Intrauterine Growth Restriction and Its Impact on Intestinal Morphophysiology Throughout Postnatal Development in Pigs

Thais Santos
Universidade Federal de Minas Gerais
Saffir Fernandes
Universidade Federal de Minas Gerais
Stefany Araujo
Universidade Federal de Minas Gerais
Fernando Felicioni
Universidade Federal de Minas Gerais
Thais Paula
Universidade Federal de Minas Gerais
Andre Caldeira Brant
Universidade Federal de Minas Gerais
Soraia Ferreira
Universidade Federal de Minas Gerais
Luciana Naves
Federal University of Lavras
Stefania Souza
Federal University of Lavras
Paulo Campos
Universidade Federal de Viçosa
Helio Chiarini-Garcia
Universidade Federal de Minas Gerais
Ana Luisa Dias
Federal University of Uberlândia
Fernanda Almeida (✉ falmeida@icb.ufmg.br)
Universidade Federal de Minas Gerais

Research Article

Keywords: IUGR, Pigs, small intestine, histomorphometry, enzymes

Posted Date: January 24th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1239812/v1

Abstract

Intrauterine growth restriction (IUGR) compromises fetal development, leading to low birth weight, and predisposes to gastrointestinal disorders. Pigs that suffered IUGR present poor postnatal development, resulting in great economic losses to the industry. The small intestine may be involved with their growth commitment, but studies investigating this issue are still limited. Thus, the present study aimed to investigate small intestine morphofunctional alterations in IUGR pigs throughout the production phases (birth to 150 days). IUGR pigs presented lower body weight from birth to the finishing phase ($P<0.05$). Although histomorphometrical parameters were not affected during the pre-weaning period, their commitment was observed specifically in the duodenum of the IUGR group at older ages ($P<0.05$). The most detrimental effects on the small intestine, such as highest duodenum crypts' depth, lowest villus height:crypt depth ratio and absorptive area, increased apoptosis and lower proliferation of the duodenum epithelium were noticed at 70 days of age ($P<0.05$). Additionally, IUGR pigs presented the lowest chymotrypsin and amylase activities at 70 and 150 days of age, respectively ($P<0.05$). These findings showed intestinal disturbances in IUGR pigs throughout the different production phases, demonstrating that poor performance is a consequence of intestinal damage mainly at the grower period.

Introduction

Genetic and nutritional strategies have been used to improve growth and reproductive performance in pigs. However, there are still some challenges that prevent animals to fully express their growth potential [1]. Among them, intrauterine growth restriction (IUGR) stands out as the main cause of low birth weight and postnatal growth commitment, affecting up to 20% of piglets within a litter in modern hyperprolific sows [2, 3]. It has been reported that IUGR has harmful impacts on neonatal survival, postnatal growth, nutrition utilization efficiency, health, and performance [2, 4–5]. Thus, growth restriction *in utero* has major implications for animal science [6].

Animal physiology, metabolism and growth are strictly related to the small intestine integrity and function, a key organ involved not only in the processes of digestion and absorption, but also in local and systemic immune responses [2, 7]. There is increasing evidence that the uterine environment modulates intestinal development, so that insults at this stage can compromise its function, which might not be reversed throughout postnatal life [8]. For instance, previous studies have reported villus atrophy and villus-crypt hyperplasia, delayed maturation of intestinal mucosa, decreased intestinal motility as well as decreased digestion and absorption of colostrum and milk in newborn and weaned IUGR pigs [9, 10]. Although information on intestinal commitment has been previously reported, it involved mainly the weaning period [11], with a lack of information on other production phases.

Morphometrical and biochemical approaches are the most used techniques in animal science to evaluate small intestine structure and function, as they provide reliable results [12, 13]. For example, intestinal maturity is extensively assessed by studying the size and number of villi, mucosal height, crypts depth, and epithelium height [4, 14, 15]. In addition, as the activity of the brush border enzymes is considered an

important indicator of maturation and digestive capacity in pigs [5, 11] its investigation in IUGR individuals also becomes essential.

An adequate development of the gastrointestinal tract is a prerequisite to ensure the individual's health. In this sense, an integrated and comprehensive understanding of growth, development and maturation of the intestine, and its dynamics throughout life, would contribute to the establishment of nutritional strategies to improve growth performance, thus providing significant contribution to the increase of productivity and profitability [16]. Hence, the aim of the present study was to investigate the morphofunctional aspects of the small intestine in IUGR pigs along their development, from birth up to 150 days of age, and establish the critical production phase in which growth restriction *in utero* would have the most detrimental effects for subsequent growth performance.

Methods

Animals and experimental design

One hundred twenty pairs of male littermate piglets (Landrace x Large White x Duroc multiple cross - Agroceres PIC), born to sows of parity 4th –6th in litters of 15–22 total born and mean litter birthweight variation of 1.25–1.65 kg, were selected immediately after birth, before colostrum intake, and were divided into two birthweight categories: normal birthweight (NW), birthweight ranging from 1.6 to 1.9 kg (n=120); and intrauterine growth restricted (IUGR), birthweight ranging from 0.7 to 1.0 kg (n=120). The NW–IUGR pair selected corresponded to the highest and the lowest birthweight males from each litter. The criteria used at selection were based on the concept of uterine crowding, according to previous studies [4, 17]. To define birthweight ranges, 1,000 piglets of the same genotype were previously weighed, and the average and standard deviation calculated (μ = 1.3; □ = 0.3). Birthweight ranges were set as μ + □ to μ + 2□ for NW and μ - □ a μ - 2□ for IUGR [4, 17–19]. Two sub-groups of 16 animals each (8 NW and 8 IUGR) were euthanized at either birth or 48 hours after weaning (26 days of age), and two other sub-groups of 20 animals each (10 NW and 10 IUGR) were euthanized at either 70 or 150 days of age, to obtain biometrical data and tissue collection.

These ages were chosen based on the characterization of postnatal gastrointestinal maturation during the main stages of the production cycle: birth (reflects placental function); after weaning (transition from milk to solid food); 70 days (grower phase) and 150 days (finisher phase).

The experimental protocol was approved by the Animal Experimentation Ethics Committee (CEUA) from the Federal University of Minas Gerais (protocol nº 2016/342). All experiments were performed in accordance with the CEUA relevant guidelines and regulations. Reporting of all experimental procedures complied with recommendations in ARRIVE guidelines.

Biometrical measures

Individual body weights were recorded at birth, 26 days, 70 days, and 150 days of age, without feed and water restriction. Immediately after euthanasia by electric stunning, the small intestine was removed from the abdominal cavity, weighed and the length was recorded. Additionally, to confirm the occurrence of IUGR, brain and liver were weighed in the newborn subgroup to obtain the brain to liver weight ratio, as performed by Alvarenga et al. [4].

Sample collection and processing

Fragments from the duodenum and jejunum were collected from the cranial and duodenum-jejunal flexures, respectively, and submitted to different processing protocols. For histological analyzes, fragments (1-2 cm length) were washed in saline and stretched on filter paper, fixed in 4% paraformaldehyde for 24 h, stored at 4^{o}C in 0.05M phosphate buffer (pH 7.4) for 24 h and embedded in Paraplast (Sigma Aldrich, São Paulo, Brazil). Histological sections (5 µm thickness) were then deparaffinized in xylene and rehydrated with graded dilutions of ethanol. Slides were stained with hematoxylin and eosin (HE) and Periodic Acid Schiff (PAS) for morphological observations and goblet cell counting, respectively.

Histomorphometrical evaluation

Histological sections were evaluated through a light microscope (Olympus BX51), and measurements performed using a ruler fitted in a 10x eyepiece, calibrated with a micrometer ruler. A total of 10 intact, well-oriented crypt-villus units from the duodenum and jejunum were randomly selected for measurements of the following parameters, as previously described [4]: (a) mucosal height (MH): from the *muscularis mucosae* up to the apex of the villus; (b) villus height (VH), from the base up to the apex of the villus; (c) depth of the intestinal crypt (DC), from the *muscularis mucosa* to the base of the villus and (d) villus (WV) and (e) crypt width (CW). Additionally, it was determined the ratio between villus height and intestinal crypt depth (VH/CD), villus surface area (S) and intestinal absorption area (AA), using the formulas described by Dong et al. [5] and Kisielinski et al. [20], as follows:

$$\mathbf{S}: \pi \frac{villuswidth}{2} \sqrt{\left(\frac{villuswidth}{2}\right)^2 + villusheight^2}$$

$$\mathbf{AA}: \frac{(villuswidth \bullet villusheight) + \left(\frac{villuswidth}{2} + \frac{cryptwidht}{2}\right)^2 - \left(\frac{villuswidth}{2}\right)^2}{\left(\frac{villuswidth}{2} + \frac{cryptwidth}{2}\right)}$$

Duodenum and jejunum histological sections were stained with Periodic Acid Schiff (PAS) to detect neutral mucins (stained in purple) present in goblet cells. The number of goblet cells was determined using the protocol standardized by Gomes et al. [21]. For each animal, 10 random fields of the intestinal crypts were selected, and the images obtained using a light photomicroscope (BX-51 Olympus)

connected to a Q-Color 3 digital camera (Olympus), at 400x magnification. All goblet cells were quantified in the crypt areas of each image using the Image-Pro Express software (Media Cybernetics, Rockville, MD, USA), and further equalized to squared millimeter (mm^2).

Expression of proliferation and apoptosis markers through Immunofluorescence (IF)

To determine whether IUGR can have effects on proliferative activity and apoptosis of the duodenal epithelium, immunofluorescence assays for Ki67, a marker of cell proliferation, and Caspase-3, a marker of apoptosis, were performed. Five animals from the IUGR and control groups were randomly selected. As the histomorphometrical parameters in the jejunum were similar in both experimental groups, proliferative and apoptotic assays were not performed. Briefly, the duodenum histological sections were dewaxed, rehydrated, and microwaved for 3 x 5 minutes in 0.1M sodium citrate buffer (pH 6) for epitope antigen retrieval, cooled down to room temperature, and rinsed with phosphate-buffered saline pH7.4 (PBS). To reduce non-specific binding, sections were incubated with 3% bovine serum albumin (Sigma-Aldrich, São Paulo, Brazil) in Tris-HCl buffer solution (PBS, pH 7.3) for 90 min at 4°C. All samples were incubated separately overnight at 4°C with either primary antibody anti-Ki67 (IgG; 1: 200; Thermo Fisher Scientific, São Paulo, Brazil) or anti-Caspase 3 (IgG; 1: 600; Thermo Fisher Scientific, São Paulo, Brazil), respectively. Staining of all slides for each assay was carried out simultaneously in a single session. After washing, secondary antibody Alexa Fluor 555 (goat antirabbit, IgG, A-21422, Thermo Fisher Scientific, São Paulo, Brazil), diluted in 1: 200 (antibody: PBS), was added to the sections and incubated for 90 min at 4°C. Finally, nuclei were stained using DAPI (Sigma-Aldrich, São Paulo, Brazil), in the proportion 1: 1,000 (DAPI: PBS) and the slides were mounted with 50% glycerol (v/v in PBS). Negative controls were obtained by omitting the primary antibody. Human testes samples were used as the positive control for Ki-67, and canine breast tumor tissue was used for Caspase-3. Immunolocalization images were acquired using a Zeiss Apotome microscope (Carl Zeiss Microscopy, São Paulo, Brazil) equipped with filters suitable for detecting Alexa Fluor 555 signals.

For the study of cell proliferation, 10 randomly selected field of intestinal gland (crypts) for each animal were photographed with a magnification of 200x. All positive cells in the epithelium of the intestinal gland were quantified using the Image-Pro Express software (Media Cybernetics, Rockville, USA) and the values expressed as number of immunostained cells per mm^2 of intestinal gland.

On the other hand, Caspase-3 analysis was performed through the determination of fluorescence intensity. Photomicrographs were obtained at 200x magnification for each animal and 10 randomly selected intestinal villi delineated using the Image J software (NIH). Before analysis, the photomicrographs were converted to black and white images and the Image J software was equalized considering white as 100% (positive areas) and black as 0% of fluorescence intensity. Values were expressed as average % of fluorescence intensity.

Determination of enzyme activity in the small intestine

For the determination of the enzymatic activities of lactase, amylase, lipase, chymotrypsin and trypsin, 2-cm samples of duodenum and jejunum were frozen in liquid nitrogen. For the preparation of the crude extract, 100 mg samples were homogenized in a Turrax type homogenizer, using 0.01 M phosphate buffered saline (NaCl 0.138 M; KCl - 0.0027 M; pH 7.4) in a 1:10 ratio (weight:volume). After centrifugation at 15.000g for 15 minutes at 4ºC, the supernatant was collected and stored at -20ºC for further analysis.

The enzymes activities were measured in the duodenum and jejunum tissue extract samples. For all of them, the quantities of proteins in the enzyme extracts were determined according to Bradford [22], using bovine serum albumin as the standard protein.

Lactase activity was determined using *O*-nitrophenyl *B*-D-galactopyranoside (ONPG, Sigma-Aldrich, São Paulo, Brazil) as an artificial substrate [23]. In the colorimetric assay, conducted at pH 7.4 and 37°C, the ONPG was cleaved by lactase releasing ortho-nitrophenol that was measured at 420 nm. The chymotrypsin activity was measured according to the Hummel method [24] based in the hydrolysis rate of the substrate *N*-Benzoyl-L-tyrosine ethyl ester (BTEE, Sigma-Aldrich, São Paulo, Brazil). The trypsin activity was determined using N*α* Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA, Sigma-Aldrich, São Paulo, Brazil) as a substrate according to the method of Erlanger et al. [25]. Finally, lipase activity was determined according to the protocol described in the Lipase assay kit (Analisa Gold, Belo Horizonte, Brazil) based on an improved dimercaptopropanol tributyrate (BALB) method, in which SH groups were formed from the lipase cleavage of BALB reaction with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to originate a yellow-colored product. The color intensity, measured at 412 nm, was proportional to the enzyme activity in the sample. For the determination of amylase activity, the Amylase assay kit was used (Analisa Gold, Belo Horizonte, Brazil) based on the modified Caraway method: the amylase contained in the sample hydrolyzed the starch by releasing sugar and dextrin molecules. After the addition of the iodine solution, the blue color was formed by complexation with the non-hydrolyzed starch. Thus, the amylase activity was inversely proportional to the intensity of the blue color formed and was calculated in comparison to the substrate control.

The specific activity of all enzymes was expressed in U/mg protein, being U defined as the amount of enzyme that hydrolyzes 1 mol of substrate per minute of reaction. All samples were analyzed in duplicate and the absorbance values were measured using the Power Wave^{TM} XS Microplate Scaring Spectrophotometer (Bio-Tek Instruments, Potton, United Kingdom).

Statistical analyses

All variables were tested for normality prior to analysis, using the univariate procedure of the Statistical Analysis System [26]. Data were analyzed as a randomized complete block design, with littermate pairs as block, and pig as the experimental unit. Data were submitted to analysis of variance (ANOVA) and means were compared by the Student-T test, with $P < 0.05$ considered significant. Important associations between performance and small intestine parameters were evaluated across birthweight groups by

correlation analysis (INSIGHT procedure of SAS). In the tables and figures, data are reported as least square means ± pooled s.e.m.

Results

Growth performance and biometrical parameters

Body weight and average daily gain from birth to 150 days of age are presented in Fig. 1. Intrauterine growth-restricted piglets showed lower body weights and average daily gains during all stages of development compared to their NW littermates ($P < 0.001$). In addition to poor performance, IUGR pigs also showed higher mortality rate throughout the production phases (18% vs 8%).

Furthermore, newborn IUGR piglets presented lower liver and small intestine weights, and shorter small intestine length, which persisted up to 70 days of age ($P < 0.05$). However, brain:liver weight ratio was higher in the IUGR experimental group, providing strong evidence of intrauterine growth restriction in this experimental group ($P < 0.05$). Although the small intestine weight and size was affected by birth weight up to 70 days of age, its relative weight and size were similar in both experimental groups at all ages evaluated except at 70 days, when those parameters were higher in IUGR animals ($P < 0.05$; Table 1).

Table 1 Body weight and intestinal biometrical parameters from normal (NW) and intra-uterine growth restricted (IUGR) male littermate pigs throughout the production phases

	Birth		26 days		70 days		150 days	
Parameters	NW (n=8)	IUGR (n=8)	NW (n=15)	IUGR (n=15)	NW (n=15)	IUGR (n=15)	NW (n=15)	IUGR (n=15)
Body weight (kg)	1.62 ± 0.04^{a}	0.87 ± 0.04^{b}	6.6 ± 0.1^{a}	4.8 ± 0.1^{b}	25.0 ± 0.4^{a}	18.5 ± 0.4^{b}	106.0 ± 1.3^{a}	90.0 ± 1.3^{b}
SI weight (kg)	0.060 ± 0.005^{a}	0.030 ± 0.005^{b}	0.2 ± 0.2^{a}	0.1 ± 0.2^{b}	1.6 ± 0.7^{a}	1.2 ± 0.7^{b}	3.1 ± 0.2^{a}	3.0 ± 0.2^{a}
SI length (m)	4.0 ± 0.1^{a}	2.6 ± 0.1^{b}	7.2 ± 0.2^{a}	6.7 ± 0.2^{b}	15.0 ± 0.4^{a}	13.6 ± 0.4^{b}	18.5 ± 0.6^{a}	18.0 ± 0.5^{a}
Relative SI weight	3.6 ± 0.4^{a}	3.3 ± 0.5^{b}	4.3 ± 0.3^{a}	4.0 ± 0.3^{a}	5.9 ± 0.6^{b}	7.8 ± 0.6^{b}	2.8 ± 0.2^{a}	3.3 ± 0.2^{a}
Relative SI length	0.25 ± 0.02^{b}	0.32 ± 0.02^{a}	0.014 ± 0.02^{a}	0.016 ± 0.02^{a}	0.055 ± 0.007^{a}	0.084 ± 0.008^{b}	0.016 ± 0.001^{a}	0.019 ± 0.001^{b}
Brain weight (g)	27.0 ± 0.4^{a}	24.0 ± 0.1^{b}	-	-	-	-	-	-
Liver weight (g)	52.5 ± 4.3^{a}	20.6 ± 4.3^{b}	-	-	-	-	-	-
Brain: liver weight	0.55 ± 0.07^{b}	1.24 ± 0.07^{a}	-	-	-	-	-	-

^{ab} Within the same age, least square means with different superscripts within a row differ ($P < 0.05$). SI: small intestine.

Small intestine histomorphometry and kinetics

The small intestine histomorphometrical data are summarized in Table 2.

Table 2 Small intestine histomorphometrical parameters from normal (NW) and intra-uterine growth restricted (IUGR) male littermate pigs throughout the production phases

Parameters	Newborn		26 days		70 days		150 days	
	NW (n=8)	IUGR(n=8)	NW(n=8)	IUGR(n=8)	NW(n=10)	IUGR(n=10)	NW(n=10)	IUGR(n=10)
Duodenum								
Villus Length (µm)	416 ± 12	398 ± 10	334 ± 47	323 ± 47	313 ± 23[a]	193 ± 23[b]	395 ± 39[a]	246 ± 39[b]
Crypt Depth (µm)	125 ± 7	111 ± 7	324 ± 15	288 ± 15	441 ± 21[a]	526 ± 21[b]	567 ± 70	555 ± 70
Mucosal Height (µm)	557 ± 53	571 ± 29	536 ± 103	486 ± 115	833 ± 37	785 ± 37	1054 ± 208	1236 ± 205
Villus/Crypt Ratio	3.4 ± 0.3	4.0 ± 0.3	1.0 ± 0.1	0.9 ± 0.1	0.7 ± 0.1[a]	0.4 ± 0.1[b]	0.7 ± 0.1	0.5 ± 0.1
Villus Surface Area (mm^2)	0.61 ± 0.07	0.47 ± 0.07	0.13 ± 0.02	0.12 ± 0.03	0.15 ± 0.02[a]	0.07 ± 0.02[b]	0.25 ± 0.02[a]	0.13 ± 0.02[b]
Absorptive Area (mm^2)	0.030 ± 0.004	0.024 ± 0.004	0.06 ± 0.01	0.05 ± 0.01	0.09 ± 0.01[a]	0.06 ± 0.01[b]	0.080 ± 0.004[a]	0.050 ± 0.004[b]
Jejunum								
Villus Length (µm)	760 ± 52	577 ± 48	249 ± 20	225 ± 9	372 ± 21	337 ± 19	429 ± 15	371 ± 80
Crypt Depth (µm)	100 ± 22	73 ± 3	201 ± 21	184 ± 13	328 ± 31	326 ± 18	312 ± 14	235 ± 58
Mucosal Height (µm)	328 ± 102	665 ± 44	560 ± 31	540 ± 36	755 ± 32	655 ± 32	767 ± 24	806 ± 45
Villus/Crypt Ratio	8.7 ± 0.7	7.8 ± 0.4	1.3 ± 0.1	1.1 ± 0.0	1.2 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	1.5 ± 0.1
Villus Surface Area (mm^2)	0.07 ± 0.10	0.06 ± 0.04	0.04 ± 0.01	0.03 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.02 ± 0.01	0.01 ± 0.01
Absorptive Area (mm^2)	0.030 ± 0.001	0.030 ± 0.001	0.040 ± 0.004	0.030 ± 0.001	0.010 ± 0.003	0.010 ± 0.002	0.050 ± 0.003	0.040 ± 0.001

[ab] Within the same age, least square means with different superscripts within a row differ ($P < 0.05$).

From birth to weaning, the duodenum was not structurally affected by IUGR. However, the negative effects of IUGR became evident at the grower (70 days) and finishing (150 days) stages, with a significant reduction in villus height, absorptive area, and villus surface area ($P < 0.01$). IUGR effects on the intestinal mucosa were more severe at 70 days since an increase in crypt depth and a decrease in the villus / crypt ratio were observed ($P < 0.05$).

Figure 2 shows aspects of the duodenal mucosa developmental kinetics (from birth to 150 days of age). Interestingly, in the NW group, villus height was relatively stable from birth to finishing. On the other hand, in IUGR pigs, duodenal villi height decreased after weaning and persisted low until 150 days of age, with the lowest height observed at 70 days.

Moreover, histomorphometrical parameters in the jejunum were similar in both experimental groups at all ages evaluated, suggesting that the duodenum is the segment of the small intestine that is more sensitive to IUGR effects on the mucosa.

Evaluation of cellular proliferation and apoptosis in the duodenum epithelium

The results of cellular proliferation and apoptosis are shown in Fig. 3. Growth restriction *in utero* negatively affected cellular proliferation of the duodenum epithelium, which was lower in IUGR piglets at birth (Fig. 3A; $P < 0.05$). However, this parameter was similar throughout the production phases (from weaning to 150 days of age).

Similar comparison performed with Caspase-3 expression showed that it also increased overtime, reaching the highest value at 70 days of age ($P < 0.05$) and coming down to similar values observed at birth, during the finisher phase (150 days of age - Fig. 3D).

Regarding apoptosis of the duodenum epithelium, it was shown a different pattern of expression. Although it was similar between the experimental groups at birth, Caspase-3 expression was higher at weaning, 70 and 150 days of age in IUGR pigs (Fig. 3B; $P < 0.05$).

When comparing the proliferative activity overtime within the same experimental group, a markedly increase in cellular proliferation was observed in the IUGR group, as Ki-67 expression was about 3 folds higher at 150 days compared to the other ages (Fig. 3C; P < 0.05). On the other hand, the expression of both markers remained relatively constant overtime in the NW experimental group (P > 0.05; Figs. 3C-D).

Body Weight and intestinal parameters correlations

The correlations between body weight and histomorphometrical parameters of the duodenum are presented in Table 3.

Table 3 Correlations between small intestine morphofunctional parameters and body weight at birth, 26 days, 70 days, and 150 days of age

Parameters	Birth BW		26 days Birthweight		26 days BW 26 days		70 days Birthweight		70 days BW 70 days		150 days Birthweight		150 days BW 150 days	
	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value
SI Weight	0.86	<0.05	0.84	<0.05	0.82	<0.05	0.71	<0.05	0.69	<0.05	0.59	0.11	0.48	0.21
SI Length	0.87	<0.001	0.85	<0.05	0.64	0.08	0.89	<0.001	0.89	<0.001	0.79	<0.05	0.89	<0.05
Villus Height	0.18	0.63	0.12	0.77	0.26	0.52	0.81	<0.05	0.68	<0.05	0.77	<0.05	0.60	0.11
Crypt Depth	0.11	0.75	0.43	0.28	0.39	0.32	-0.56	0.08	-0.64	<0.05	0.03	0.93	0.22	0.59
Villus / Crypt Ratio	-0.23	0.50	0.26	0.56	0.35	0.43	0.82	<0.05	0.76	<0.05	0.66	0.07	0.56	0.14
Absorptive Area	-0.08	0.82	0.40	0.32	0.50	0.20	0.75	<0.05	0.60	0.06	0.93	<0.001	0.67	0.09
Villus Surface Area	0.30	0.39	0.08	0.83	0.23	0.57	0.79	<0.05	0.67	<0.05	0.87	<0.05	0.72	<0.05
Ki-67	0.69	<0.05	0.69	<0.05	0.72	<0.05	0.64	<0.05	0.68	<0.05	-0.37	0.35	0.18	0.65
Caspase-3	0.23	0.50	-0.87	<0.05	-0.65	0.07	-0.88	<0.05	-0.80	<0.01	-0.61	0.10	-0.71	<0.05

Intestinal weight and length, as well as villus height, villus height:crypth depth ration, absorptive area and Ki-67 expression were positively correlated with body weight at all ages. Conversely, Caspase-3 expression was negatively correlated to body weight, as it was higher when body weight was lower (P < 0.05). Interestingly, these associations seemed more evident particularly at 70 days of age, reflecting late IUGR effects on the small intestine.

A positive correlation was observed between body weight and cell proliferation at all ages evaluated. On the other hand, for the apoptosis marker, a negative correlation with body weight from weaning to 150 days was observed, indicating that, in this period, the lower the body weight, the higher the apoptosis index in the duodenal epithelium.

Digestive enzymes activity assays

Table 4 presents the results of specific enzymes activity in the duodenum and jejunum. In the duodenum, a decrease in amylase activity was observed in IUGR pigs at 150 days of age (P < 0.05), whereas the activities of lactase, lipase, trypsin and chymotrypsin were not affected by birthweight at the other ages evaluated. In the jejunum, there was a decrease in the activity of chymotrypsin in the IUGR group at 70 days of age (P < 0.05), while the other enzymes (lactase, lipase, amylase and trypsin) activities were similar between both experimental groups. No changes in enzymes activities were observed at birth or at weaning (P > 0.05).

Table 4 Concentrations of the small intestine enzymes from normal (NW) and intra-uterine growth restricted (IUGR) male littermate pigs throughout the production phases

Parameters	Birth		26 Days		70 Days		150 Days	
	NW	IUGR	NW	IUGR	NW	IUGR	NW	IUGR
Duodenum								
Lactase (U/mg)	383 ± 93	417 ± 93	162 ± 35	132 ± 35	92 ± 13	73 ± 12	71 ± 11	84 ± 11
Amylase (U/mg)	1.0 ± 0.4	1.6 ± 0.3	1.7 ± 0.4	0.4 ± 0.5	1.8 ± 0.2	1.6 ± 0.2	1.3 ± 0.1[a]	0.9 ± 0.1[b]
Lipase (U/mg)	0.12 ± 0.10	0.2 ± 0.10	0.08 ± 0.10	0.05 ± 0.10	0.04 ± 0.10	0.06 ± 0.10	0.03 ± 0.10	0.02 ± 0.10
Chymotrypsin (U/mg)	579 ± 150	201 ± 28	237 ± 53	267 ± 59	330 ± 80	376 ± 76	731 ± 104	532 ± 127
Trypsin (U/mg)	779 ± 170	400 ± 113	406 ± 91	242 ± 105	382 ± 103	635 ± 98	295 ± 73	115 ± 79
Jejunum								
Lactase (U/mg)	287 ± 97	407 ± 97	227 ± 75	275 ± 75	234 ± 57	216 ± 40	88 ± 23	128 ± 21
Amylase (U/mg)	1.0 ± 0.3	1.2 ± 0.4	1.0 ± 0.4	1.3 ± 0.3	0.9 ± 0.2	0.6 ± 0.2	0.6 ± 0.1	0.5 ± 0.1
Lipase (U/mg)	0.04 ± 0.10	0.04 ± 0.10	0.04 ± 0.10	0.05 ± 0.10	0.08 ± 0.10	0.05 ± 0.10	0.04 ± 0.10	0.07 ± 0.10
Chymotrypsin (U/mg)	461 ± 77	387 ± 41	402 ± 301	663 ± 301	367 ± 55[a]	190 ± 62[b]	340 ± 75	402 ± 86
Trypsin (U/mg)	555 ± 79	460 ± 62	494 ± 116	354 ± 150	474 ± 99	372 ± 99	272 ± 48	199 ± 64

[ab] Within the same age, least square means with different superscripts within a row differ ($P < 0.05$).

Discussion

There is evidence that intestinal integrity is a key factor for intestinal health in humans and pigs [7, 27]. In the present study, we focused on the changes in the small intestine mucosa and enzymes activities, which led to growth development commitment in IUGR pigs throughout the production phases (from birth to the finishing period). In general, studies performed to investigate the functionality of the small intestine are limited to the weaning phase [11]. To the best of our knowledge, this is the first study to demonstrate intestinal morphofunctional alterations in growth restricted pigs from birth to 150 days of age, showing evidence that the most detrimental effects on the small intestine occur at the grower period (70 days of age) and not at weaning.

We found that weight and length of the small intestine in IUGR animals remained lower up to 70 days. This finding may indicate lower use of nutrients and provide the physiological basis for impaired postnatal growth in those animals [28]. In addition, intestinal length in relation to body weight was greater in IUGR pigs, probably due to the compensatory hypertrophy of this organ, suggesting a potential metabolic priority in relation to body growth [27].

Interesting, our data show that IUGR did not affect the duodenal morphology of piglets during the preweaning period. However, villus height and surface area, absorption area and villus / crypt ratio decreased during the postweaning period. Such commitment was evident when villus height was compared overtime within the same experimental group (kinetics), as a significant drop was observed, in particular, at 70 days of age. This difference may be due to a higher rate of apoptosis, and is extremely relevant, since intestinal commitment during that production phase may compromise the proper digestion of feed ingredients, thus altering nutrient availability [1].

As the immune and digestive systems of the newly weaned pig are still immature, IUGR piglets are more susceptible to enteric antigenic challenges that can also cause a reduction in body weight. It is worth

mentioning that both experimental groups shared the same environmental conditions and received the same diets. The greater crypt depth in the IUGR group at 70 days suggests that the proliferative activity of stem cells occurs, but does not compensate for cell losses, since the villi remained shorter.

To clarify the mechanism of intestinal epithelium commitment, expressions of proliferation and apoptosis markers were evaluated. Caspase-3 is a marker of cell apoptosis and plays an important role in the exchange of intestinal epithelial cells [29]. Higher rates of apoptotic cells in the villus of the duodenum were observed in IUGR piglets from weaning to the finishing phase. This finding suggests that the great removal of old enterocytes may cause shortening of the villi [30], which might be a factor involved in the delay of the intestinal epithelium maturation. Furthermore, higher apoptosis may affect the integrity of the intestinal mucosa as it increased the permeability of the intestinal barrier [10], which might be the cause of higher incidence of diarrhea in low birthweight piglets.

Our data show a significant decrease in the expression of Ki-67, the cell proliferation marker, in the intestinal glands of IUGR newborns. Since proliferative cells of the intestinal epithelium include stem / progenitor cells, stem cells may have reduced regenerative capacity in the immature intestine of IUGR individuals [31]. This limited ability of damage repair can further aggravate intestinal injury, in particular when the intestine is submitted to insults during postnatal development.

When comparing the expressions of Ki-67 and Caspase-3 overtime within the same experimental group, they were similar throughout postnatal life in normal birthweight animals, whereas there were variations for both markers in their IUGR littermates. For instance, the expression of Ki-67 was greater at 150 days, whereas, at that same age, the expression of Caspase-3 decreased. These events may reflect a compensatory mechanism of mucosal recovery, after the renewal disorders observed at 70 days of age.

Although morphological changes have been observed, impairments in enzymes activities were mild. We demonstrated that, in the duodenum and jejunum of IUGR piglets, lactase activity was not significantly affected. In addition to lactase, lipase and trypsin were not affected by growth restriction *in utero* at any of the production phases, but the jejunum chymotrypsin decreased 48% at 70 days of age and duodenal amylase secretion was 35% lower at 150 days. Chymotrypsin and trypsin catalyze the hydrolysis of the peptide bonds of dietary protein, while amylase degrades carbohydrates in the mammalian intestine [14]. In this sense, the lower enzymatic activity of both enzymes in the grower and finishing phases can be a limiting factor for efficient digestion of proteins and carbohydrates, essential nutrients for optimal muscle growth, which may also explain the low growth performance of IUGR animals. In fact, weaning and diet composition often influence enzyme secretion in young pigs [32]. However, this study indicates that IUGR may intensify these effects at older ages and may be the cause of permanent effects of IUGR on postnatal growth performance.

Conclusion

The post weaning is a crucial period of intense duodenal disorders, particularly the transition from the nursery to the grower periods, expressed by reduction of villi size, decreased absorption area and activity

of important enzymes in the processing of proteins and carbohydrates. Thus, this transition period deserves special attention, and management and nutritional practices should be implemented to minimize those deleterious effects. These findings may contribute to the elucidation of morphofunctional disturbances in the main stages of swine production, suggesting that permanent low growth performance of IUGR pigs may be a consequence of duodenal damage, not at weaning, but at the grower period.

Declarations

Acknowledgements

We would like to thank all the support from the workers of Fazenda São Paulo, where the experiment was conducted. T.G. Santos was awarded a scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and F.R.C.L. Almeida was awarded a CNPq research fellow.

Authors' contributions

FRCLA, HCG and ALNAD conceived the study. FRCLA analyzed the data. TGS, SDF, SBOA, FF, TMDP, ALCB and SVF performed the experiments. LPN and SPS performed the enzymatic activity evaluations. TGS, ALNAD and FRCLA wrote the manuscript. PHRFC, HCG, ALNAD and FRCLA supervised the study and approved the final version. All authors read and approved the final manuscript.

Funding

This work was funded by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES) - finance code 001; Fundação de Amparo à Pesquisa do Estado de Minas Gerais and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - 422410/2016-0).

Availability of data and materials

All data supporting our findings are included in the manuscript.

Additional information

The experimental protocol was approved by the Animal Experimentation Ethics Committee from the Federal University of Minas Gerais (protocol nº 2016/342).

Competing interests

The authors declare that they have no competing interests.

References

1. Zhang W, Ma C, Xie P, Zhu Q, Wang X, Yin Y, Kong X. Gut microbiota of newborn piglets with intrauterine growth restriction have lower diversity and different taxonomic abundances. J. Appl.

Microbiol. **127**, 354-369. https://doi.org/10.1111/jam.14304 (2019).

2. Li Y, Zhang H, Su W, Ying Z, Chen Y, Zhang L, Lu Z, Wang T. Effects of dietary *Bacillus amyloliquefaciens* supplementation on growth performance, intestinal morphology, inflammatory response, and microbiota of intra-uterine growth retarded weanling piglets. J. Anim. Sci. Biotechnol. **9**, 22. https://doi.org/10.1186/s40104-018-0236-2 (2018).
3. Lynegaard JC, Hales J, Nielsenm MN, Hansen CF, Amdi C. The stomach capacity is reduced in intrauterine growth restricted piglets compared to normal piglets. Animals **10**, 1291-1298. https://doi.org/10.3390/ani10081291 (2020).
4. Alvarenga ALN, Chiarini-Garcia H, Cardeal PC, Moreira LP, Foxcroft GR, Fontes DO, Almeida FRCL. Intrauterine growth retardation affects birthweight and postnatal development in pigs, impairing muscle accretion, duodenal mucosa morphology and carcass traits. Reprod. Fertil. Dev. **25**, 387-395. https://doi.org/10.1071/RD12021 (2013).
5. Dong L, Zhong X, Ahmad H, Li W, Wang Y, Zhang L, Wang, T. Intrauterine Growth Restriction Impairs Small Intestinal Mucosal Immunity in Neonatal Piglets. J. Histochem. Cytochem. **62**, 510-518. https://doi.org/10.1369/0022155414532655 (2014).
6. He J, Dong L, Xu W, Bai K, Lu C, Wu Y. Dietary Tributyrin Supplementation Attenuates Insulin Resistance and Abnormal Lipid Metabolism in Suckling Piglets with Intrauterine Growth Retardation. Plos One **10**, e0136848. https://doi.org/10.1371/journal.pone.0136848 (2015).
7. Celi P, Cowieson AJ, Fru-Nji F, Steinert RR, Kluenter AM, Verlhac V. Gastrointestinal functionality in animal nutrition and health: new opportunities for sustainable animal production. Anim. Feed Sci. Technol. **234**, 88-100, https://doi.org/10.1016/j.anifeedsci.2017.09.012 (2017).
8. Meyer AM., Caton JS. Role of the Small Intestine in Developmental Programming: Impact of Maternal Nutrition on the Dam and Offspring. Adv. Nutr. **7**, 169-178. https://doi.org/10.3945/an.115.010405 (2016).
9. Budiño FEL, Thomaz MC, Kronka RN, Júnior JMP, Santana AE, Tucci FM et al. Influência da adição de probiótico e/ou prebiótico em dietas de leitões desmamados sobre as atividades das enzimas digestivas e parâmetros sanguíneos. Acta Sci. Anim Sci. **26**, 529-536. https://doi.org/10.4025/actascianimsci.v26i4.1743 (2004).
10. Ferenc K, Pilżys T, Skrzypek T, Garbicz D, Marcinkowski M, Dylewska M. Structure and Function of Enterocyte in Intrauterine Growth Retarded Pig Neonates. Dis Markers 5238134. https://doi.org/10.1155/2017/5238134 (2017).
11. Su W, Zhang H, Ying Z, Li Y, Zhou L,Wang F, Zhang L, Wang T. Effects of dietary L-methionine supplementation on intestinal integrity and oxidative status in intrauterine growth-retarded weanling piglets. Eur. J. Nutr. **57**, 2735-2745. https://doi.org/ 10.1007/s00394-017-1539-3 (2018).
12. Helke KL, Ezell PC, Duran-Struuck R, Swindle MM. Biology and Diseases of Swine. Lab. Anim. Med. 695-769. https://doi.org/10.1016/B978-0-12-409527-4.00016-X. (2015).
13. Gupta SK, Behera K, Pradhan CR, Mandal AK, Sethy K, Behera D, Shinde KP. Studies of the macroscopic and microscopic morphology (hippocampus) of brain in Vencobb broiler. Vet. World **9**,

507-511.www.veterinaryworld.org/Vol.9/May-2016/13.pdf (2016).

14. Mickiewicz M, Zabielski R, Grenier B, Le Normand L, Savary G, Holst JJ et al. Structural and functional development of small intestine in intrauterine growth retarded porcine offspring born to gilts fed diets with differing protein ratios throughout pregnancy. J. Physiol. Pharmacol. **63**, 225-239. http://www.jpp.krakow.pl/journal/archive/06_12/pdf/225_06_12_article.pdf (2012).
15. Che L, Hu L, Liu Y, Yan C, Peng X, Xu Q et al. Dietary Nucleotides Supplementation Improves the Intestinal Development and Immune Function of Neonates with Intra-Uterine Growth Restriction in a Pig Model. PloS one **11**, e0157314. https://doi.org/10.1371/journal.pone.0157314 (2016).
16. Li T, Huang S, Lei L, Tao S, Xiong Y, Wu G et al. Intrauterine growth restriction alters nutrient metabolism in the intestine of porcine offspring. J. Anim. Sci. Biotechnol. **12**, 15-27. https://doi: 10.1186/s40104-020-00538-y (2021).
17. Auler PA, Moreira GHFA, Hogg CO, Ashworth CJ, Bortolozzo FP, Chiarini-Garcia H, Almeida FRCL. Testicular parameters and spermatogenesis in different birthweight boars. Reprod. Fertil. Dev. **29**, 1720-1728. https://doi.org/10.1071/RD16164 (2017).
18. Alvarenga ALN, Sousa RV, Parreira G, Chiarini-Garcia H, Almeida FRCL. Fatty acid pro le, oxidative stability of pork lipids and meat quality indicators are not affected by birth weight. Animal **8**, 660-666. https://doi.org/10.1017/S1751731114000093 (2014).
19. Felicioni F, Pereira AD, Caldeira-Brant AL, Santos TG, Paula TMD, Magnabosco D et al. Postnatal development of skeletal muscle in
pigs with intrauterine growth restriction: morphofunctional phenotype and molecular mechanisms. J. Anat. **236**, 840-853. https://doi.org/10.1111/joa.13152 (2020).
20. Kisielinski K, Willis S, Prescher A, Klosterhalfen B, Schumpelick V. A simple new method to calculate small intestine absorptive surface in the rat. Clin. Exp. Med. **3**, 131-5. https://doi.org/10.1007/s102380200018 (2002).
21. Gomes JR, Ayub LC, Dos Reis CA, Machado MJ, da Silva J, Omar NF et al. Goblet cells and intestinal Alkaline phosphatase expression (IAP) during the development of the rat small intestine. Acta Histochem. **119**, 71-77. https://doi: 10.1016/j.acthis.2016.11.010 (2017).
22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. **72**, 248–254. https://doi.org/10.1006/abio.1976.9999 (1976).
23. Leksmono CS, Manzoni C, Tomkins JE, Lucchesi W, Cottrell G, Lewis PA. Measuring Lactase Enzymatic Activity in the Teaching Lab. J. Vis. Exp. **6**, 54377-54382. https://doi: 10.3791/543772018 (2018).
24. Hummel BCW. A modified spectrophotometric determination of chymotrypsin, trypsin and thrombin. Can. J. Biochem. Physiol. **37**, 1393-1399. https://doi.org/10.1139/o59-157 (1959).
25. Erlanger BF, Kokowsky N, Cohen W. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. **95**, 271–278. https://doi.org/10.1016/0003-9861(61)90145-X (1961).

26. SAS-Statistical Analysis System Institute, Inc. SAS user's guide: Statistics. Cary: SAS Inst., Inc. 1998, 956p.
27. Shanklin, DR, Cooke RJ. Effects of intrauterine growth on intestinal length in the human fetus. Biol Neonate. **64**, 76-81. https://doi.org/10.1159/000243974 (1993).
28. Che L, Zhou Q, Liu Y, Hu L, Peng X, Wu C et al. Flaxseed oil supplementation improves intestinal function and immunity, associated with altered intestinal microbiome and fatty acid profile in pigs with intrauterine growth retardation. Food Funct. **10**, 8149-8160. https://doi.org/10.1039/c9fo01877h (2019).
29. Delgado ME, Grabinger T, Brunner T. Cell death at the intestinal epithelial front line. FEBS J. **283**, 2701-2719. https://doi: 10.1111/febs.13575 (2016).
30. Vereecke L, Beyaert R, van Loo G. Enterocyte death and intestinal barrier maintenance in homeostasis and disease. Trends Mol. Med. 17, 584-593. https://doi: 10.1016/j.molmed.2011.05.011 (2011).
31. Fung CM, White JR, Brown AS, Gong H, Weitkamp JH, Frey MR. et al. Intrauterine growth restriction alters mouse intestinal architecture during development. PloS One **11**, e0146542. https://doi: 10.1371/journal.pone.01465422016 (2016).
32. Cera KR, Mahan DC, Reinhart GA. Effect of weaning, week postweaning and diet composition on pancreatic and small intestinal luminal lipase response in young swine. J. Anim. Sci. **68**, 384-91. https://doi: 10.2527/1990.682384x (1990).

Figures

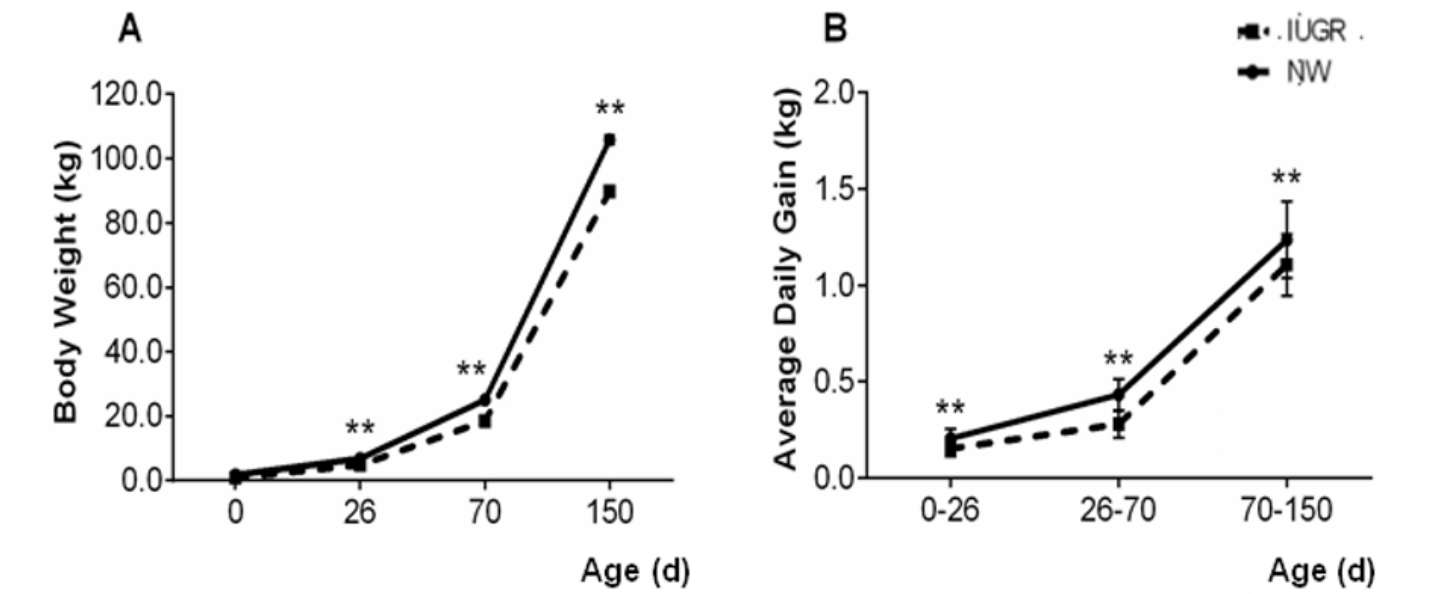

Figure 1

Growth curve (A) and average daily gain (B) in growth restricted (IUGR) and normal (NW) birth weight male littermate pigs from birth (0) to 150 days of age. IUGR pigs grew slower and gained less body weight throughout the production phases. ** Means differ statistically between groups ($P < 0.001$).

Figure 2

Villus height in duodenum throughout postnatal growth (kinetic) in growth restricted (IUGR) and normal (NW) birth weight male littermate pigs. Villus height decreased sharply from birth to 70 days of age in IUGR pigs (black square), reaching its lowest height at this age. On the other hand, villus height remained relatively stable in the NW littermates in the same period. [a,b] Means differ statistically between groups ($P < 0.05$).

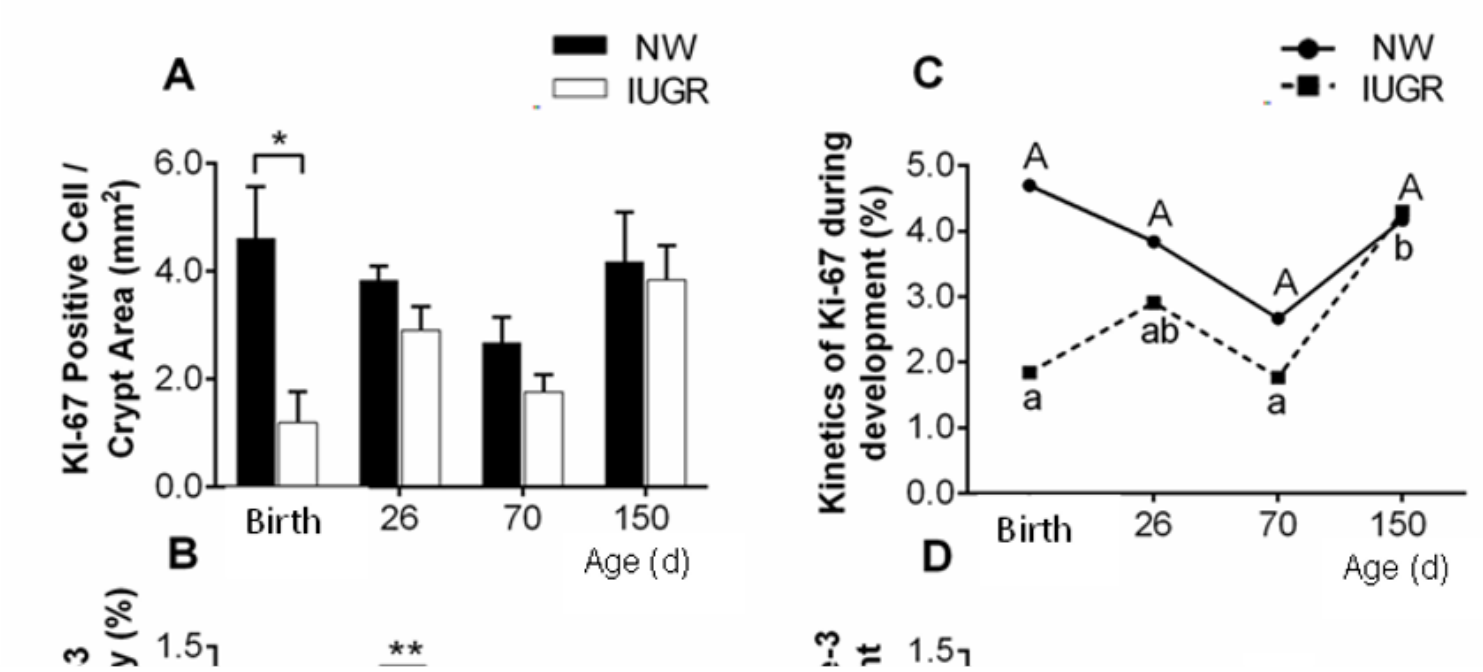

Figure 3

Characterization of Ki-67 and Caspase-3 expressions in the duodenum of intrauterine growth restricted (IUGR) and normal (NW) birth weight male littermate pigs. (A) Number of Ki-67 positive cells per area of epithelium of intestinal glands (density). Cellular proliferation was higher in NW piglets at birth but remained similar between the experimental groups throughout the production phases. (B) Mean percentage of Caspase-3 fluorescence cells in the duodenal villi. IUGR pigs presented higher apoptosis after weaning, which remained higher until 150 days of age. (C) The pattern of cell proliferation overtime (from birth till 150 days of in NW pigs remained relatively constant throughout postnatal development. On the other hand, in IUGR pigs, cell proliferation significantly increased from 70 days to 150 days of age (D) Regarding the pattern of apoptosis overtime, mean percentage of Caspase-3 fluorescence in the duodenal villi remained constant in the NW animals, but it increased drastically until 70 days in the IUGR pigs. ^{a,b} LSMeans differ statistically between groups ($P < 0.05$).