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Age-related differences in mucosal barrier function and morphology of the small intestine in low and normal birth weight piglets

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ABSTRACT: To test the hypothesis that the mucosal maturation of the small intestine is altered in low birth weight piglets, pairs of naturally suckled low birth weight (LBW, n = 20) and normal birth weight (NBW, n = 20) littermate piglets were selected and sampled after 0, 3, 10, and 28 d of suckling. In vivo intestinal permeability was evaluated via a lactulose-mannitol absorption test. Other indirect measurements for mucosal barrier functioning included sampling for histology and immunohistochemistry (intestinal trefoil factor [ITF]), measuring intestinal alkaline phosphatase (IAP) activity, and immunoblotting for occludin, caspase-3, and proliferating cell nuclear antigen (PCNA). The lactulose-mannitol ratio did not differ between NBW and LBW piglets, but a significant increase in this ratio was observed in 28-d-old piglets (P = 0.001). Small intestinal villus height did not differ with age (P = 0.20) or birth weight (P = 0.20). In contrast, villus width (P = 0.02) and crypt depth (P < 0.05) increased gradually with age, but no birth-weight-related differences were observed. LBW piglets had significantly more ITF immunoreactive positive cells per villus area compared to NBW piglets, whereas no age (P = 0.82) or region-related (P = 0.13) differences could be observed. The activity of IAP in the small intestine was higher in newborn piglets compared to the older piglets. No significant differences in cell proliferation in the small intestine was observed (P = 0.47) between NBW and LBW piglets; the highest proliferation was seen in piglets of 28 d of age (P = 0.01). Newborn piglets had significantly fewer apoptotic cells, whereas more apoptotic cells were seen in piglets of 10 d of age (P < 0.01). In conclusion, birth weight did not affect the parameters related to intestinal barrier function investigated in this study, suggesting that the mucosal barrier function is not altered in LBW piglets. Nevertheless, these results confirm that the mucosal barrier function in the small intestine of piglets alters with age.

Key words: intestinal mucosa, low birth weight, neonatal piglets, small intestine

INTRODUCTION

One of the most fundamental functions of the intestinal mucosa is to provide a barrier against luminal toxins and pathogens while allowing the digestion and absorption of nutrients (Podolsky, 1999). In this regard, the balance between epithelial cell proliferation, differentiation, migration, and apoptosis is pivotal (Hall et al., 1994; Potten, 1997). Moreover, the intestinal barrier consists of a mucus layer overlaying a single layer of epithelium sealed together by tight junctions (Pacha, 2000). The intestinal trefoil factor (ITF), secreted by goblet cells into the mucus overlaying the intestinal epithelium, contributes to epithelial protection by enhancing epithelial integrity and promoting mucosal restitution (Taupin and Podolsky, 2003). Intestinal alkaline phosphatase (IAP) is expressed in villus-associated enterocytes and is essential for maintaining intestinal homeostasis (Goldberg et al., 2008). In the neonatal intestine, occludin is essential for creating and maintaining a functional intestinal barrier (Clark et al., 2006). Because of genetic selection and improved management techniques in the pork industry, litter sizes have increased, resulting in a higher birth weight variation and reduced mean piglet
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12 ± 0.9, mean litter birth weight 1.37 ± 0.25 kg). Piglets with a birth weight less than 1 kg (Pardo et al., 2013) that deviated a maximum of ±0.5 SD from the mean birth weight of the litter were considered LBW (0.84 ± 0.21 kg). Those piglets that deviated a maximum of ±0.5 SD from the mean birth weight of the litter were considered NBW (1.47 ± 0.29 kg). Piglets with a birth weight less than 1 kg (Pardo et al., 2013) that deviated 1.5 SD from the mean birth weight of the litter were considered LBW (0.84 ± 0.21 kg). Those piglets that deviated a maximum of ±0.5 SD from the mean birth weight of the litter were considered NBW (1.47 ± 0.29 kg). Piglets were either euthanized at 0 d (within 24 h after birth; n = 10) or remained suckling the sow until d 3 of age (n = 10), d 10 of age (n = 10), or d 28 (n = 10). During the suckling period, piglets had free access to water and creep feed (starting at 10 d of age).

Permeability Measurements

In vivo gut permeability was evaluated using a functional lactulose-mannitol absorption test. After fasting, piglets were dosed intragastrically with 0.75 g lactulose/kg BW and 0.3 g mannitol/kg BW (Sigma-Aldrich, Steinheim, Germany) 4 h before euthanasia. Concentrations of lactulose and mannitol in urine collected by cystocentesis at the time of euthanasia were measured using an enzymatic spectrophotometric method (Behrens et al., 1984; Blood et al., 1991).

Sample Collection

Piglets were euthanized at 0 d, 3 d, 10 d or 28 d after parturition by severing the carotid arteries under deep barbiturate anesthesia (intraperitoneal, sodium pentobarbital, 200 mg/kg, Kela Laboratoria, Hoogstraten, Belgium). After euthanasia, the gastrointestinal tract was immediately removed and rinsed, and its length and wet weight were recorded. The small intestine was divided into a proximal part (at 25% of total small intestinal length) and a distal part (at 75% of total small intestinal length). Multiple 2- to 3-cm-long tissue samples were snap frozen in liquid nitrogen and stored at −80°C or immersed in 4% paraformaldehyde solution for 2 h. After fixation, samples were rinsed with PBS (pH 7.4) for 24 h and paraffin embedded.

Small Intestinal Morphology

Crypt depth, villus height, and villus width were measured (Olympus BX 61, analySIS Pro, Olympus Belgium, Aartselaar, Belgium) in 30 longitudinally villus-crypt units per animal in 5-µm-thick paraffin sections that were conventionally stained with haematoxylin and eosin.

Immunohistochemical Determination of ITF

In the rehydrated paraffin sections endogenous peroxidase was depleted with 3% hydrogen peroxide in Tris-buffered saline (TBS) for 15 min at room temperature. Subsequently, sections were incubated for 1 h with a polyclonal rabbit anti-ITF antibody (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:100 in TBS enriched with 0.3% Triton X-100 and 1% BSA. Next, after washing with TBS, sections were incubated with biotinylated goat anti-rabbit antibody (1:200, diluted in TBS with 0.3% Triton X-100 and 1% BSA, Dako, Glostrup, Denmark) for 30 min at room temperature. After rinsing with TBS, sections were incubated with streptavidin-horseradish peroxidase (1:200, diluted in TBS with 0.3% Triton X-100 and 1% BSA, Dako) for 30 min at room temperature. After 2 wash steps for 5 min with TBS and 1 wash step for 5 min with distilled water, a positive reaction was visualized by incubating the sections with the 3,3′-diaminobenzidine chromogen (Dako) at room temperature. Sections were counterstained with haematoxylin and cover slipped. Negative controls were obtained by omitting the primary antibody and replacing it by normal serum.

Immunoreactive (IR) ITF positive cells were quantified in 30 well-oriented crypt-villus units. To calculate villus surface area, villus length and villus width were measured (Olympus BX 61, analySIS Pro, Olympus Belgium). Accounting for the variable villus shape and position in which each villus is sectioned, the midvillus width was used to calculate villus surface area.
Intestinal Alkaline Phosphatase Assay

Frozen small intestinal tissue was homogenized in 1% Triton X-100 solution. Intestinal alkaline phosphatase activity was determined in the homogenates via a colorimetric assay (Alkaline Phosphatase Assay Kit, Abcam, Cambridge, UK). Enzyme activities were expressed as units per gram wet tissue. One unit of activity was defined as the amount of enzyme that hydrolyses 1 µmol substrate per min.

Western Immunoblotting: Occludin, Proliferating Cell Nuclear Antigen, Caspase-3

Protein expression was determined using SDS-PAGE and Western blotting analysis. Frozen samples of the proximal small intestinal region were homogenized in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P40 [vol/vol], 0.5% deoxycholate [wt/vol]) complemented with a complete protease inhibitor cocktail tablet (Roche Applied Science, Mannheim, Germany). Protein extracts (10 µg) were resolved by Mini protean TGX 4% to 15% gradient gels (Bio-Rad Laboratories, Hercules, CA) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). After blocking nonspecific protein binding (Dako) was applied, and for proliferating cell nuclear antigen (PCNA) (1:5000, Dako, Glostrup, Denmark), anti-caspase-3 (1:1000, Sigma-Aldrich, Bornem, Belgium), and anti-β-actin (1:10000, Sigma-Aldrich, Bornem, Belgium). Subsequently, secondary antibodies were applied at room temperature: For occludin a biotin-conjugated anti-rabbit antibody (1:1000, Dako, Glostrup, Denmark), anti-caspase-3 (1:1000, Sigma-Aldrich, Bornem, Belgium), and anti-β-actin (1:10000, Sigma-Aldrich, Bornem, Belgium). Protein band intensities were quantified using densitometry (GeneSnap and GeneTools software, Syngene, Cambridge, UK). A normalized OD was obtained by dividing the protein density by the density of the loading control β-actin (Sigma-Aldrich, Steinheim, Germany).

Statistical Analysis

A mixed model was built to analyze the data. The model included birth weight, age (and, if applicable, region), and their interactions as fixed effects. To account for the dependence between observations within the same litter and animal, nested within litter, were added to the model. The significance of the fixed effect terms was tested using an F test with Kenward-Roger correction for the number of degrees of freedom. In case the effect of age was significant, a post hoc test was performed with a Tukey correction for multiple testing.

All calculations were performed in the statistical package R, version 2.13.1 (http://www.R-project.org/). Mixed models were fitted using the lmer function in the lme4 package. Post hoc tests were performed using the multcomp package. The F test with Kenward-Roger correction was performed as implemented in the pbkrtest package. In case data were nonnormal, regression models were fitted on log-transformed data. Descriptive statistics and results presented in tables and figures represent the nontransformed data.

RESULTS

Growth Performance

Daily growth was, on average, 80 g/d lower in LBW piglets compared to NBW piglets. In addition, the results of the BW data at d 3 (LBW: 1.01 ± 0.35 kg; NBW: 1.73 ± 0.36 kg), at d 10 (LBW: 2.40 ± 0.47 kg; NBW: 3.77 ± 0.23 kg), and at d 28 (LBW: 5.3 ± 0.84 kg; NBW: 8.2 ± 0.95 kg) demonstrated that piglets born with lower weights were still lighter than those born with normal weights, indicating that the LBW piglets did not catch up with NBW piglets during the suckling period. Moreover, we showed that the total body compositions of LBW and NBW piglets were similar during the suckling period (M. De Vos et al., unpublished data).

Permeability Measurements

Since data were nonnormal, hypothesis testing was performed on log-transformed data. The lactulose-mannitol ratio did not differ between LBW and NBW piglets (P = 0.78). The ratio was highest in piglets of 28 d of age compared to newborns and piglets of 3 and 10 d of age (P = 0.001; Table 1).

Small Intestinal Morphology

Upon sampling, none of the piglets showed signs of gastrointestinal disorders. For absolute small intestinal length, effects of birth weight (P < 0.01) and age (P < 0.01) were observed. NBW piglets had a longer small intestine compared to LBW piglets (Table 2). A significant increase in small intestinal length was seen with increasing age, except for newborns and piglets of 3 d of age in which the increase in length was not significant. For relative small intestinal length (adjusted
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for BW), a significant interaction was observed between age and weight ($P = 0.03$). Newborn and 28-d-old NBW piglets had relatively shorter small intestines compared to LBW piglets ($P < 0.001$). In the other age groups, the difference between NBW and LBW was not significant.

Intestinal villus height did not differ according to birth weight ($P = 0.20$) or age ($P = 0.20$; Fig. 1A). However, villus width differed significantly in the different age groups ($P = 0.03$). Newborn piglets had significantly more slender villi than d 10 ($P = 0.03$) and d 28 ($P = 0.003$) piglets. Piglets of 3 d of age had thinner villi than piglets aged 28 d ($P = 0.003$; Fig. 1B). A significant interaction between age and region ($P = 0.02$) for crypt depth was observed. In the proximal part of the small intestine, crypt depth significantly increased from d 3, with the deepest crypts seen in 28-d-old piglets. In the distal region of the small intestine, the crypts of 28-d-old animals were significantly deeper than those of newborn and 10-d-old piglets ($P = 0.01$; Fig. 1C).

**ITF Measurements**

ITF immunoreactive (IR) positive cells were detected along the crypt-villus axis in the small intestine of piglets. Irrespective of birth weight, newborn piglets had significantly fewer ITF immunoreactive cells compared to 10-d-old and 28-d-old piglets ($P < 0.01$; Fig. 2A).

No age-related ($P = 0.82$) or region-related ($P = 0.13$) differences were observed when ITF was expressed per villus surface area (Fig. 2B), nor did we find an interaction between age and region (age × region interaction; $P = 0.78$). When lumping all regions and age groups, LBW piglets had, on average, a significantly higher number of ITF IR positive cells per mucosal surface area compared to NBW piglets ($P = 0.03$).

**Intestinal Alkaline Phosphatase Assay**

In the proximal region, newborn piglets had higher ($P < 0.001$) IAP activity than the other age groups, whereas in the distal region of the small intestine differences between age groups were smaller (age × region interaction; $P = 0.001$; Fig. 3). No effect of BW was observed ($P = 0.65$).

**Western Immunoblotting**

Since data were nonnormal, hypothesis testing was performed on log-transformed data. No differences between NBW and LBW piglets could be detected regarding the protein expression of occludin ($P = 0.19$;

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**Table 1. Lactulose and mannitol of LBW and NBW piglets at different ages**

<table>
<thead>
<tr>
<th>Item</th>
<th>BWT</th>
<th>d 0</th>
<th>d 3</th>
<th>d 10</th>
<th>d 28</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactulose-mannitol ratio</td>
<td>NBW</td>
<td>0.68 ± 0.13</td>
<td>0.78 ± 0.21</td>
<td>0.65 ± 0.12</td>
<td>3.42 ± 1.16</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>LBW</td>
<td>0.36 ± 0.09</td>
<td>0.67 ± 0.19</td>
<td>0.58 ± 0.10</td>
<td>6.49 ± 1.39</td>
<td>0.03</td>
</tr>
<tr>
<td>Lactulose, mmol/L</td>
<td>NBW</td>
<td>44.86 ± 9.75</td>
<td>67.49 ± 24.41</td>
<td>37.27 ± 8.45</td>
<td>55.57 ± 10.48</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>LBW</td>
<td>23.09 ± 5.76</td>
<td>29.50 ± 7.31</td>
<td>34.63 ± 10.23</td>
<td>65.63 ± 14.30</td>
<td>0.06</td>
</tr>
<tr>
<td>Mannitol, mmol/L</td>
<td>NBW</td>
<td>73.80 ± 10.59</td>
<td>82.86 ± 19.93</td>
<td>69.57 ± 7.2</td>
<td>54.74 ± 13.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>LBW</td>
<td>67.81 ± 7.61</td>
<td>50.51 ± 7.90</td>
<td>52.64 ± 8.60</td>
<td>9.98 ± 0.64</td>
<td>0.48</td>
</tr>
</tbody>
</table>

1Values are presented as mean ± SEM. BWT = birth weight; NBW = normal birth weight; LBW = low birth weight.

**Table 2. Absolute and relative length and weight of the small intestine (SI) of LBW and NBW piglets at different ages**

<table>
<thead>
<tr>
<th>SI parameter</th>
<th>BWT</th>
<th>d 0</th>
<th>d 3</th>
<th>d 10</th>
<th>d 28</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute SI length, cm</td>
<td>NBW</td>
<td>439.40 ± 25.04</td>
<td>443.17 ± 18.70</td>
<td>638.20 ± 27.61</td>
<td>944.20 ± 34.31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>LBW</td>
<td>334.24 ± 27.59</td>
<td>318.17 ± 18.80</td>
<td>523.60 ± 33.34</td>
<td>848.80 ± 47.40</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Relative SI length, cm/kg BW</td>
<td>NBW</td>
<td>257.46 ± 46.67</td>
<td>255.66 ± 46.70</td>
<td>166.78 ± 20.88</td>
<td>116.28 ± 7.59</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>LBW</td>
<td>406.07 ± 24.38</td>
<td>322.73 ± 76.68</td>
<td>231.17 ± 40.85</td>
<td>164.53 ± 18.21</td>
<td>0.03</td>
</tr>
<tr>
<td>Absolute SI weight, g</td>
<td>NBW</td>
<td>64.80 ± 7.10</td>
<td>69.17 ± 7.27</td>
<td>138.20 ± 3.97</td>
<td>269.20 ± 18.63</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>LBW</td>
<td>33.74 ± 4.19</td>
<td>39.50 ± 5.57</td>
<td>88.60 ± 7.31</td>
<td>196.40 ± 15.11</td>
<td>—</td>
</tr>
<tr>
<td>Relative SI weight, g/kg BW</td>
<td>NBW</td>
<td>37.28 ± 5.83</td>
<td>35.77 ± 5.21</td>
<td>36.15 ± 2.41</td>
<td>33.02 ± 2.50</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>LBW</td>
<td>39.81 ± 7.34</td>
<td>37.65 ± 5.18</td>
<td>38.06 ± 2.52</td>
<td>37.66 ± 3.73</td>
<td>0.73</td>
</tr>
</tbody>
</table>

1Values are presented as mean ± SEM. BWT = birth weight; NBW = normal birth weight; LBW = low birth weight.

2A significant interaction between age and BW was observed. This indicates that the effect of BW is not uniform across age groups and, conversely, that the difference in outcome between age groups is different for the LBW and NBW piglets.

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Fig. 4A) or PCNA (P = 0.47; Fig. 4B). A significantly higher expression of occludin (P < 0.001) and PCNA was observed in piglets of 28 d of age (P = 0.01; Fig. 4C). For caspase-3 no significant differences between BW could be detected (P = 0.61). Newborn piglets had the lowest number of apoptotic cells, whereas the highest number of apoptotic cells was seen in piglets of 10 d of age (P < 0.01; Fig. 4C). Immunohistochemical stainings with anti-PCNA and anti-caspase-3 were performed to locate the proliferating/apoptotic cells (Fig. 5).

**DISCUSSION**

Nutritional status markedly influences gut mucosal growth and function. Low birth weight piglets have lower energy reserves and are therefore less successful in gaining nutrients and, as a consequence, weight. In addition, a LBW, as a result of intrauterine growth retardation, alters gastrointestinal morphology and function in the immediate postnatal period in piglets (Xu et al., 1994; Wang et al., 2005; D’Inca et al., 2010). However, it is not known whether these alterations persist in later stages of the postnatal period. We hypothesized that disruption of the small intestinal barrier function may be causally related to the increased neonatal mortality and morbidity seen in piglets born with a LBW (Lay et al., 2002). To test whether these alterations persist in later stages of the postnatal period, we investi-
gated the intestinal barrier function in naturally suckling piglets of 0, 3, 10, and 28 d of age.

In our study, small intestinal histomorphological variables (villus height, villus width, and crypt depth) were not affected by birth weight, suggesting that the small intestine of these LBW piglets develops normally. Overall, these results confirm what has already been reported (Wiyaporn et al., 2013), but these results do not exclude the possibility that in the first hours after birth differences can exist. Indeed, others have reported that nonsuckled (Xu et al., 1994) or LBW newborn piglets (D’Inca et al., 2011) have shorter villi immediately after birth. A gradual increase in crypt depth and villus width occurred with age, whereas villus height remained relatively constant.

The in vivo intestinal barrier function was evaluated using a dual-sugar absorption test (Bjarnason et al., 1995). The lactulose-mannitol ratio is inversely related to the intestinal paracellular barrier function. The highest lactulose-mannitol ratio was observed in piglets of 28 d of age, suggesting a decrease of intestinal paracellular barrier function. However, it should be kept in mind that data based on a ratio are difficult to interpret, as changes in both the numerator and denominator will affect the result (Nejdfores et al., 2000; Wijtten et al., 2011). In piglets of 28 d of age the lowest levels of mannitol were recovered; LBW piglets tended to have lower urinary mannitol levels, indicating less transcellular transport in the small intestine of these piglets. Although lactulose levels did not differ significantly, occludin expression was increased in piglets of 28 d of age. Besides ontogenic maturation, this decrease might be attributed to decreased milk intake at the end of the suckling period, as the highest peak for milk production during lactation is 18.7 d postpartum (Hansen et al., 2012). Indeed, it has been shown that reduced feed intake around weaning is associated with reduced intestinal barrier function (Spreeuwenberg et al., 2001).

In the neonatal intestine, the tight junction protein occludin is essential for creating and maintaining a functional epithelial intestinal barrier (Clark et al., 2006). In our study, we observed a significant increase in occludin protein expression in the small intestine of 28-d-old piglets.
Figure 5. Immunohistochemical stainings with (A) anti-PCNA and (B) anti-caspase-3 were performed to locate the proliferating/apoptotic cells. PCNA = proliferating cell nuclear antigen. See online version for figure in color.
This result indicates that changes in occludin protein expression are not responsible for the increased permeability of the small intestine. It should be taken into account that the in vivo dual-sugar permeability test is a marker of whole small intestinal permeability and that the Western blot data of occludin only give insight into a specific small intestinal site. In addition, it should be pointed out that tight junctions are composed of several proteins, including occludin, claudins and junctional adhesion molecule. (Mitic and Anderson, 1998).

Intestinal alkaline phosphatase is expressed in villus-associated enterocytes and is essential for maintaining proper gut homeostasis (Goldberg et al., 2008). Moreover, IAP detoxifies bacterial lipopolysaccharide (LPS), reduces LPS-induced inflammation, and restricts transmucosal passage of bacteria (Poelstra et al., 1997; Geddes and Philpott, 2008). To our knowledge IAP activity has not been studied before during the suckling period in LBW piglets. The decline in IAP activity with increasing age in our study might in part explain the decrease in intestinal barrier function that was observed at the end of the suckling period. Indeed, it has been shown that the decrease in IAP activity, which occurs during reduced feed intake, is a key component of the gut mucosal barrier dysfunction (Goldberg et al., 2008). Along the gastrointestinal tract, expression of ITF peptides and generation of an ITF-containing mucus layer has been reported (Madsen et al., 2007). The results of our study show that the absolute number of ITF IR positive cells in the small intestine increases until the age of 10 d. If villus surface area is taken into account, LBW piglets have higher ITF IR cells per villus surface area compared to NBW piglets. These data suggest a mucosal insult has occurred in the small intestine of LBW pigs because ITF is upregulated on mucosal damage (Ng et al., 2013). Also, it was reported that ITF expression increases during fasting (Fernandez-Estivariz et al., 2003) and around weaning in rats (Lin et al., 1999) and pigs (Scholven et al., 2009). Therefore, reduced feed intake in these LBW piglets might be responsible for the upregulation in ITF.

The small intestine is characterized by a high turnover rate (Hall et al., 1994). The daily loss of cells by apoptosis needs to be compensated by mitotic divisions of stem cells in the crypts to preserve the intestinal barrier. In the present study PCNA was used to evaluate cell proliferation on whole tissue homogenates. An increase in PCNA expression was seen in piglets of 28 d of age. This increase is in accordance with the deeper crypt depth seen in these piglets. Apoptosis, measured by caspase-3 expression, was lowest in the newborn piglets and highest in piglets of 10 d of age. Comparably, it was shown that the extrusion zone located on the top of villi was not active during the first few days after birth. This observation is supported by data showing that the apoptotic activity gradually increases until a maximum is reached 21 d after birth (Skrzypek et al., 2005).

In conclusion, the presented data demonstrate that the mucosal barrier function in the small intestine of LBW piglets develops similar to those of NBW piglets during the suckling period. The age-dependent increased intestinal permeability could possibly be attributed to the attenuated IAP activity and could be counteracted by an increase in occludin and cell turnover.

**LITERATURE CITED**


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