (VIP(MP): 18.6 ± 2.5%; GFAP(ISP): 36.0 ± 1.8%) but not after formula feeding (VIP(MP): 29.2 ± 3.1%; GFAP(ISP): 40.7 ± 1.6%) ($P<0.05$).

The diet-dependent changes of the volume density of eNOS-IR in the tunica mucosa were influenced by the intestinal region in a way the density of eNOS-IR is significantly decreased in the distal small intestine of enterally-fed piglets (SOW: 33.3 ± 7.3%; FOR: 31.9 ± 7,1%) compared with NB (57.8 ± 5.3%) and TPN (56.3 ± 2.7%) ($P<0.05$).

The presence of nuclear HIF1α-IR only correlated with the administration of formula ($P<0.01$).

4. Discussion

During the postnatal period, the ENS and endothelium grew at the same pace as the intestine. However, parenteral or formula feeding increased the density of VIP- and GFAP-IR. Since both play a role in mucosal defence (Ekblad and Bauer, 2004; Rühl et al., 2004), this could indicate an alerted state. General defence mechanisms could be triggered by the bacterial colonization during the TPN-period and by the predominantly Gram-negative bacterial flora in formula-fed piglets compared to a balanced flora in colostrum-fed piglets (Van Haver et al., 2009).

Specifically, lipopolysaccharides could either directly (von Boyen et al., 2004; Arciszewski et al., 2008) or indirectly via raised levels of interleukin-1ß (Neunlist et al., 2003; von Boyen et al., 2004) upregulate VIP and GFAP. In addition IL-1ß could lower eNOS (Kofler et al., 2005), which is seen in the caudal small intestine. This

predisposes this region to hypoxia when enteral feeding of inferior diets is installed. The latter was confirmed by the high prevalence of HIF-1α-IR in the formula-fed group.

5. Conclusion

The data presented suggest feeding formula to immature piglets results in a pro-inflammatory response of the ENS and primes the caudal small intestine for hypoxia. Colostrum-fed piglets receive multiple bioactive molecules via sow's milk, which blocks this harmful condition.

Conflict of interest

There is no conflict of interest regarding the manuscript.

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