Parenteral and enteral feeding in preterm piglets differently affects extracellular matrix proteins, enterocyte proliferation and apoptosis in the small intestine

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The preterm intestine is immature and responds differently to total parenteral nutrition (TPN) and enteral nutrition, compared with the term intestine. We hypothesised that in preterms, diet composition and feeding route affect mucosal morphology, enterocyte mitosis and apoptosis, and the distribution of laminin-1, fibronectin and collagen IV (extracellular matrix proteins (ECMP)). Preterm piglets (93.5\% of gestation) were delivered via caesarean section and birth weight-matched allocated to one of the four experimental groups: the piglets were either euthanised immediately after delivery, after 3 d of TPN or after 2 d enteral feeding with colostrum or milk formula, following 3 d of TPN. We combined immunohistochemistry, image analysis and stereological measurements to describe the intestinal mucosal layer. No significant changes occurred after 3 d of TPN. Feeding colostrum or milk replacer for 2 d after TPN was associated with an increased crypt depth. Only enteral feeding with colostrum resulted in an increased villus height and mitotic index. Neither TPN nor enteral feeding changed the distribution pattern of ECMP or the occurrence of bifid crypts. The immature distribution pattern of ECMP in TPN-fed piglets, coupled with unchanged enterocyte mitosis and apoptosis indices, illustrates that feeding preterm pigs 3 d TPN does not lead to mucosal atrophy. Despite the invariable distribution of ECMP, colostrum was associated with crypt hyperplasia resulting in an increased villus height. These data illustrate that some mechanisms regulating cell turnover are immature in preterms and may in part explain the abnormal gut responses to TPN and enteral feeding in prematurely born pigs.

Preterm piglets: Crypt fission: Extracellular matrix proteins: Cell turnover

The newborn’s digestive tract faces the challenge of an abrupt transition from intra-uterine parenteral nutrition support via the umbilical cord to extra-uterine enteral nutrition with mother’s milk or formula. In order to meet this goal, the gastrointestinal tract undergoes intensive growth several weeks before delivery\textsuperscript{(1)}. In consequence, preterm neonates have an immature gastrointestinal tract and often require a period of total parenteral nutrition (TPN) – in combination with minimal enteral feeding – before total enteral nutrition can be administered\textsuperscript{(2)}. Fortunately, it was shown in a study using the preterm piglet as a model of human preterm nutrition that the preterm intestine is less sensitive to TPN-induced mucosal atrophy compared with the term gut\textsuperscript{(1,3,4)}. A study conducted by Niinikoski \textit{et al.} attributed the TPN-induced mucosal atrophy seen in term-born neonates to a dramatic decrease in intestinal blood flow lowering protein synthesis, cell survival and proliferation\textsuperscript{(5)}. This detrimental effect of TPN was already visible after 48 h of TPN.

The prenatal growth of the mucosa continues after birth in term and preterm piglets, especially after the administration of colostrum\textsuperscript{(1,3–8)}. In term piglets, milk formula is well tolerated and induces normal intestinal growth and villus elongation without the development of intestinal atrophy and inflammation\textsuperscript{(6,7)}. In contrast, in preterm piglets, feeding formula induces mucosal atrophy and intestinal dysfunction both with and without a previous TPN period\textsuperscript{(1,3,4)}. In contrast to the situation in the term piglet, the mechanisms – enterocyte proliferation, enterocyte apoptosis, crypt fission, extracellular matrix proteins (ECMP) – supporting these changes in mucosal morphology and their relation to diet or feeding route are not yet documented in detail in the preterm piglet.

Mucosal surface growth is coupled to the enterocyte proliferation rate. Wolinski \textit{et al.} observed a transient elevation of the mitosis:apoptosis ratio in jejunal enterocytes, resulting in growth of the gut mucosa after enteral feeding for 6 d after delivery\textsuperscript{(8)}. In addition to proliferation, crypt fission could add to this increase of the epithelial surface\textsuperscript{(9,10)}. Already it has been shown that dietary luminal factors influence the proliferation and apoptosis rate of enterocytes\textsuperscript{(8,11–14)} and – albeit not always in the same manner – the process of

\textbf{Abbreviations:} COL, oral nutrition with sow’s colostrum; ECMP, extracellular matrix proteins; FOR, oral nutrition with an infant milk formula; TPN, total parenteral nutrition; UFD, no nutrition; $V_\text{apopt}$, volume density of apoptotic cells; $V_\text{prol}$, volume density of mitotic (proliferative) cells.

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crypt fission\(^{9,14\text{--16}}\). In addition, interactions between the enterocytes and components of the extracellular matrix affect epithelial cell mitosis\(^{17\text{--20}}\). The role of the mesenchymal environment in the process of regulating epithelial cell proliferation via crypt fission seems minor\(^{20}\). ECM form an organised network of glycoproteins, proteoglycans and glycosaminoglycans of which type IV collagen, laminins and fibronectin are the main representatives and constituents of the basement membrane\(^{21}\). A balanced distribution of ECMP steers the differentiation and migration of enterocytes along the crypt–villus axis\(^{17\text{--20}}\). However, their composition and distribution can be altered during gastrointestinal disease processes, resulting in delayed mucosal healing and thus lesion formation\(^{22\text{,23}}\).

Considering the importance of an adequate cell renewal cycle for gut mucosal growth and repair in preterm newborns, we aimed to investigate the underlying processes: mitosis and apoptosis of the enterocytes, crypt fission and the concurrent alteration in the ECMP in preterm piglets subjected to TPN immediately after delivery, followed by enteral nutrition. We used image analysis techniques and morphometry based upon stereological principles in order to generate unbiased quantitative data.

We hypothesise that the relative insensitivity of preterm pigs for mucosal atrophy is due to a low proliferation and apoptotic capacity of immature enterocytes and a non-gradient distribution of the ECMP along the villus axis. Introduction of enteral feeding might affect these processes depending on the nature of the enteral feed (formula v. sow’s colostrum).

### Experimental methods

#### Animals

Delivery, incubation, surgery and assignment to feeding patterns of the premature piglets were implemented according to methods described earlier\(^{3\text{,4}}\). In short, a total of twenty-nine crossbred (Large White \times Danish Landrace) piglets from six sows were delivered by caesarean section at 107–108 d (93–94 %) of gestation. After delivery, the piglets were placed in infant incubators with regulated temperature, moisture and extra O\(_2\) supply. Throughout the experiment rectal temperature and O\(_2\) saturation were monitored. In contrast to man, transplacental transport of immunoglobulins does not occur in pigs, which makes neonatal pigs dependent on intravenous fluids (Vamin \(18 \, \text{g N/litre electrolyte free};\) Fresenius Kabi, Uppsala, Sweden) and sterile water. The nutritional goal was to provide the pigs with sufficient energy and protein to allow for a slightly positive energy balance. The infusion rate was \(4 \, \text{ml/kg per h}\) for the first 24 h post-partum, gradually increasing to \(6–8 \, \text{ml/kg per h}\) during the subsequent 2 d. In this way, the pigs received an average of \(450 \, \text{kJ/kg per d}\), \(6.5 \, \text{g amino acids/kg per d}\) and a fluid intake of \(160 \, \text{ml/kg per d}\) during the 3 d period.

#### Experiment 1

After delivery, the piglets were birth weight-matched assigned to two different groups. One group of piglets was killed within 12 h of delivery and received no nutrition (UFD; \(n\) 3). A second group of piglets was given an elemental TPN (\(n\) 6) fluid (Nutriflex Lipid plus; Braun, Melsungen, Germany) for 3 d. The parenteral nutrient solution was prepared aseptically (Nutriflex Lipid plus; Braun). The composition was based on that used previously for term and preterm pigs\(^{3\text{,4}}\). In order to achieve this composition, part of the glucose and lipid was withdrawn and substituted with amino acids (Vamin \(18 \, \text{g N/litre electrolyte free};\) Fresenius Kabi, Uppsala, Sweden) and sterile water. The nutritional goal was to provide the pigs with sufficient energy and protein to allow for a slightly positive energy balance. The infusion rate was \(4 \, \text{ml/kg per h}\) for the first 24 h post-partum, gradually increasing to \(6–8 \, \text{ml/kg per h}\) during the subsequent 2 d. In this way, the pigs received an average of \(450 \, \text{kJ/kg per d}\), \(6.5 \, \text{g amino acids/kg per d}\) and a fluid intake of \(160 \, \text{ml/kg per d}\) during the 3 d period.

#### Experiment 2

After TPN (similar to the groups in experiment 1), piglets were given oral nutrition (\(15 \, \text{ml/kg per 3 h}\)) with either sow’s colostrum (COL; \(n\) 9) or an infant milk formula (FOR; \(n\) 11) for 32–42 h. The milk diets were collected and prepared as described previously\(^{3\text{,4}}\).

#### Tissue preparation

The small intestine from the pyloric sphincter to the ileocecal junction was excised by cutting along the mesenteric border. It was carefully emptied of its contents, 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 taken. These were rinsed in 0·01M-PBS solution, pH 7·4 (PBS) and fixed at random positions, one (3–5 mm diameter) biopsy was processed for paraffin embedding. From these tissue blocks, \(3 \, \mu\)m paraffin vertical sections were made to be either conventional (haematoxylin–eosin) or immunohistochemically stained after dewaxing and rehydrating with distilled water or PBS, respectively.

#### Histological scoring and basic morphometry

The paraffin sections stained with haematoxylin–eosin were used for microscopic scoring of the gastrointestinal lesions according to the presence of atrophic or inflammatory morphological lesions\(^{24}\): 0, normal morphology; 1, slight tunica mucosa and lamina propria separation; 2, increased tela submucosa and lamina propria separation with oedema in the...
tela submucosa; 3, severe separation of the tela submucosa and lamina propria, blood congestion and regional loss of villi; 4, blood congestion, transmural necrosis and generalised loss of villi (Fig. 1). In addition, these sections were used for measuring the depth of the crypts and the height of the intestinal villi in thirty well-orientated villi and crypts (per tissue block) at a low magnification (10×) and the percentage of crypt fission at a higher magnification (20×). Crypt fission is expressed as the percentage of crypts having a split or fissure in their base. Of each tissue block, approximately 100 crypts were scored in a total of thirty sections taken systematically at random. Only crypts sectioned longitudinally were evaluated.

**Immunohistochemistry**

Immunohistochemistry (Table 1) was used to detect the ECMP: laminin-1, fibronectin, collagen IV, apoptotic (cleaved caspase-3 protein) and mitotic (proliferating cell nuclear antigen) cells. After antigen retrieval or biotin blocking, non-specific staining was blocked successively by incubation in 3 % H₂O₂ and 20 % normal serum. All immunohistochemical incubations were carried out in a closed humidified container. All immunohistochemical steps were alternated by washings in 0.01 % 2-amino-2-hydroxymethyl propane-1,3-diol (Tris)-buffered saline (TBS; pH 7.4). All antisera were diluted in 0.01 % TBS. A positive immunohistochemical reaction was visualised by incubating the sections with 3-amino-9-ethylcarbazole (DakoCytomation, Glostrup, Denmark) for 5 min. Finally, the sections were counterstained with haematoxylin.

**Stereological measurements and image analysis**

An Olympus BX50 microscope, equipped with a Sony CCD camera connected to a computer system running the software program Cast-grid (Olympus, Copenhagen, Denmark), was used for the quantitative analyses. The optimal density of the stereological grid (number of points), the number of sections and the number of sample fields were estimated as described previously(25) and resulted in the analysis of approximately thirty fields of vision in thirty sections taken systematically at random of each tissue block.

The volume densities (Vᵥ) of the apoptotic cells (Vᵥapop) and mitotic (proliferative) cells (Vᵥprol) in the mucosal layer of the intestinal wall were estimated by counting the number of grid points (P) hitting the space of interest (P(I)) (immunoreactive cells lying within or intersecting the margins of the mucosal epithelium of the intestinal wall) and the number of points hitting the reference space (P(ref)) (the enterocytes of the mucosal layer). The following equation yielded the Vᵥapop and Vᵥprol: Vᵥ(I;ref) = P(I)/P(ref).

The measurements of the Vᵥapop and Vᵥprol are comparable with the apoptotic and mitotic index measured with a previously described technique by Wright et al(26). In order to verify our protocol we performed a pilot study comparing both methods, which yielded statistically indifferent results (data not shown). The mitosis:apoptosis ratio was determined by dividing the Vᵥprol by the Vᵥapop.

The immunoreactive intensity of the ECMP-stained slices was evaluated using an image analysis program AnalySIS.

*Fig. 1.* Haematoxylin and eosin-stained sections of the preterm small intestine, illustrating: (a) no lesions; (b) mild dilated submucosal vessels; (c) severely dilated submucosal vessels; (d) regional loss of villi, severely dilated submucosal vessels and blood congestion. Scale bar = 100 μm.
Table 1. Immunohistochemistry

<table>
<thead>
<tr>
<th>Target</th>
<th>Pretreatment</th>
<th>Primary antibody, dilution, time, temperature</th>
<th>Secondary antibody, dilution, time, temperature</th>
<th>Peroxidase system, dilution, time, temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin-1</td>
<td>Trypsine (MP Biomedical, Montréal, Canada), pH 7.8, 20 min, 37°C</td>
<td>Anti-laminin (Sigma, Bornem, Belgium), 1/25, 90 min, 37°C</td>
<td>Biotinylated swine anti-rabbit (DakoCytomation, Glostrup, Denmark), 1/200, 30 min, RT</td>
<td>Streptavidin horseradish peroxidase (DakoCytomation), 1/200, 30 min, RT</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>EDTA, pH 8, 30 min, 92–97°C, 30 min CD</td>
<td>Anti-fibronectin (Sigma), 1/800, ON, RT</td>
<td>Anti-rabbit Envision® (DakoCytomation), 30 min, RT</td>
<td>Anti-rabbit Envision® (DakoCytomation), 30 min, RT</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Target retrieval solution (DakoCytomation), pH 6, 10 min, pressure cooker, 20 min CD</td>
<td>Anti-human collagen IV (DakoCytomation), 1/25, 90 min, 37°C</td>
<td>Anti-mouse Envision® (DakoCytomation), 30 min, RT</td>
<td>Anti-mouse Envision® (DakoCytomation), 30 min, RT</td>
</tr>
<tr>
<td>PCNA</td>
<td>Target retrieval solution (DakoCytomation), pH 6, 10 min, microwave, 10 min CD</td>
<td>PCNA (Sigma), 1/500, 45 min, RT</td>
<td>Biotinylated goat anti-mouse (DakoCytomation), 1/200, 30 min, RT</td>
<td>Streptavidin horseradish peroxidase (DakoCytomation), 1/200, 30 min, RT</td>
</tr>
<tr>
<td>Cleaved caspase-3 protein</td>
<td>Target retrieval solution (DakoCytomation), pH 6, 20 min, microwave, 20 min CD</td>
<td>Cleaved caspase 3 (Sigma), 1/250, 120 min, 37°C</td>
<td>Anti-rabbit Envision® (DakoCytomation), 60 min, RT</td>
<td>Anti-rabbit Envision® (DakoCytomation), 60 min, RT</td>
</tr>
<tr>
<td>EGFR</td>
<td>Biotinin blocking (DakoCytomation)</td>
<td>Anti-EGFR (Sigma), 1/70, ON, 4°C</td>
<td>Biotinylated goat anti-mouse (DakoCytomation), 1/400, 30 min, RT</td>
<td>Streptavidin horseradish peroxidase (DakoCytomation), 1/200, 30 min, RT</td>
</tr>
</tbody>
</table>

RT, room temperature; CD, crypt depth; ON, overnight; PCNA, proliferating cell nuclear antigen; EGFR, epidermal growth factor receptor.

Pro 5.0 (Olympus). The images were converted into greyscale (black: grey-value = 0 to white: grey-value = 254). The average of the grey-value of the different parts of the intestinal wall (crypt region and proximal part, middle part and distal part of the villus) was determined. The difference between the grey-value of different parts measured on the same section (for example, grey-value of the distal part of the villus minus grey-value of the proximal part of the villus) was taken and statistically these values were compared between the various experimental groups in order to detect staining differences along the crypt–villus axis. Of each tissue block, approximately sixty crypt–villus axes were scored in a total of thirty sections taken systematically at random.

Statistical evaluation

The volume densities were arcsine-transformed to turn percentages into an infinite data range. Since no interaction was detected between region or feeding group, the morphological data were analysed by repeated-measures ANOVA (region as in-between factor) to detect significant effects of feeding type. The possibility for regional differences to exist was explored via ANOVA analyses. Post hoc comparisons were performed using a least significant difference (LSD) test.

The grey-value difference of the ECMP did not meet the requirement for using parametric statistical methods and therefore Kruskal–Wallis tests were performed. Pearson correlation tests were used to detect correlations between the different parameters.

All analyses were carried out using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA).

Unless stated otherwise, the values given are the means of the three intestinal regions. Results are expressed as mean values with their standard errors. *P*<0.05 was considered significant.

Results

Villus height, crypt depth, crypt fission and lesions

Feeding parenteral nutrition for 3 d was not associated with significant changes in villus height and crypt depth relative to the UFD piglets (*P*>0.05). In contrast, 2 d of enteral nutrition with sow’s colostrum after 3 d of TPN induced a significant increase in villus height in the small intestine compared with TPN and formula-fed pigs (values irrespective of intestinal region: TPN, 519 (SEM 35) μm; COL, 624 (SEM 29) μm; FOR, 521 (SEM 32) μm) (*P*=0.02). Irrespective of feeding regimen, villus height was higher in the middle small intestine (549 (SEM 21) μm) compared with in the proximal small intestine (483 (SEM 27) μm) (*P*=0.04) (Table 2).

Crypt depth in enterally fed pigs was higher relative to in the TPN-fed group (values irrespective of intestinal region: TPN, 52 (SEM 3) μm; COL, 63 (SEM 3) μm; FOR, 65 (SEM 3) μm) (*P*=0.03). Irrespective of feeding regimen, crypt depth was higher in proximal (62 (SEM 3) μm) compared with in middle (54 (SEM 2) μm) and distal small intestine (52 (SEM 2) μm) (*P*=0.03) (Table 2).

Crypt fission (Fig. 2) apparently did not differ between the various groups (*P*=0.06). Irrespective of feeding regimen, crypt fission was lower in proximal (13·6 (SEM 2·5) %) compared with in middle (19·9 (SEM 8·0) %) and distal small intestine (21·9 (SEM 6·7) %) (*P*=0.02) (Table 2).

After 3 d of TPN no significant increase in lesion score was seen compared with the UFD piglets (*P*<0.05). COL piglets (0·21 (SEM 0·08)) had a lower lesion score than FOR piglets (1·04 (SEM 0·18)) (*P*=0.05). The microscopic lesion score was positively correlated with the V vapop (*P*=0·03), and in consequence with the mitosis:apoptosis ratio (*P*=0·03). Examples of tissues having the characteristics of lesion scores 0–4 are shown in Fig. 3.
Volume density of proliferative and apoptotic cells

Proliferating cell nuclear antigen immunoreactivity was almost entirely confined to the nucleus. The cells in mitotic division were restricted to the crypt compartment in all feeding groups except for occasional mitoses at the transition between crypt and intervillus floor in COL piglets (Fig. 3(a)). In accordance with this observation, the Vvprol – irrespective of intestinal region – tended to be higher in the intestine of COL piglets (14·1 (SEM 1·2) %) compared with TPN (10·4 (SEM 1·2) %) and FOR piglets (10·1 (SEM 0·7) %) (P < 0·05) (Table 2). Irrespective of feeding regimen, Vvprol was higher in the proximal (12·8 (SEM 0·8) %) than in the middle intestine (10·6 (SEM 1·0) %) (P < 0·05). A positive correlation of Vvprol was seen between crypt depth (P < 0·05), microscopic lesion score (P < 0·05), V vapop (P < 0·01) and mitosis:apoptosis ratio (P < 0·05). In contrast, a negative correlation was seen between Vvprol and villus length (P < 0·01).

Cleaved caspase-3, a specific marker for apoptotic cells, was used to detect and localise apoptotic changes in the small intestine of the piglets. Immunoreactivity was found mainly in the nuclei of apoptotic cells. Apoptotic epithelial cells were present over the entire length of the villi but concentrated at the top of the villi (Fig. 3(b)). Few scattered apoptotic cells were seen in the crypts and the intervillus floor (Fig. 3(a)). Scale bar = 50 μm.

Active caspase-3 immunoreactivity (b) in the proximal small intestine of the formula-fed piglet is present at the level of the villus (a). Scale bar = 200 μm.

Table 2. Villus height (μm), crypt depth (μm), crypt fission (%), volume density of epithelial proliferating cells (Vvprol) (%) and apoptotic cells (V vapop) (%) according to small-intestinal region and feeding group (Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Region</th>
<th>Proximal</th>
<th>Middle</th>
<th>Distal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>Villus length (μm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UFD</td>
<td>359</td>
<td>63</td>
<td>414</td>
</tr>
<tr>
<td>TPN</td>
<td>514</td>
<td>51</td>
<td>536</td>
</tr>
<tr>
<td>FOR</td>
<td>559</td>
<td>40</td>
<td>564</td>
</tr>
<tr>
<td>COL</td>
<td>636</td>
<td>44</td>
<td>674</td>
</tr>
<tr>
<td>Crypt depth (μm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UFD</td>
<td>45·7</td>
<td>5·7</td>
<td>41·5</td>
</tr>
<tr>
<td>TPN</td>
<td>58·5</td>
<td>4·7</td>
<td>49·0</td>
</tr>
<tr>
<td>FOR</td>
<td>77·4</td>
<td>3·6</td>
<td>59·7</td>
</tr>
<tr>
<td>COL</td>
<td>70·3</td>
<td>4·1</td>
<td>62·4</td>
</tr>
<tr>
<td>Crypt fission (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UFD</td>
<td>14·1</td>
<td>1·01</td>
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<tr>
<td>TPN</td>
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<td>0·67</td>
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<tr>
<td>FOR</td>
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<td>17·4</td>
</tr>
<tr>
<td>COL</td>
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<tr>
<td>UFD</td>
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</tr>
<tr>
<td>TPN</td>
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<td>0·75</td>
<td>7·33</td>
</tr>
<tr>
<td>FOR</td>
<td>10·81</td>
<td>0·08</td>
<td>9·64</td>
</tr>
<tr>
<td>COL</td>
<td>12·63</td>
<td>0·32</td>
<td>10·28</td>
</tr>
<tr>
<td>V vapop (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UFD</td>
<td>0·756</td>
<td>0·007</td>
<td>1·299</td>
</tr>
<tr>
<td>TPN</td>
<td>0·622</td>
<td>0·071</td>
<td>0·964</td>
</tr>
<tr>
<td>FOR</td>
<td>0·603</td>
<td>0·034</td>
<td>1·096</td>
</tr>
<tr>
<td>COL</td>
<td>0·715</td>
<td>0·048</td>
<td>1·061</td>
</tr>
</tbody>
</table>

UFD, no nutrition; TPN, total parenteral nutrition; FOR, oral nutrition with an infant milk formula; COL, oral nutrition with sow’s colostrum.

Fig. 2. Crypt fission (1) in the distal small intestine of a piglet fed orally with sow’s colostrum. Scale bar = 50 μm.

Fig. 3. Proliferating cell nuclear antigen immunoreactivity (a) in epithelial cells in the middle small intestine in an unfed piglet. These epithelial cells were confined to the crypts and the intervillus floor (1). Scale bar = 100 μm. Active caspase-3 immunoreactivity (b) in the proximal small intestine of the formula-fed piglet is present at the level of the villus (1). Scale bar = 200 μm.
(0.75 (SEM 0.07) %) and distal small intestine (0.75 (SEM 0.08) %) (P=0.01). The $V_{\text{vapop}}$ was positively correlated with the microscopic lesion score (P=0.03) and $V_{\text{vapop}}$ (P=0.01).

The $V_{\text{vprol}}$: $V_{\text{vapop}}$ ratio did not significantly differ between the feeding groups. However, the mitosis:apoptosis ratio was significantly lower in the middle (9.3 (SEM 1.4) %) compared with the proximal (23.4 (SEM 1.4) %) and distal (24.8 (SEM 1.3) %) small intestine (P<0.01) irrespective of feeding group.

The extracellular matrix proteins

Collagen IV immunoreactivity was uniformly stained along the crypt–villus axis (Fig. 4(a)). In some small-intestinal segments of the TPN-fed piglets collagen IV immunoreactivity was less intense. Laminin-1 immunoreactivity (Fig. 4(b)) and fibronectin immunoreactivity (Fig. 4(c)) were homogeneously distributed in the crypt and villus basement membrane. In some small-intestinal segments laminin-1 immunoreactivity and fibronectin immunoreactivity revealed a gradient in their staining intensity. The staining intensity of laminin-1 immunoreactivity expressed a decrease from the villus to the crypt whereas the staining intensity of fibronectin immunoreactivity increased from the apex of the villus towards the crypts in some of the sections. However, these distribution patterns could not be assigned to a specific feeding pattern or histopathological scoring (P>0.05).

Discussion

Numerous studies have shown that TPN induces mucosal atrophy in term neonates, which has been attributed to the absence of trophic signals from the lack of luminal nutrients\(^\text{(27–29)}\) and a drop in intestinal blood flow\(^\text{(5)}\). Nevertheless, TPN is often instituted in preterm neonates because the immature intestine may not tolerate enteral feeding\(^\text{(2)}\). Fortunately, the TPN-fed preterm intestine does not appear to suffer from the same degree of mucosal atrophy as the term intestine although TPN remains inferior in stimulating gut growth and maturation compared with enteral nutrition\(^\text{(1,3,4)}\). In the present study using the preterm piglet, the lack of any effect of 3 d of TPN on villus height and crypt depth confirms these previous observations. In addition, we found that the underlying mechanisms such as the distribution of ECMP, apoptosis, mitosis, and crypt fission remained unchanged in the TPN fed.

Quantitative image analysis revealed that type IV collagen, fibronectin and laminin-1 were uniformly distributed along the crypt–villus axis. This contrasts with the results of Groos\textit{ et al.}, who describe specific changes in the distribution pattern of laminin-1, fibronectin and collagen IV in human adult intestine after parental feeding\(^\text{(30)}\). Taken together, the present results suggest that preterm piglets do not exhibit TPN-induced mucosal atrophy after 3 d of TPN, partly due to indifferent proliferation and apoptosis indices. The latter could be the result of the distribution pattern of the ECMP that resembles mainly that of a fetal (immature) intestine\(^\text{(31,32)}\).

In term piglets, 1 d of suckling rapidly increases the enterocyte proliferation rate and decreases the apoptotic rate\(^\text{(8,33)}\). In contrast, 2 d enteral feeding with colostrum induced an increase of the $V_{\text{vprol}}$ but no change in the $V_{\text{vapop}}$ in the preterm piglet’s intestine. Significant apoptosis-inducing mechanisms during normal physiological development of porcine gut epithelium are the local deprivation of growth factors such as epidermal growth factor\(^\text{(15,34)}\), insulin-like growth factor\(^\text{(35)}\) and leptin\(^\text{(8)}\) and the establishment of an intestinal microbiota\(^\text{(36)}\). This is supported by the observation that a higher apoptosis rate was seen in formula-fed neonatal piglets\(^\text{(12)}\) which received no milk-borne growth factors and...
are known to harbour a different microbiota compared with sow-reared piglets\textsuperscript{1,37}. However, a difference in apoptotic index between formula-fed piglets and piglets fed colostral milk was not observed in our experimental setting using preterm piglets. Perhaps, the preterm small intestine is lacking certain receptors for food bioactive components or microbiota? It has already been reported that short-term epidermal growth factor treatment was not able to preserve villus architecture in the preterm pig small intestine\textsuperscript{12}, probably due to a lack of epidermal growth factor receptors\textsuperscript{138}.

An additional explanation for the unaltered \(V_{vapop}\) in both enterally fed groups could be the unaffected distribution pattern of the ECM, especially laminin-1. In the mature small intestine, laminin-1 is highly concentrated in the villus region\textsuperscript{18,19,30}. This is in agreement with the task assigned to this glycoprotein, namely to promote intestinal epithelial proliferation and differentiation\textsuperscript{17–20}. To facilitate this, the expression of laminin-1 increases from the crypt to the villus region\textsuperscript{18,19,30}. This gradient was absent in the preterm piglets. Thus, cell differentiation and ultimate shedding from the villus tips is equally possible along the villus axis. Similar to laminin-1, the distribution pattern of fibronectin and collagen IV was unaffected by diet and feeding route. Fibronectin is normally expressed concomitantly with cell migration\textsuperscript{18,39}. In the preterm piglets, fibronectin immunoreactivity evenly delineates the epithelial basement membrane along the crypt–villus axis. The latter is comparable with the expression pattern in the fetal intestine of piglets\textsuperscript{39}. The homogeneous distribution of subepithelial collagen corresponds to its role as a biomechanical scaffold\textsuperscript{18}, and accords to its presence in the human\textsuperscript{34} and rat\textsuperscript{32} developing gut. The absence of any gradient in ECM distribution along the villi and equal distribution patterns between the feeding groups can – at least in part – be held responsible for a slow cell migration along the crypt–villus axis.

In conclusion, the present morphometric study demonstrates that quantitative differences exist with respect to morphological (villus height and crypt depth) and functional (\(V_{vprol}\) and \(V_{vapop}\); mitosis:apoptosis ratio, crypt fission) parameters between differently fed preterm piglets. TPN feeding for a short period does not affect mitosis and apoptosis rates of enterocytes that perhaps could be related to the preserved fetal distribution pattern of ECM. Formula feeding to preterm piglets was associated with a shorter villi, a lower \(V_{vapop}\) and a higher microscopic lesion score, \(V_{vprol}\) and \(V_{vapop}\).

The observation of an increase in villus length could indicate, however, that changes in crypt depth, fission and \(V_{vprol}\) have occurred in an early phase of enteral feeding. Zhang \textit{et al.} revealed that 6h of suckling is sufficient to cause significant changes in intestinal structure and function\textsuperscript{45}.

Irrespective of the feeding regimen, typical regional differences could be seen along the small intestine. The mitosis:apoptosis ratio was significantly higher in the proximal intestine, resulting in deeper crypts, fewer bifid crypts and smaller villi. These findings reflect a more efficient proximal absorption of enteral nutrients, resulting in diminished exposure in the distal intestine. In addition, in the proximal small intestine of the preterm piglet, the process of crypt hyperplasia seems to be more important than crypt fission, whereas in the distal small intestine this seems to be reversed.

Consistent with earlier results\textsuperscript{1,3,4}, TPN fluid infused parenterally is well tolerated by preterm piglets, resulting in an unchanged microscopic lesion score compared with the unfed piglets. Compared with colostrum-fed piglets, formula-fed piglets had less preserved villus architecture and in some cases even complete destruction of the villi (resembling lesions that are associated with those of necrotising enterocolitis). Studies with pigs born at term showed that formula was associated with only minor gastrointestinal dysfunctions at a subclinical level. In addition, feeding formula to term piglets mimicked many of the short-term postnatal trophic effects observed in colostrum-fed piglets\textsuperscript{1,3,4}. Thus, preterm delivery compromises the intestinal responses to feeding, perhaps resulting in the development of intestinal lesions. During the development of these mucosal lesions, apoptosis may account for the initial loss of cells in the apical villi\textsuperscript{46}. Moreover, in order to preserve villus architecture, a higher \(V_{vprol}\) is needed, resulting in a positive correlation between microscopic lesion score, \(V_{vprol}\) and \(V_{vapop}\).

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M. O. gathered the morphometrical data and wrote the manuscript with significant contributions by M. D. V., T. T., P. S. and C. V. G.; M. D. V. gathered and interpreted the

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data regarding crypt fission; E. V. H. contributed to the statistical analyses; L. V. B. and A. W. conducted the histopathological scoring; T. T., P. S. and C. V. G. – together with M. O. – designed and conducted the animal experimental protocol. In addition, the authors gratefully acknowledge the excellent technical assistance of Katty Huybrechts and Gunther Vrolix.

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