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MECHANISMS INVOLVED IN THE DEVELOPMENT OF THE SMALL INTESTINE MUCOSAL LAYER IN POSTNATAL PIGLETS

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The use of complementary visualization and measurement techniques allowed accurate description and quantification of changes in the intestinal mucosal architecture and provided a comprehensive outlook on the dynamics of remodeling and maturation processes of the mucosal layer taking place in the small intestine of piglets from birth to weaning. The aim of the study was to examine the early postnatal development of the small intestine in pigs. Three techniques were used: scanning electron microscopy (measurements of villus density and shape, height of enterocytes and microvilli, cell exfoliation, and location of extrusion zones), optical microscopy (cross section, measurement of structures: villus length and width; crypt depth; mucosal thickness), and confocal microscopy (cell localization, apoptosis, exfoliation and migration). The postnatal development of the mucosal layer of the small intestine was reflected in changes in the density, length, width, and shape of villi, crypt depth, replacement of enterocyte population, and arrangement. The presence of deep transverse furrows on villus corpus and vacuolated fetal-type enterocytes in the mucosal layer of the small intestine, which are able to engulf large amounts of colostrum shortly after birth, appears to play an important role in the observed phenomenon of straightening of the villus height and increasing of the villus diameter shortly after birth. We hypothesized that the intestinal mucosal layer is compressed before birth and ready to unfold within a short time after birth.

Key words: *development of the small intestine, scanning electron microscopy, villi, enterocytes, apoptosis, extrusion zones, maturation, confocal microscopy*

INTRODUCTION

Changes in the structure and digestive function of the small intestine start prenatally (1-3). At birth, the structure of the small intestine is almost complete and ready to initiate digestive functions after the first colostrum intake (4-14). Microscopy studies have shown, however, a sequence of subtle changes in the intestine structure in the early postnatal period, especially in the mucosal layer, associated with colostral macromolecule uptake, 'gut closure', shift from domination of intracellular digestion to extracellular digestion by means of digestive juices, and finally changes associated with the transition from mother's milk to solid feed (5, 8-9). These changes are dynamic, e.g., the small intestine in neonatal pigs increases its relative weight by 30 – 40% just within the first postnatal 24 hours and exhibits the greatest growth in weight and length within the first 2 – 3 days after birth (5, 12, 13, 15). The growth of the small intestine involves mostly the intestinal mucosal layer, but considering the kinetics of proliferation of intestinal crypt cells it seems unlikely that the intestine achieves such an immediate growth by hyperplasia alone (5, 12).

Accumulation of colostral molecules in the enterocyte vacuole system and a rapid increase in mucosal blood flow are important factors contributing to the muscosal layer growth (16). The intestinal mucosal layer of an unsuckling neonate is already prepared to absorb large volumes of colostral macromolecules by the presence of vacuolated enterocytes not only on the villus surface, but also in the transversal furrows at villus corpuses (17,

18). Unfolding of the transversal furrows disappears in synchrony with macromolecule uptake (19).

At the same time, the entire small intestine grows in length and diameter, each functional part i.e. the duodenum, jejunum, and ileum with its own specific kinetics (15). The aim of the present paper was to demonstrate the early neonatal development of the small intestine ultrastructure in pig neonates using three complementary microscopy techniques: optical microscopy (OM), scanning electron microscopy (SEM), and confocal microscopy (CM). The development of the small intestine was studied at both tissue and cell levels. The simultaneous use of three microscopy methods along with quantitative analysis of various tissue and cell structures allowed us to draw conclusions regarding the development of gut function.

MATERIAL AND METHODS

The experimental protocols were approved by the Local Ethical Committee. The studies were carried out on 60 neonatal piglets (Polish landrace/Pietrain, age range postnatal days (PD) 0 – 38) from 12 litters. The mean number of piglets in the litter was 12.4 ± 0.9 (mean ± SE). The average birth weight of all animals in all litters was 1329.3 ± 18.5 g (mean ± SE). The examined piglets were born naturally. They were collected on the basis of similar body weight (the body weight of the unsuckling neonates ranged between 1.2 and 1.6 kg) and housed in standard farming conditions. The average weight of animals in the experimental groups was 1382.9 ± 15.3 g (mean ± SE). There were no statistically significant differences between the average birth weight of all piglets and the birth weight of the experimental piglets (all) [t-test, $t(207) = 1.971$, $P = 0.083$]. There were no statistically significant differences between the birth weight of piglets selected for the individual study groups (ANOVA, $F(5,54) = 0.266$, $P = 0.930$). The piglets were kept with their sows from birth to weaning at PD 28. Suckling piglets received commercial pre-starter (creep feed) from PD 14 and, following weaning, a starter solid food *ad libitum*. Piglets from different sows were killed by pentobarbiturate overdose (1 ml/kg b. wt. intraperitoneally) immediately after birth (day 0 – unsuckling neonates) and at PD 3, 7, 14, 21, and 38 (n = 10 for each group). The amount of active substance in 1 ml of the product used was 133.3 mg of pentobarbital sodium and 26.7 mg of pentobarbital.

For histological analysis, small 5-cm segments of the small intestine were collected: the duodenum (2 cm in front of the pancreatic duct), the initial jejunum (about 45 cm behind the Treitz ligament), the middle part of the jejunum (in the middle of the length), the end of the length of the jejunum (about 45 cm in front of the iliac-iliac ligament), and the iliac ileum (about 10 cm in front of the mouth to the caecum). For optical microscopy, the samples were fixed in Bouin solution, dehydrated, and embedded in paraffin. The histological sections (5-µm thick) were cut and stained with hematoxylin and eosine. The slides were examined using an Olympus BX50 microscope. Mucosal parameters: villus length and width, crypt depth, and mucosal thickness were measured in the slides. Minimum 30 measurements were taken for each gut segment. For SEM, the gut segments were rinsed 5 times with 0.9% NaCl, cut into square-shaped fragments, washed, and fixed (24 h) in 5% buffered formaldehyde. After fixation, the samples were washed in saline solution, dehydrated in a series of alcohols (20, 30, 50, 70, 80, 90, 96%, and absolute ethanol), dried in CPD (CPD7501, Polaron Range), and sputter coated (SC 7620, Polaron Range) with a ~20 nm Au/Pd layer. The samples were examined with the use of a LEO 1430 VP SEM. The density of villi (number of villi/1mm^2 of mucosal surface), shape of villi, and enterocyte and microvillus height were calculated or measured based on SEM images. Properly straightened and oriented samples of the intestine were used to count the average number of villi on the mucosal surface. Twenty five random measurements were made from each part of the pig intestines.

For confocal microscopy, the gut samples were rinsed in 0.9% NaCl, placed in OCT Embedding MatriX (Cell Path), and cut into 15 µm sections in a cryostat (MTC). The histological sections were placed on silane-coated glass slides. For confocal analysis, the cells were labeled with secondary Alexa Fluor 488 conjugated antibodies and 7-AAD (7-aminoactinomycin D) to counterstain the DNA. The following primary antibodies were used: anti-caspase 3, anti-transforming growth factor beta (anti-TGF-β1), anti-p53 (FITC-conjugated), and anti-MAP I LC-3. Next, the gut mucosal layer was visualized using the FV-500 confocal laser scanning microscope system (Olympus). Observations were made on at least 14 fields of vision. The confocal microscopy technique was used to confirm that the cells observed in SEM pictures were apoptotic cells.

Statistical analysis

Before analysis, all data were normalized by log (measurements) or arcsine square root (indices) transformations. The normality of data distribution was determined by the Shapiro-Wilk test and homogeneity of variance was assessed by Levene's test. The data were analyzed for the postnatal days with the intestine part as a cofactor. Analysis of variance and means separation with Tukey HSD test were used for comparisons. Differences among means were considered significant at $P < 0.05$. Backtransformed means ± standard errors are presented. All analyses were conducted using SPSS Statistics 24 software (IBM).

RESULTS

Duodenum

At birth (PD 0), the villi of unsuckling piglets were regular and finger-shaped (*Fig. 1*, *0a*). Divided and incompletely divided villi were observed. The density of the villi on the day of birth was the highest ($P < 0.001$) during the examined postnatal development of the duodenum (*Fig. 2*). There were numerous transversal furrows on the duodenal villus corpus (*Fig. 1*: *0a*). Transversal furrows are defined as up to 20-µm incisions into the villus interior not interrupting the integrity of the epithelium. Two populations of enterocytes were observed: vacuolated fetal-type enterocytes (VFE) located at the upper and lateral part of the villi, often at the tips of duodenal villi, and mature enterocytes. The VFE were present in the duodenum only on the day of birth. The VFE were easily recognizable in SEM since they were larger than mature enterocytes and highlighted by the swelling of the apical part of the cell. Single vacuolated enterocytes were present only in the apical part of a few villi at PD 0 (*Fig. 1*: *0c*). The height of the duodenal enterocytes was significantly the lowest ($P < 0.001$), compared to that noted in the examined period (*Fig. 3*). A few exfoliated cells were visible on the upper and lateral parts of the villus body in SEM and confocal microscopy. The finger-like shaped villi were regularly arranged on the mucosal surface at PD 3 (*Fig. 1*: *3a* and *3d*). The mucosal surface resembled the surface observed at birth, but the number of transversal furrows decreased (*Fig. 1*: *3a* and *3d*). The disappearance of transverse furrows corresponded with the increase in the villus length and width (*Fig. 2*). The mucosal arrangement at PD 7 was comparable to that observed on the earlier days. Not many villi were divided (*Fig. 1*: *7d*). A few leaf-like villi were observed between the prevailing finger-like villi. The villus corpus became smoother than on the earlier days and the transversal incisions were less

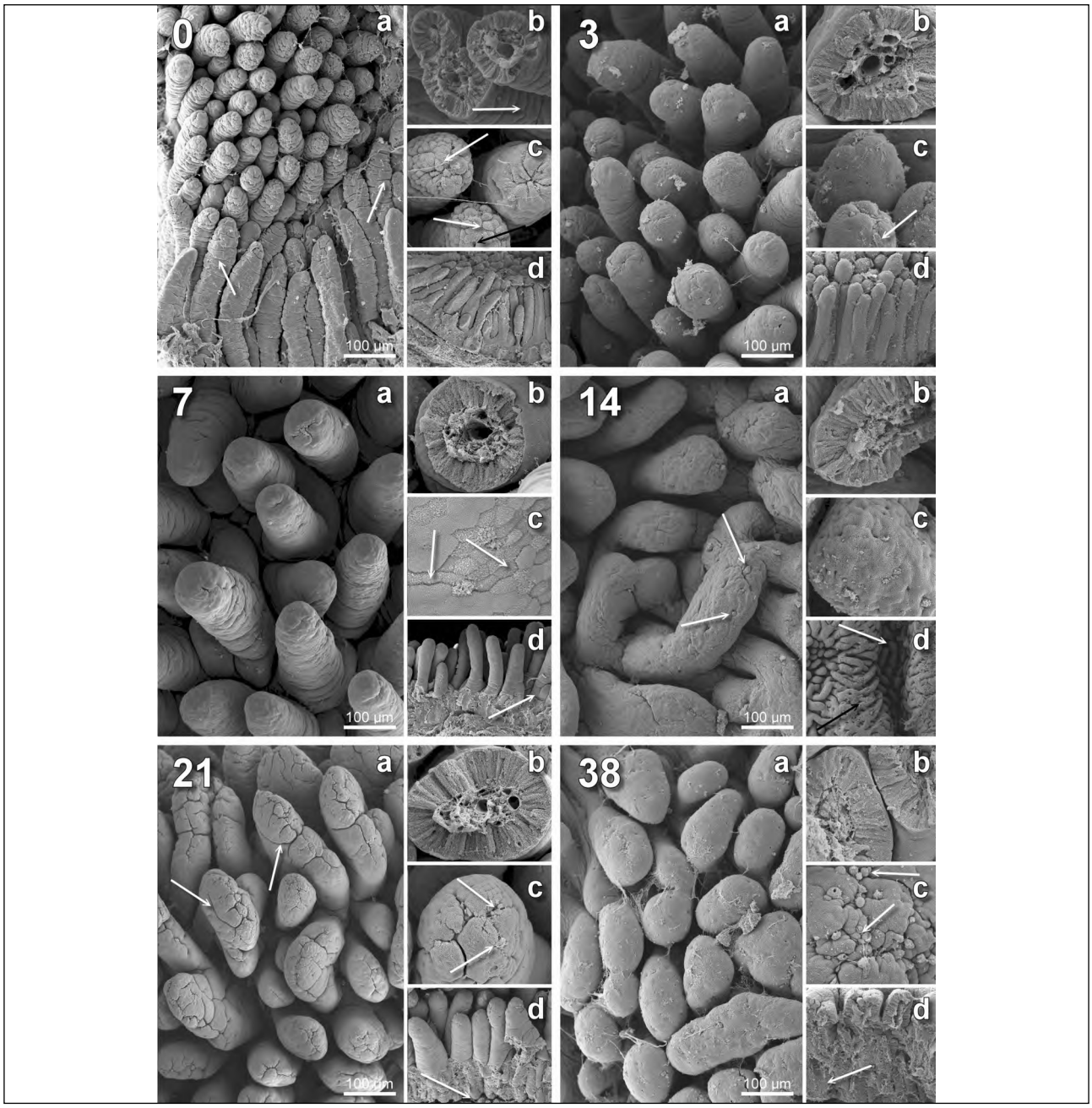

Fig. 1. (*0a*) SEM micrograph (500 ×) of the duodenal villi at birth; the villi are finger-like shaped, densely packed, and rich in many transverse furrows along the villus body (arrows). The small inserts show: b (2,5 kx): the cross section of the lower part of the villi, mature enterocytes are present, furrows on the villius body are clear (arrow); c (2 kx): the apical part of the villi. Vacuolated fetal enterocytes are located in the apical part of the villi (bulged enterocytes, white arrows); incisions in the villus apical parts appear after enterocyte shedding (black arrow); d (250 ×): cross section of the duodenal intestine, the villi have different lengths, high density, and numerous transverse furrows. (*3a*) Duodenal villi on day 3, the villi are finger-like and smooth, and their density is decreased. Inserts; b: the section of the upper part of the villus exhibits the presence of only mature enterocytes, the lacteal is bigger than on the day of birth; c: tips of the villi, only a few extruded cells are present (arrow); d: section of the intestine, note the lack of furrows and increased villus length. (*7a*) Duodenal villi on day 7; the villi are finger-like, different in length, the density is decreased. Inserts; b: the section shows mature, well arranged enterocytes; c: numerous epithelial cells at initial stages of apoptosis on the lateral part of the villus body (arrows); d: section of the intestine, the villi are not packed so densely as on earlier days, note the presence of some dividing villi (arrow). (*14a*) Duodenal villi on day 14, the villi are irregular in shape and length; most of them are leaf-like, partly divided, only a few shedding cells are visible (arrow). Inserts; b: the section of the villus; c: the apical part of the villus, d: mucosal folds; note the difference in the shape between the villi present on the folds (black arrow) and between them (white arrow). (*21a*) Duodenal mucosal layer on day 21. The villi are flat and wide, numerous 'newly grown' villi are present, incisions between groups of enterocytes are present (arrow). Insert; b: villus section, the villus is covered by regular enterocytes with a well-developed brush border; c: top of the villus with incisions and a few shedding cells (arrow); bottom: section of the intestinal villi and exposed crypts (arrow). (*38a*) Duodenal mucosa on 38 day. The leaf-like villi and tongue-like villi are dominant. Inserts; b: villus sections with regular enterocytes; c: the apical part of the villus with a well-developed extrusion zone (arrows); d: section of the intestine; note, the longest crypts in all the examined periods (arrow).

numerous and substantially shallower. The apical hexagonal outlines of the enterocytes were clearly visible on the villus body and the height of the enterocytes significantly increased ($P < 0.001$) (*Fig. 3*). There were numerous epithelial cells at the initial stages of apoptosis on the villus body (confirmed by confocal microscopy observations, (*Fig. 1*: *7c*). The surface of the villi was covered by regular enterocytes with a well-developed brush border. At PD 14, the rearrangement of the villi had already started (*Fig. 1*: *14a*). There was a significant difference in the shape of the villi on the mucosal folds and between the folds. The villi present on the folds were irregular in shape, leaf-like, partly divided, partly flat, and branched (*Fig. 1*: *14d*). The villi between the folds were more regular than the villi present on the folds. The average density of the villi decreased significantly ($P < 0.008$) compared to the earlier postnatal days. Several shedding cells were present on the villus body, with some localized on the apex of the villi (*Fig. 1*: *14a*). Numerous flat and wide villi were observed in the duodenal mucosal layer at PD 21 (*Fig. 1*: *21a*). Numerous lower villi were present in the mucosal overview (*Fig. 1*: *21a*). Groups of enterocytes separated by incisions (from 20 to 100) were present on the apical and lateral surface of the villus body (*Fig. 1*: *21a*). The integrity of the epithelium in the apical part of the villi was retained, irrespective of the presence of apoptotic cells. Extrusion zones were present and often limited to the apical part of the villi (*Fig. 1*: *21c*). Shedding cells were observed on the lateral surfaces of the villus body. The changes in the architecture of the duodenal mucosal layer were emphasized at 38 PD, compared to the earlier days (*Fig. 1*: *38a* and *38d*). The shapes of the villi on and between the folds changed. The folds were covered by flattened, leaf-like, and tongue-shaped villi.

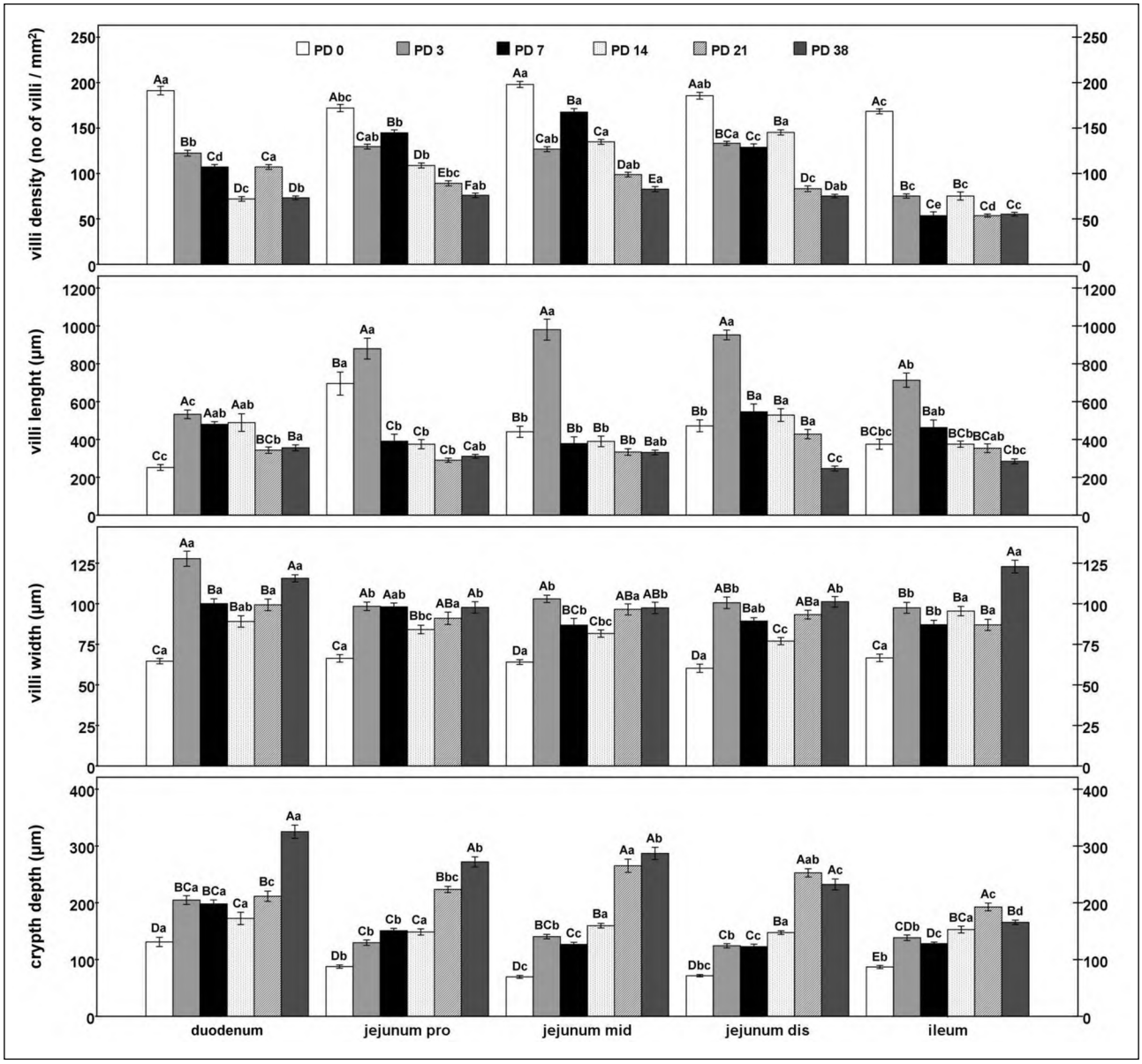

Fig. 2. Morphometric characteristic of the duodenum, jejunum (segments were taken from the jejunum: prox-25%, mid-50%, dist-75% of the jejunum length), and ileum in piglets. Analysis of variance and separation of means with Tukey HSD test were used to analyze comparisons. Columns with the same uppercase letter are not significantly different within the postnatal day. Columns with the same lowercase letter are not significantly different within the intestine part ($P < 0.05$, Tukey HSD test). Differences among the means were considered significant at $P < 0.05$. Backtransformed means ± standard errors are presented. PD 0; 3; 7; 14; 21; 38 means postnatal day 0; 3; 7; 14; 21; 38, respectively.

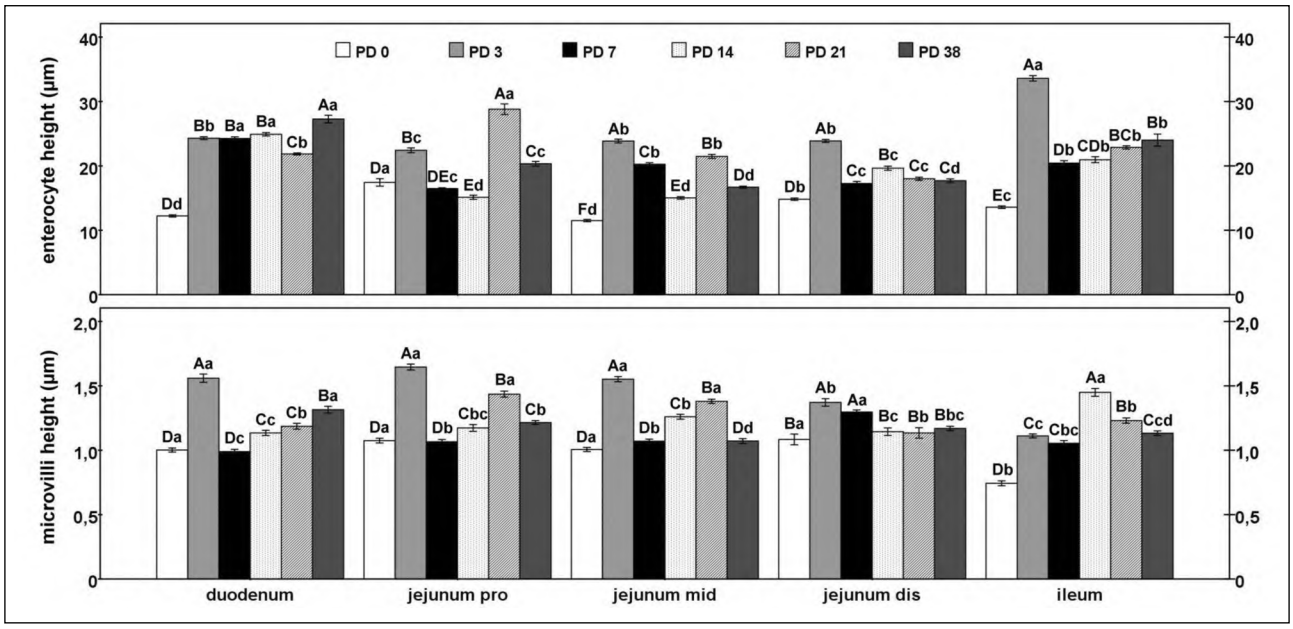

Fig. 3. Morphometric characteristic of the duodenum, jejunum (segments were taken from the jejunum: prox-25%, mid-50%, dist-75% of the jejunum length), and ileum in piglets. Analysis of variance and separation of means with Tukey HSD test were used to analyze comparisons. Columns with the same uppercase letter are not significantly different within the postnatal day the intestine part. Columns with the same lowercase letter are not significantly different within the intestine part ($P < 0.05$, Tukey HSD test). Differences among the means were considered significant at $P < 0.05$. Backtransformed means ± standard errors are presented. PD 0; 3; 7; 14; 21; 38 means postnatal day 0; 3; 7; 14; 21; 38, respectively.

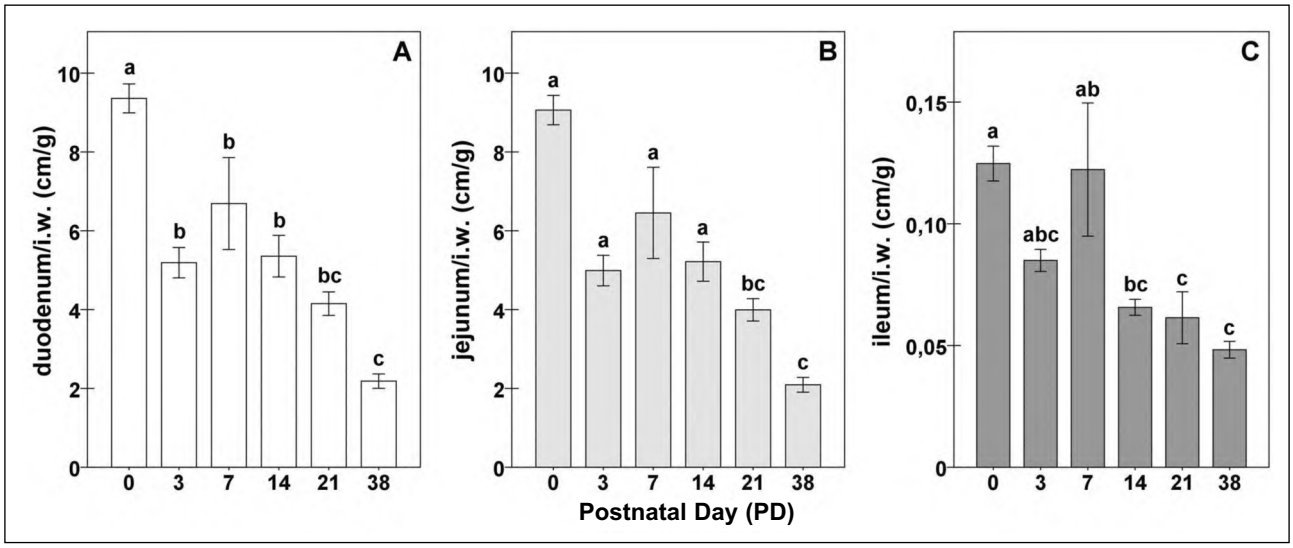

Fig. 4. Length of the duodenum (A), jejunum (B), and ileum (C) relative to the total weight of the small intestine (cm/g). Analysis of variance and separation of means with Tukey HSD test were used to analyze comparisons. Columns with the same lowercase letter are not significantly different within the intestine part ($P < 0.05$, Tukey HSD test). Differences among the means were considered significant at $P < 0.05$. Backtransformed means ± standard errors are presented. PD 0; 3; 7; 14; 21; 38 means postnatal day 0; 3; 7; 14; 21; 38, respectively. *Abbreviation:* i.w., intestine weight

Predominant and numerous leaf-like villi were present also between the folds. Numerous shedding cells were present on the villus body and localized at the villus apex; some of them were present in mature extrusion zones (*Fig. 1*: *38c*).

Jejunum

At birth, the tunica mucosal layer of the jejunum was different in all examined parts: the proximal, middle, and distal regions. The length of the jejunum relative to the total weight of small intestine (*Fig. 4*) and the length of the jejunum relative to body weight significantly decreased at PD 3 ($P < 0.001$) (*Fig. 5*). The jejunum was covered by long, finger-like villi (*Fig. 6*: *0a*). Numerous transverse furrows were observed on the surface of the villi; on the longest villi, they were located mostly at the upper parts. There were VFE populations of enterocytes (*Fig. 6*: *0b* and *0c*). The VFE enterocytes were most numerous at the distal part of the jejunum. There were no extrusion zones. The villi at PD 3 were closely packed in the proximal segment of the jejunum (*Fig. 6*: *3d*). The length of the villi was highly variable,

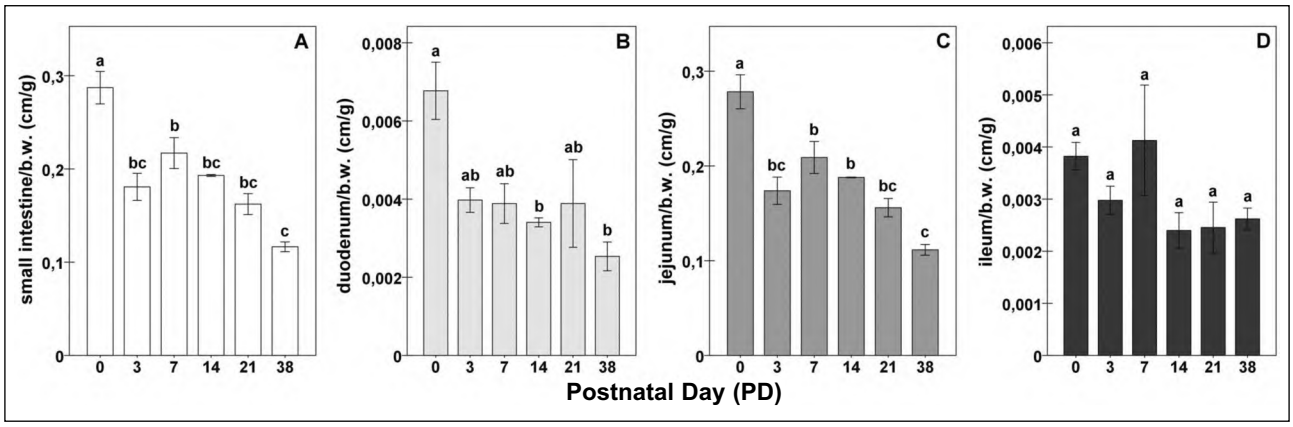

Fig. 5. Length of the small intestine (A), duodenum (B), jejunum (C), and ileum (D) relative to the body weight (cm/g b.w.). Analysis of variance and separation of means with Tukey HSD test were used to analyze comparisons. Columns with the same lowercase letter are not significantly different within the intestine part ($P < 0.05$, Tukey HSD test). Differences among the means were considered significant at $P < 0.05$. Backtransformed means ± standard errors are presented. PD 0; 3; 7; 14; 21; 38 means postnatal day 0; 3; 7; 14; 21; 38, respectively.

and exhibited the greatest value at PD 3 in all the jejunum parts ($P < 0.001$) in the study period (*Fig. 2*). The furrows were sparse on the low villi, but occurred abundantly on the longer villi. The highest microvilli were observed at 3 PD (*Fig. 3*). Most VFE were localized in the apical part of the villi in the distal part of the jejunum. Several shedding cells were present on the villus apices (*Fig. 6*: *3c*). The jejunum at PD 7 was covered by finger-like and leaf-shaped villi. The shedding cells were present on the villus apex (*Fig. 6*: *7c*). Deep furrows were observed on the surface of the finger-shaped villi in the apical part. At PD 14, the villi were finger-like in shape, and some of them showed a tendency to flattening (*Fig. 6*: *14a*). The extrusion zones with shedding cells were present on the villus apex, and some of them were present on the lateral surface of the villi (*Fig. 6*, *14c*). The shape of the villi at PD 21 changed, i.e. it was predominantly leaf-like. Some of the villi resembled opuntia sprouts (*Fig. 6*: *21a* and *21d*); additionally, there were villi with a hardly recognizable shape. The apical villus parts were rich in shedding cells (*Fig. 6*: *21c*). The villus size and shape changed significantly at PD 38 (*Fig. 6*: *38a* and *38d*). Flattened villi were predominant. Numerous shedding cells were present in the apical parts of the villus body (*Fig. 6*: *38c*).

Ileum

Most villi at PD 0 were flattened and finger- and leaf-shaped (*Fig. 7*: *0a* and *0d*). The surface of the villi was rich in transverse furrows, which were irregular due to the presence of numerous fetal-type enterocytes. This was corroborated by the cross sections of the villi, with empty spaces left after large vacuoles, nucleus, and the structure of the apical canalicular system (ACS) observed within the enterocytes (*Fig. 7*: *0b* and *0c*). There were no shedding cells on the villus body and the apex (*Fig. 7*: *0a* and *0c*). There were many VFE (*Fig. 7*: *3a* and *3b*). The villi at PD 3 were finger-shaped with numerous transverse furrows on the villus body (*Fig. 7*: *3a* and *3d*). The villi at PD 7 were finger-shaped. The villus surface was rich in furrows at PD 7. VFE were present (*Fig. 7*: *7b*). Peyer's patches were observed between the villi in the ileum (*Fig. 7*: *7a and 7d*). At PD 7, Peyer's patches formed *circa* 36% of all the 'convex structures' present on the mucosal surface. The villi covering the mucosal layer at PD 14 were short and flat (*Fig. 7*: *14a*). Peyer's patches encompassed 28% of the 'convex structures' present on the mucosal surface. The villi at PD 21 were flattened, resembling structures undergoing division (*Fig. 7*: *21a*). A few single finger-shaped villi were observed. There were active extrusion zones in the apical villus part. The epithelium covering Peyer's patches was rich in M cells, and several receptors cells were also observed (*Fig. 7*: *21c*). Peyer's patches were less numerous and accounted for *circa* 16% of the 'convex structures' on the mucosal surface. The receptors and M cells were present in the epithelium covering Peyer's patches. The shape of the villi at PD 38 was different from that observed in the pre-weaning period (*Fig. 7*: *38a*). There were extrusion zones with several shedding cells (*Fig. 7*: *38c*, *Fig. 8*).

DISCUSSION

The development of intestinal tissues is defined as a sum of growth and maturation processes (4, 5, 7-8, 12-13). Growth, defined conservatively as intense growth of the number and size of epithelial cells and maturation involve changes in the digestion and absorption functions of the epithelium and the turnover of epithelial cells (4-5). The postnatal development of the mucosal layer depends on external regulators present in colostrum and milk, because its ability to produce own growth factors is impaired (6-8).

The villi in the piglet's small intestine observed in SEM at birth were dense and short, compared to those on the subsequent days of postnatal development. The length of the villi rapidly extended with age, while their density decreased. For example, the density of the villi in the middle part of the jejunum decreased by 55% unit/mm^2 of the serosal surface between PD 0 and PD 3.

The length of the duodenum and jejunum relative to body weight between birth and PD 3 decreased significantly, which may partly explain the decrease in the density of the villi, while the relative length did not show significant changes. The villus length and width in the middle part of the jejunum increased by 122% and 61%, respectively, between birth and PD3. Xu *et al.* (7) showed a 70% increase in the small intestine mass, a 24% increase in the length, a 15% increase in the width, a 24% increase in the depth of intestinal crypts, and elongation of intestinal villi by 33% during the first day of piglets' life. The changes in the villus density, length, and width are related to the rapid growth of the small intestinal absorptive area shortly after birth (5, 8, 9, 20-21), if the word growth is appropriate in this context.

The widely accepted term growth is used to describe development-related changes. The growth is linked with organ enlargement by increasing the number of cells and/or the size of

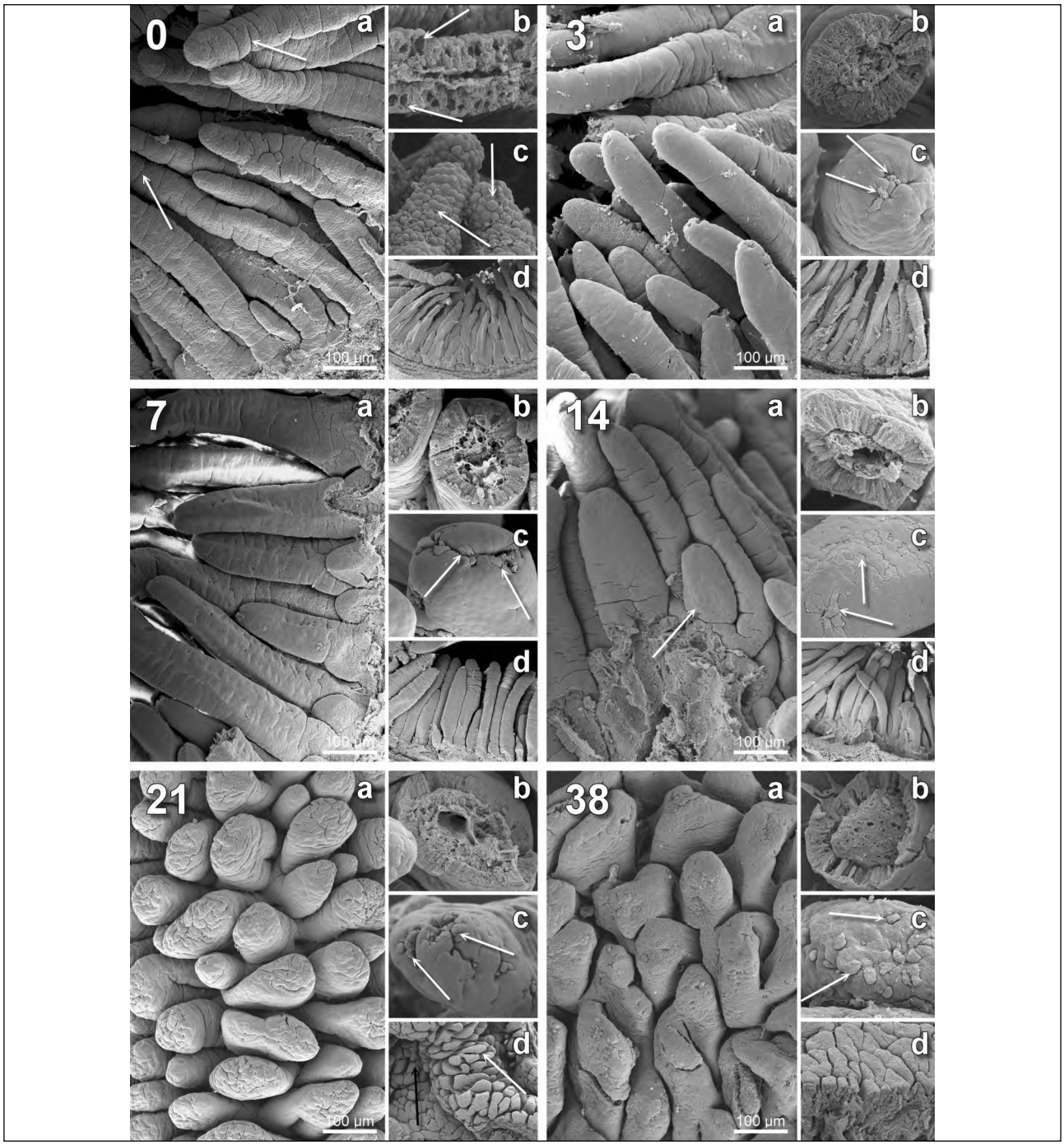

Fig. *6*. (*0a*) SEM micrograph of villi from the middle part of the jejunum at birth; the villi are densely packed, finger-like shaped with numerous transverse furrows (arrows). Enterocytes present on the villus body represent the fetal population. Inserts; b: the cross section along the villus length (the distal jejunum) revealed numerous vacuolated enterocytes (arrows) (2 kx); c (1kx): the villi (the middle jejunum) are covered by vacuolated, distended enterocytes (arrows); d (250 ×): cross section of the middle jejunum intestine showing villi of different length. (*3a*) Middle jejunum villi on day 3; the surface of finger-like shaped villi is smooth. Inserts; b: the section of the upper part of the villus shows the presence of mature enterocytes; c (2kx): extruded enterocytes present in the apical part of the villus (arrows); d: section of the intestine; the villus height is variable. (*7a*) Middle jejunum on day 7; the villi are finger-like with a tendency to flattening, variable in length. Inserts; b: the section shows mature enterocytes; c: shedding epithelial cells are mostly present in the apical villus part (arrows); d: section of the mid intestine; note the physiological cramps of the villi in their upper part. (*14a*) Middle jejunum villi on day 14; the villi are finger-like and flat, different in size. Inserts; b: the section of the villus from the distal part of the jejunum, the enterocytes are well developed; c: extruded epithelial cells are present on the villus body (arrows); d: section of the mid intestine, note the ratio between the villi and crypt length. (*21a*) Middle jejunum villi on day 21. The villi are leaf-like; incisions with shedding cells in the apical parts are present. Inserts; b: the section of the villus showing mature enterocytes; c: top of the villus with shedding cells in apical incisions (arrows); d: mucosal folds; note the difference in the shape between villi present on the folds (white arrow) and between the folds (black arrow). (*38a*) Middle jejunum villi on 38 day; note the leaf-like, tongue-like, and ‘opuntia-like’ villi. Inserts; b: villus section with regular enterocytes; c: the apical part of the villus; numerous shedding cells are present in a vaguely defined extrusion zone (white arrows); d: section of the mid jejunum; note the ‘merged’ villus architecture.

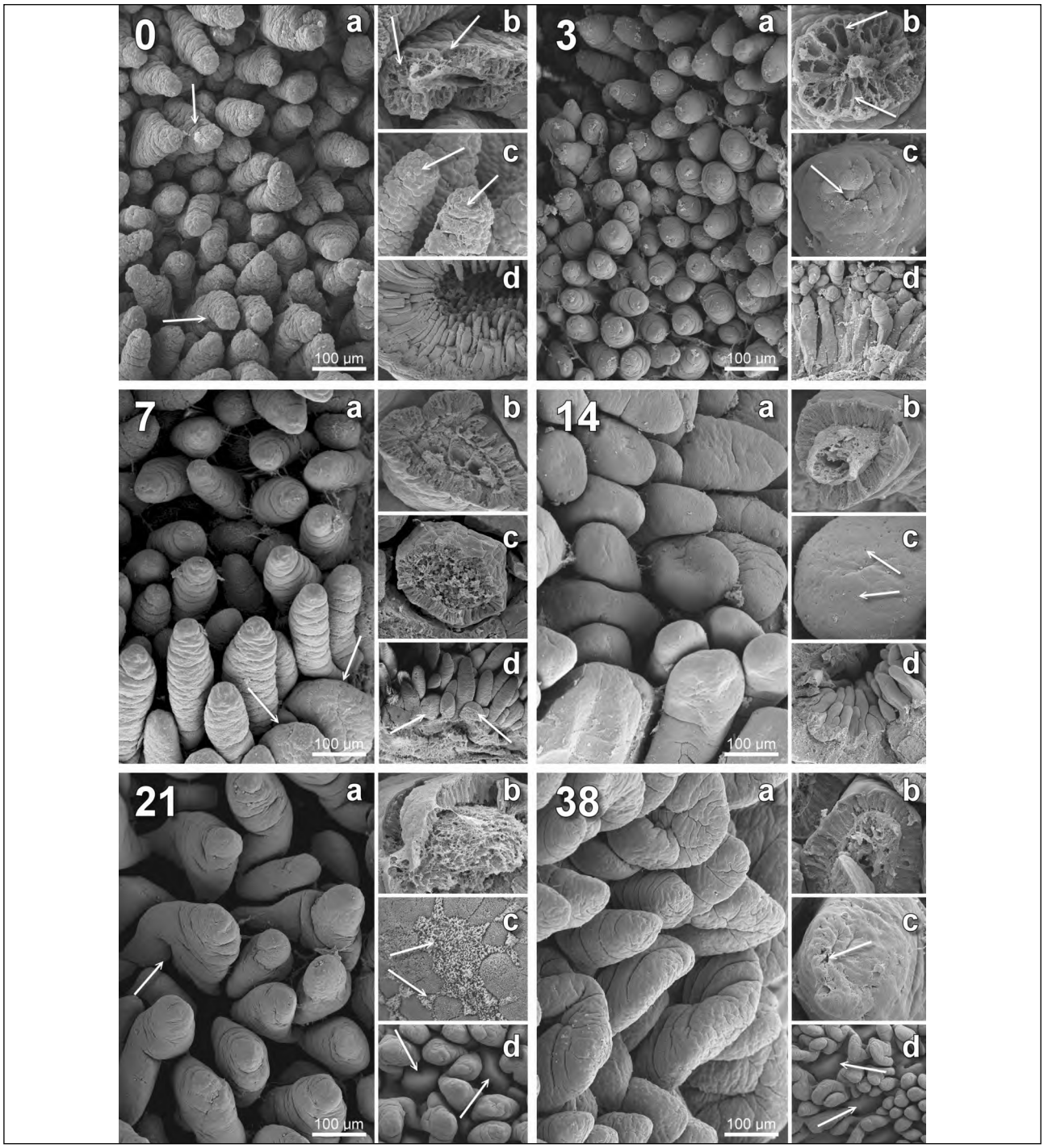

Fig. 7. (*0a*) SEM micrograph (500 ×) of ileal villi at birth; the villi are finger-like and flattened, densely packed; transverse furrows are present (arrows). Distended enterocytes of the fetal population cover the ileal villi. Inserts; b (2.5 kx): the cross section of the apical part of the villus with vacuolated enterocytes (arrows); c (2 kx): the apical part of villi covered by bulging fetal enterocytes (arrows); the furrows are present; d (250 ×): cross section of the ileal intestine showing densely packed villi of different length. (*3a*) Ileal villi on day 3; the villi are finger-shaped. Inserts; b: the section of the upper part of the villus revealed the presence of vacuolated enterocytes (arrows); c: shedding cells are rare in the apical part of the villi (arrow); d: section of ileal intestine, note the ratio between the villus and crypt length. (*7a*) Ileal villi on day 7; the villi are different in length; Peyer's patches are present at the base of the villi (arrows). Inserts; b: the section shows both vacuolated and mature adult enterocytes; c: cross section of Peyer's patch; d: sections of the intestine; the tall, dome-like structures between the taller villi are Peyer's patches (arrows). (*14a*) Ileal villi on day 14; the villi are flattened and different in size. Inserts; b: the section of the apical part of the villus shows the presence of mature enterocytes; c: the apical part of the villus, the numerous dark spots are the outlets of mucus cells (arrows); d: section of the ileal intestine, note the different size of the villi and deep crypts. (*21a*) Ileal mucosal surface on day 21. The villi are flat; some of them undergo division (arrow). Inserts; b: villus wall covered by regular enterocytes; c: the surface of Peyer's patch with M cells (arrows); d: section of the intestine; note the presence of Peyer's patches (arrows). (*38a*) Ileal mucosal surface on day 38; the villi are leaf-like and tongue-like. Inserts; b: villus section with regular enterocytes; c: the apical part of the villus with the extrusion zone (arrow); d: section of the intestine; note the dome-like Peyer's patches (arrows).

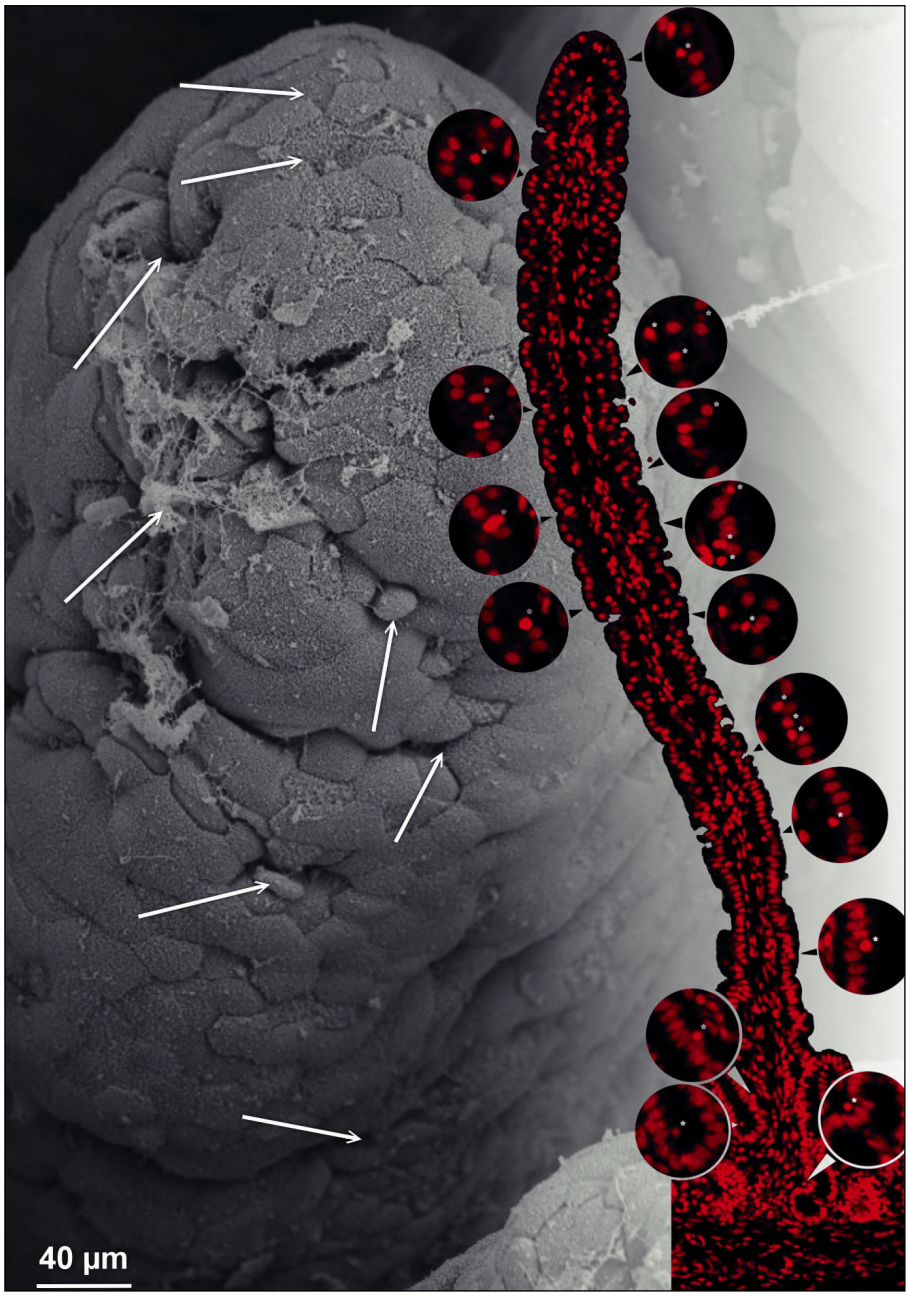

Fig. 8. SEM microphotograph depicting the duodenal villus from a 38-day-old weaned pig. The post weaning period is associated with intensive remodeling of the intestine mucosal surface; hence the multitude of dying cells, both single as well as in pockets (arrows). During the post-weaning period, apoptotic cells are present on the whole villus length and in crypts, as shown in the CM insert (asterisks). For CM, cell nuclei were visualized by 7-AAD (7-aminoactinomycin D). Apoptotic cells characterized by chromatin condensation and pyknosis were identified by laser scanning cytometry. Insert adapted with permission from the *Journal of Physiology and Pharmacology* (Godlewski MM, Slupecka M, Wolinski J, Skrzypek T, Skrzypek H, Motyl T, Zabielski R. Into the unknown - the death pathways in the neonatal gut epithelium. *J Physiol Pharmacol* 2005; 56: 7-24).

the cells. Since the mucosa of the small intestine in the short neonatal period is solely responsible for supplying nutrients to the whole organism, the presence of transverse furrows just after birth suggests that we should consider not only dynamic growth (commonly linked with increasing the number of epithelial cells), but also mechanisms causing extension of villus length within a short time of postnatal life. The extension is associated with the presence of numerous transverse furrows on the villus body shortly after birth, before the first feeding (21), and probably before birth (8). Numerous furrows seem to be a functional reserve for the mucosa.

We hypothesized that the small intestine mucosal surface was ‘compressed’ and ready to unfold immediately after birth and colostrum intake. The presence of a great number of transverse furrows on the villi at birth (21), prolonged sustenance of the great number of VFE (5), and changes in microcirculation (22) strongly support this hypothesis.

The rapid growth observed as a sequence of succeeding changes in the mucosal morphology suggests the presence of an earlier arrangement, probably developed during the prenatal period, even several weeks before birth. Trahair *et al*. (23) reported that the oral intake of amniotic fluid by the fetus is important for the growth of the small intestine. A pig fetus swallows and absorbs large amounts of amniotic fluid before birth. The fluid contains growth factors, peptides, hormones, proteins, cytokines, immunomodulatory factors, and other components and is probably responsible for accelerated fetal growth before birth (3-5). Sangild *et al*. (3) demonstrated marked increases in piglets’ body weight (200%) and relative weight of the small intestine (80%) during the last 20% of gestation. In pig experimental models where substrate supply from the fluid has been surgically restricted, small intestine growth was reduced. Ligated fetuses showed significantly reduced body weight (20%) and a reduction of intestinal weight

(43%), compared with sham-operated fetuses (3). Moreover, the above-mentioned authors observed villi with irregular shapes; some of them were branching and blunted, the apical part of enterocytes developed abnormally, and epithelial cell differentiation was reduced. The changes in the mucosal architecture probably caused reduction of the mucosal surface.

The 'after-birth straightening' of numerous deep, transverse furrows on the villus body can support the rapid enlargement of the length of the villi. There are two types of furrows: those present after birth that disappear directly after the first feeding and furrows appearing on the villus body depending on its physiological stage (24). We discussed the first type of furrows in this paper. The enterocyte height between the birth and PD 3 increased significantly for all the intestine segments analyzed. The process that leads to smoothing of 'crumpled' villi probably starts from enterocyte incrustation by colostrum proteins (4, 5, 18), and the open intestinal barrier is the basis of this process.

The most impressive feature of fetal enterocytes is the ability to transfer whole proteins across the cell into circulation without losing their biological activity (18). This phenomenon is described as an open intestinal barrier. This feature allows uncontrolled absorption of colostrum proteins and bioactive factors. Before birth, small and 'unfilled' VFE were packed on the surface of a furrowed villus. Following the first suckling, the VFE uptake colostrum and rapidly enlarge, 'pushing' at the neighboring cells and gradually unfolding the villus (19).

The significant increase in the height of the enterocytes in the jejunum at PD 3 and the decrease at PD 7 seem to confirm the important function of VFE on the first few days after birth. The SEM technique allowed us to observe gradual disappearance of VFE in time. The VFE change into mature enterocytes is a key feature of mucosal maturation. The timing of the gradual disappearance differed in the segments of the small intestine. The VFE containing an ACS were observed in the piglets from birth to PD 21 (19). The presence of VFE in piglets shortly before birth has been reported as well (2). In the duodenum, single VFE were located in the upper part of a few villi after birth. In the distal jejunum and ileum, VFE covered the entire villus body. After birth, they were replaced by mature enterocytes. VFE were present in the proximal jejunum up to PD 3, whereas single VFE were present in the middle and distal jejunum and ileum up to PD 14. At PD 21, there were no VFE in the entire small intestine.

Mickiewicz *et al*. (25) reported that maturation of the small intestine in intrauterine growth retarded piglets (IUGR) was delayed and VFE enterocytes were present for a longer time in IUGRs compared to normal weight born piglets. It is likely that the development of the small intestine mucosa was adapted in piglets born with low birth weight.

Acceleration of gut microcirculation stimulated by production of nitric oxide and endothelin (26) leads to more efficient absorption of colostrum and milk particles. Pappenheimer & Michel (16) demonstrated that increases in the villus capillary blood flow and permeability-surface area product were necessary components of absorptive mechanisms: the epithelial transport of normal digestive loads could not be sustained without concomitant increases in the capillary blood flow and area product. Transmission of colostrum proteins from enterocytes to the villus lumen leads to an increase in the lymph and blood volume. The intense increase in local intestinal blood flow and lymph formation caused by production of endothelin and nitrogen oxide (26) can facilitate the extension of the villi and smoothing of their surface. The intestinal microcirculation of the newborn infants was characterized by lower resting vascular resistance. This feature contributes to a higher rate of blood flow and increases delivery of nutrients and oxygen (27). These processes suggest a strict relationship between passive engulfment of colostrum components, hemodynamic conditions, and intense villus extension in the neonatal gut during early postnatal days.

Generally, the shedding of cells (mainly enterocytes) in all parts of the small intestine during the first week of postnatal life was negligible. There were only a few villi observed at birth in the duodenum, jejunum, and ileum, where intense shedding of cells took place. A greater number of shedding cells along the villus body was observed after PD 7. Biernat *et al*. (5, 28) reported a phenomenon of a decrease in apoptosis in suckling pig neonates 24 hours after birth. Furthermore, a decrease in apoptosis corresponded with an increase in the mitosis rate during two days after birth. In pig neonates, the mitotic index in the crypt region increased by 40 – 50% during the first two postnatal days (5, 28-30).

The increasing mucosal DNA content in the neonate intestinal mucosal layer also suggested elevated cell proliferation in crypts (17). The crypt depth increased significantly within the first 3 days of postnatal life in all the examined segments. A similar finding was reported by Marion (9): the intestinal crypt depth increased by 40%. Besides the deepening of crypts, villus enlargement was also observed. The intense cell proliferation in the neonatal crypts also caused shortening of the renewal cycle from 20 days during pregnancy to 2 – 3 day in newborn piglets (18). In newborn piglets, microscopic analysis evidenced the presence of apoptotic cells, as single cells and groups of cells, not only in the extrusion zones, but also along the entire villus body and in crypts (29).

There are two means of elimination of epithelial cells in neonatal piglets: shedding into the lumen and bringing under the epithelium for phagocytosis (29). We observed epithelial cells primed for apoptosis and elimination in packets (cell groups), containing up to 7 neighboring cells. The packets were detectable in SEM due to the collapse of their microvilli or cell lifting above the neighboring epithelium. This fact prompted the participation of auto- and paracrine factors in the regulation of enterocyte apoptosis. The main role of cytokines transforming growth factor beta1 and tumor necrosis factor alpha was postulated by Iwanaga (30) and Godlewski *et al*. (31). The presence of receptors for those cytokines in the basal and apical membrane showed that apoptotic signals could be transferred through lumen and tissue continuity.

The scant numbers of shedding cells on the villus body in the duodenum and jejunum in the early postnatal period was probably related to reduction of apoptosis within a short time after birth, as reported by Biernat (5). This strategy of prolonged sustenance of the great number of enterocytes rich in the apical canalicular system may increase the transfer time of intact bioactive substances across the epithelial barrier in newborn piglets. The widely established and accepted pattern of villus functional zones with easily observed extrusion zones seems to be corresponding only to adult animals, as strictly expressed extrusion zones were present only after the first week of life.

During the postnatal development, the small intestine is colonized by diverse microbiota, which is also a critical factor of intestine mucosal development (32). Chen *et al*. (33) suggested that microbes from the maternal and surrounding environments may play an important role in the microbial succession of newborn piglets after birth. Pasternak *et al*. (32) reported that decreased MAMDC4 (the MAM domain-containing 4) surface expression in the neonatal intestine is not functionally or temporally-driven physiological development, but rather a result of bacterial colonization. Colonization of the intestine by different bacteria, especially those rich in lipopolysaccharides (LPS) like *E. coli* or *S. typhimurium*, can contribute to increased DNA damage and induce repair processes by LPS-induced long-term oxidative stress in the intestine of three-week-old rats (34).

Research on colonization of the small intestine by bacteria during the postnatal period is likely to bring new information about the development and maturation of the mucosal membrane.

The small intestine of newly weaned piglets exhibited reduction in villus height and increase (35, 36). The alterations are thought to reduce the digestive and absorptive function of the small intestine mucosal layer. The changes occurring in the post-weaning period were limited to the change in the villus shape from the finger-shaped to leaf-shaped villus and the decrease in villus density. Some parameters also changed depending on the small intestine segment. The changes observed in the mucosal membrane were not as pronounced as those reported by Wang *et al*. (37). They reported the most serious damage to the intestinal morphology between 3 – 5 day post weaning. This was probably related to the day of observation; in our studies, the changes in the small intestinal mucosa were analyzed 10 days after weaning, which enabled relieving the stress to which the newborn and its digestive system were exposed at the time.

In conclusion, the process of neonatal mucosal development seems to be an effect of two co-existing mechanisms based on physical and physiological processes. The observations suggest that the most pronounced changes took place after the first colostrum intake. Absorption of colostrum, which is rich in bioactive substances, immunoglobulins, and relatively large protein particles, triggers a cascade of changes in all systems of the organism. The colostrum components influence the intestinal mucosal layer at two levels. The first group of factors comprising hormones, peptides, cytokines, and other bioactive particles such as colostral and milk epidermal growth factor, insulin-like growth factor-1 (IGF-1), insulin-like growth factor - 2 (IGF-2), TGF-β, glucagon-like peptide-2 (GLP-2), insulin, and leptin acts *via* a biochemical pathway, leading to epithelial cell proliferation and maturation. Simultaneously, the uptake of large amounts of colostrum proteins, electrolytes, and water physically fills the enterocytes, lacteal, and capillaries causing enlargement of villi. Both 'active' biochemical and 'passive' physical modes of growth and enlargement of epithelial cells are possible by the presence of vacuolated fetal-type enterocytes (equipped with the apical canalicular system and large vacuoles) in the open intestinal barrier.

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