Single-blinded Study Highlighting the Differences between the Small Intestines of Neonatal and Weaned Piglets

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Abstract

Background: The gut is the body's major immune structure, and the gut mucosa, which contains intraepithelial lymphocytes (IELs) and subepithelial natural immune cells, is considered the primary site for eliciting local immune responses to foreign antigens. Pigs are susceptible to intestinal infections at all life stages; however, neonates tend to be the most susceptible. This study compared the small intestine of neonatal and weaned piglets to provide a theoretical basis for preventing intestinal infectious diseases in neonatal piglets.

Results: Histological analyses of weaned piglet intestines showed increased crypt depth, higher IEL count, and larger ileal Peyer's patches compared with those of neonates. Additionally, the ileal villi of weaned piglets were longer than those of neonatal piglets. The expression of claudin-3 and occludin protein was remarkably higher in weaned piglets than in neonatal piglets. The numbers of CD3 + T cells, goblet cells, and secretory cells were also higher in the small intestine of weaned piglets than in those of neonates. The number of secretory IgA-positive cells in the jejunum was not significantly different between neonatal and weaned piglets. The gene expression of 12 pattern recognition receptors (PRRs), such as TLR1–10, MDA5, and RIG-I in the small intestines of both neonatal and weaned piglets was also examined. The mRNA expression of most pattern recognition receptors genes in the duodenum and jejunum was higher in weaners than in neonates; however, the inverse was true in the ileum. Compared with that in weaned piglets, there were significantly fewer CD3 + , CD4 + , and CD8 + T cells from peripheral blood-mononuclear cells in neonatal piglets.

Conclusions: In this study, the physical and immunological components of small intestines of neonatal and weaned piglets were investigated. Our results provide preliminary data on differences in the immune mechanisms between the small intestines of 0- and 21-day-old piglets. Future studies could focus on additional developmental stages of pigs and how the differences in their small intestines affect the animal's response to pathogens

Background

The gut is critical for the maintenance of good health and production efficiency in pigs[1, 2]. The small intestine is divided into three sections—duodenum, jejunum, and ileum. The intestinal mucosa in pigs is not only involved with the digestion and absorption of nutrients and energy but also plays an important role in combating foreign antigens, including food proteins, natural toxins, and pathogenic and commensal microorganisms[3, 4]. Hence, the gut is a major immune organ and the gut mucosa is thought to be the primary site for eliciting and mediating local immune responses[3, 5].

Infectious diseases of the digestive tract are the most frequent and recurrent conditions in the swine industry. Various viruses, such as coronavirus transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhoea virus(PEDV) and rotavirus A (RVA), have been primarily infect neonatal piglets [6-8], but the underlying mechanism is not yet fully understood. Infections due to TGEV, PEDV, RVA and other

pathogens can lower the feed conversion efficiency by causing diarrhea; hence, they represent major threats to the swine industry.

In pigs, changes in the intestinal morphology and structure mainly occur at birth and during weaning[9]. The porcine intestinal immune system is immature at birth[10], and thus, piglets are most susceptible to pathogenic organisms at the neonatal stage[11]. The intestinal mucosal barrier has physical, biochemical, and immunological components [12]. Many studies have reported on the development of intestinal structures of the pig small intestine[13]. However, few studies have compared the intestinal structures between neonatal and weaned piglets. In the present study, we compared the physical and immunological components of small intestines of neonatal and weaned piglet intestines to provide preliminary data for further studies in the future.

Methods

Animals

A total of six—three 21-day-old (weaners; weighing 8-10 kg) and three 0-day-old (neonates; weighing 1.10-1.30 kg)—male cross-bred Duroc/Landrace/Yorkshire piglets were obtained from the Jiangsu Huai'an Pig Farm (Huai'an, China). The piglets were housed indoors at the Jiangsu Huai'an Pig Farm, under constant conditions of 60 % humidity, 26 °C, and 12 h light/dark cycle as well as free access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University, and the National Institutes of Health guidelines for the performance of animal experiments were followed.

Material collection and preparation

Each piglet was euthanized by an intravenous injection of pentobarbital sodium (100 mg/kg) before sample collection. To determine complete anesthetization of the piglets before opening the abdomen, their palpebral and withdrawal reflexes were checked. Duodenum, jejunum, and ileum tissue samples were collected from the piglets and fixed in 4 % paraformaldehyde for 48 h at 25 °C. After fixation, the samples were sectioned into small pieces. Then the small pieces (1cm) dehydrated using a graded ethanol series (75, 85, 95, 100, and 100 %, each for 1 min in that order). The dehydrated blocks were embedded in paraffin wax and kept at room temperature to dry. Next, the tissue samples were sliced longitudinally into 5-µm-thick sections using a microtome. The sections were dried horizontally on a warming tray (37 °C) overnight, dewaxed in xylene, rehydrated in a graded series of ethanol (100, 90, 80, and 70 %, each for 1 min in that order), and washed in phosphate-buffered saline (PBS). These tissue sections were then used in the subsequent experimental analyses. All observations were single-blinded.

Histological analysis

The prepared tissue sections were stained with hematoxylin and eosin (H&E) and examined using light microscopy (BH-2, Olympus). The size of the ileal Peyer's patches (PPs), villus height and crypt depth

were measured using computer-assisted morphometry (Image-Pro Plus software).

Immunohistochemistry

Antigens were retrieved from the tissue sections by placing them in a citrate buffer (pH 6; 90-95 °C) for 15 min. Then, the sections were treated with 0.3 % hydrogen peroxide at room temperature for 15 min and washed with PBS to quench endogenous peroxidase activity. To avoid the non-specific binding of antibodies, the sections were blocked using 5 % bovine serum albumin for 30 min at room temperature. After incubating with the primary antibodies overnight at 4 °C, the sections were treated with biotinylated secondary antibodies for 1 h at room temperature (Table I). To visualize the immuno-positive cells, the sections were stained with diaminobenzidine (DAB) for 60 min at room temperature and sealed with neutral balata. The respective isotypes were used as negative controls. The sections were visualized using a light microscope (Olympus CX23; Olympus Corporation, Tokyo, Japan) at a magnification of 400× or 100×. Different fields (n=10) of each tissue in each piglet were counted for the statistical analysis.

Immunofluorescence

The tissue sections were incubated with 0.4 % Triton X-100 in PBS for 5 min. After blocking with 5 % bovine serum albumin in PBS for 1 h, the sections were stained with Ulex europaeus agglutinin-1 (UEA-1) antibodies at room temperature for 2 h. PBS was used instead of the antibodies in control samples. After staining with DAPI, the sections were observed using confocal laser scanning microscopy (LSM-710; Zeiss, Oberkochen, Germany).

RNA isolation and Real-time quantitative PCR

Total RNA was extracted from the tissues using a TRIzol® Plus RNA Purification kit (Thermo Fisher Scientific, Inc.). One microgram of total purified RNA was reverse transcribed to cDNA, using the PrimeScript™ RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China), as follows: one cycle of 37 °C for 15 min followed by 85 °C for 5 s. RT-qPCR analysis was performed with 2 µl of diluted cDNA (vol:vol, 1:5) was used to perform RT-qPCR analysis, by ABI 7500 PCR system (Life Technologies; Thermo Fisher Scientific, Inc.) and SYBR-Green qPCR Master Mix (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturers' protocols. The thermocycler reaction involved a pre-incubation period of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. Specific primers referred from the studies by Zong et al. (2019), Temeeyasen et al. (2017), and Wang et al. (2020) are shown in Table II [14, 15] [16]. Data were normalized against GAPDH mRNA levels and are expressed as fold differences between control and treated cells, calculated using the $2^{-\Delta\Delta CT}$ method.

Flow cytometry

Isolated single mononuclear cells were obtained from the piglet peripheral blood-mononuclear cells (PBMCs) by density centrifugation using a porcine peripheral blood lymphocyte separation kit (Solarbio).

PBMCs were stained with anti-CD3-APC (BD Biosciences, California, USA), anti-CD4-FITC (BD Biosciences, California, USA), and anti-CD8-PE (BD Biosciences, California, USA) (1:100 dilution) for 30 min at 4°C in the dark. The cells were then washed twice with PBS, and the expression of surface markers was observed using flow cytometry (FACSC6, BD Biosciences). The flow cytometry data were analyzed using the FlowJo software. A total of 10 000 lymphocytes were acquired per sample.

Statistical analysis

Analysis of variance and unpaired Student's t-tests were employed to determine statistically significant differences among multiple groups. The differences were considered significant at * $P < 0.05$, ** $P < 0.01$. Results are expressed as mean $\pm$ SEM.

Results

1. Histological differences between neonatal and weaned piglet intestines

As shown in Fig. 1a, microvilli in the small intestine of neonatal piglets appeared different from those of weaned piglets. Weaned piglets showed shortening, clubbing, and blunting of the duodenal villi, whereas the ileal villi were longer than those of the neonatal piglets. Jejunal villi showed no difference in length between neonatal and weaned piglets. In weaners, both the crypt depth (Fig. 1b) and number of IELs (Fig. 1c) were markedly higher than those in neonates. Weaners also had significantly larger and more mature PPs than those of neonates; moreover, the boundaries between the partial PPs were obscure in neonates (Fig. 1d).

2. Weaners have superior intestinal barrier function than neonates

Tight junctions are important determinants of epithelial barrier functions[17]. Consequently, we compared the expression of genes coding for tight junction proteins in various areas of the small intestine between the neonatal and weaned piglets. As shown in Fig. 2a, compared with that in neonates, the expression of claudin and occludin transcripts in the intestinal mucosa was remarkably high in weaners; however, the expression of E-cadherin and ZO-1 transcripts showed no significant difference. We stained the tissue sections with anti-claudin-3 antibodies and occludin to further compare the expression of tight junction proteins in various gut areas between the neonates and weaners. The expression of claudin-3 and occludin proteins was also higher in weaners than in neonates, especially in the duodenum (Fig. 2b).

3. Characteristics of mucosal immunity in neonatal and weaned piglet intestines

CD3⁺ T lymphocytes are the major T lymphocyte subtype[18, 19]; therefore, the number of CD3⁺ T lymphocytes could be considered as the absolute T lymphocyte count. We compared the number of CD3⁺ T lymphocytes in the small intestine between neonatal and weaned piglets. The CD3⁺ T lymphocyte distribution patterns were examined using immunohistochemistry. Immuno-positive cells were stained brown. Weaned piglets had more CD3⁺ T lymphocytes in the duodenum, jejunum, and ileum than

neonatal piglets did (Fig. 3a). SIgA is the main immunoglobulin isotype in animals and is primarily secreted across the intestinal mucosal surface, especially in the small intestine[20]. SIgA plays an important role in intestinal mucosal immunity and is an index of intestinal mucosal immunity[21]. The number of IgA⁺ B cells on the small intestine mucosal surface were examined by immunohistochemistry. Immuno-positive cells were stained brown. Weaned piglets had more IgA⁺ B cells in the duodenum and ileum than neonatal piglets did, although there was no significant difference in the IgA⁺ B cells between neonates and weaners (Fig. 3b). Goblet cells (GCs) could secrete anti-microbial proteins, chemokines, and cytokines and have important innate immunity-related functions besides intestinal barrier maintenance[22]. that PAS-stained intestinal sections showed the presence of GCs in the intestinal mucosal surface epithelia of neonatal and weaned piglets. These cells, which appeared purple after PAS staining, were typically circular and cup-shaped and were located in the intestinal lamina propria. As shown in Fig. 3c, weaned piglets had more GCs in the duodenum, jejunum, and ileum than neonatal piglets did. UEA-1, a universal marker of secretory cells, was also examined to determine the activation status of the intestinal cells[23]. Weaners had a higher proportion of intestinal secretory cells than neonates did (Fig. 3d).

4. Differences in pattern recognition receptor (PRR) genes in the intestinal mucosa of neonatal and weaned piglets

PRRs are expressed on various mucosal cell types, such as toll-like receptors (TLRs) and RIG-I-like receptors (RLRs), are important sensors in host-microorganism crosstalk. PRRs play a critical role in the detection of pathogen and in the elicitation of inflammatory and immune responses[24]. The expression of 12 PRR genes (TLR1–10, MDA5, and RIG-I) along the small intestine (duodenum, jejunum, and ileum) was examined in both neonatal and weaned piglets. In the duodenum, the expression of TLR3, TLR5, MDA5, and RIG-I genes was higher in weaners than in neonates. In contrast, duodenal TLR6 expression was lower in weaners than in neonates (Fig. 4a). In the jejunum, the expression of TLR1, 2, 4, 5, 10, and MDA5 was higher in weaners than in neonates (Fig. 4b). In the ileum, only the expression of TLR 4 was high; while the expressions of TLR3, 5, 6, 7, MDA5, and RIG-I were lower in weaners than in neonates (Fig. 4c).

5. Percentage of CD3⁺, CD4⁺, and CD8⁺ T lymphocytes in peripheral blood mononuclear cells

Lymphocytes are a major component of the peripheral innate immune system and are responsible for engulfing and killing pathogens during an infection[25]. Neonatal lymphocytes have quantitative deficiencies. As shown in Fig. 5, the percentage of CD3⁺ cells in the blood of neonatal and weaned piglets was 15.4 % and 41.5%, respectively, whereas the percentage of CD4⁺ CD8⁺ T cells in the blood of neonatal and weaned piglets was 2.17 % and 6.35 %, respectively. Therefore, the percentage of lymphocytes was significantly lower at birth than at weaning, at which point it reaches higher levels. These results suggest that neonatal piglets are more susceptible to contracting infection.

Discussion

Pigs are one of the most economically important livestock species worldwide. Pigs, like humans, are true omnivores and share similar anatomy and physiology of the digestive system, which favors the use of pigs as a suitable animal model to investigate human intestinal diseases and to understand the biological pathways underlying mucosal functions and development[26-28]. Therefore, the use of pigs as an animal model can aid in revealing the structure and function of the intestine in humans.

There are two major transition periods during the development of the immune system in pig; one at birth and one at weaning [29]. The weaning age of modern pig systems has declined over time[30]. It has been reported that weaning of Wuzhishan piglets at 21 days of age old may have positive effects on the adaptive immune system [31]. Some researchers have explored the quantitative changes in the two main stem cell populations between the small intestines of 0- and 21-day-old piglets[32]; however, the difference on the physical and immunological components of small intestines between neonatal and weaned piglets remains unknown. Therefore, neonatal (0 day) and weaned piglets (21 days) were chosen as the two important time points for examination in this study.

In animal production, enteric diarrhoea is one of the most frequent early clinical signs of outbreak, with high morbidity and mortality[11, 33]. Various enteric pathogens can cause intestinal diseases in piglets or adult pigs, leading to tremendous economic losses[8]. Neonatal piglets are highly susceptible to infection with viral and/or bacterial pathogens[34]. Our study compared the small intestine morphology, immune cell composition, and expression of tight junction protein and PRRs gene in neonatal and weaned piglets to investigate the causes of susceptibility of neonatal piglets to infection.

The small intestine has numerous villi that increase its internal surface area and improve the efficiency of nutrient absorption. We found that the duodenal villi of neonatal piglets were significantly longer than that of weaned piglets. However, a contrasting observation was noted in the ileum. This might have been due to different dietary patterns. Neonatal piglets only consume milk, whereas weaning pigs are provided with solid feed. Pigs have different dietary requirements at different stages of development. Hence, it can be expected that pigs' intestines change morphologically as they age, to accommodate the changes in their diets. Feed additives (probiotics and prebiotics) reportedly affect on the morphometric characteristics of the intestines in piglets[35, 36]. Our study showed that the shape, length, and other characteristics of these villi differed significantly between neonates and weaners.

Intestinal crypts have large numbers of stem cells, which help maintain the integrity of the intestinal epithelium and protect it from injury due to passing food [37, 38]. Our study showed that the intestinal crypt depth of weaned piglets was significantly higher than that of neonatal piglets, possibly to produce more stem cells. In their study, Villagómez et al. showed that IELs act as sentinels for maintaining the integrity of the mucosal barrier and thus protect the body from infection [39]. In our study, the number of IELs in each villus and PP of weaners was markedly higher than that of neonates. In addition, compared with that in weaned piglets, the immune system in neonatal piglets is not fully developed. This is made apparent in the lesser numbers of immune cells, such as CD3⁺ T cells, IgA⁺ cells, GCs, secretory cells, found in intestinal of neonates when compared with those found in weaners.

Tight junctions are cell adhesion apparatuses that act as barriers and/or channels in the spaces between adjacent epithelial cells [40]. The expression of tight junction protein in the intestinal tract is age-specific. For example, claudin is normally expressed in the human fetal small intestine, but not in the adult colon under homeostatic conditions[41]. We examined the expression of genes coding for major intestinal tight junction proteins in neonatal and weaned piglet small intestinal tissue. We found no significant difference in ZO-1 and E-cadherin gene expression. However, the expression of claudin and occludin genes were different in the jejunum, and claudin-3 expression showed obvious dissimilarity at the protein level. These results indicate that weaners have better intestinal epithelial integrity than neonates.

PRRs are either expressed on cell surfaces or associated with intracellular vesicles that specifically bind to pathogen-associated molecular patterns (PAMPs)[42, 43]. RNA viruses, including PEDV, can interact with a large number of PRRs in the intestinal mucosa, such as TLRs and RLRs[44]. This interaction plays a critical role in the activation of the innate immune response. When microbes breach physical barriers such as the skin and mucosa, TLRs recognize their PAMPs and activate an immune response[45]. Compared with post-weaning, the immune system of newborns is less developed, which may be related to TLR expression[46]. Gene expression of 12 pattern recognition receptors (PRRs) (TLR1–10, MDA5, and RIG-I) in the small intestines of neonatal and weaned piglets was also examined. We found that the number of TLRs in the duodenum and jejunum of weaners was relatively higher than in those of neonatal piglets; however, the ileum showed the opposite results for TLR expression.

Lymphocytes are a major subclass of white blood cells. Lymphocytes enter the blood circulation and facilitate the detection and elimination of pathogens and the dissemination of immunologic memory[47]. Differences observed in peripheral blood lymphocyte subpopulations in children compared to adults have prompted similar studies in animals. From birth until weaning piglets, CD4 + "naive" T cells counts remain higher than CD8 + T cells. Our results are consistent with previous reports. Moreover, we found that the percentage of lymphocytes was significantly lower in neonates than in weaners. On the basis of this results, we suggest that diagnosis of the disease should be based on the size rather than the percentage of lymphocyte subpopulations, according to appropriate age-matched reference values.

Conclusion

Our study is the first to evaluate the differences in the structure and immune capabilities of the small intestine between neonatal and weaned piglets. Our findings suggest that the difference on the physical and immunological components of small intestines between neonatal and weaned piglets may be cause the susceptibility of neonatal piglets to infection. This study provides useful reference data on the immune mechanisms of the small intestine for future studies.

Abbreviations

IEL: Intraepithelial lymphocyte; PRR: Pattern recognition receptor; PEDV: Porcine epidemic diarrhea virus; H&E: Hematoxylin and eosin; PP: Peyer's patch; GC: Goblet cell; TLR: Toll-like receptor; RLR: RIG-I-like

receptor; PAMP: Pathogen-associated molecular pattern.

Declarations

Ethics approval and consent to participate

All efforts were made to minimize suffering. The animal protocol was approved by the University of Nanjing Agriculture University Committee on Animal Resources Committee (Permit Number: SYXK2011–0036).

Consent to publish

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing financial interests.

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Authors' Contributions

CY participated in design of the study, analyzed the data and prepared the manuscript. YJ and AUS carried out the experiments. EZ and PZ raised piglets and collected the samples, conducted the experiment. QY designed the study and revised the manuscript. All the authors read, revised, and approved the final manuscript.

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Tables

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Figures

Figure 1

Histological and morphometrical differences between the small intestines of neonatal and weaned piglets. Tissue sections were stained using hematoxylin and eosin and certain parameters were compared between neonatal and weaned piglets. a. Intestinal villus height. b. Intestinal crypt depth. c. Intestinal intraepithelial lymphocytes (→). d. Ileal Peyer's patches. Scale bars: 20 μm in (b-c) and 100 μm in (a and d). The certain parameters were calculated in 10 random fields. (n=10 per group). Differences were considered significant at (*) 0.01 < p < 0.05 and (**) p < 0.01.

Figure 2

Differences in intestinal barrier function between neonatal and weaned piglets. a. mRNA levels of claudin, E-cadherin, occludin, and ZO-1 in pig small intestine. All samples were tested in triplicate and the results are expressed as fold changes relative to the control animals. Data are presented as means $\pm$ standard errors. b and c. Immunohistochemical staining of claudin-3 positive cells and Occludin positive cell, respectively, Positive cells were quantified using densitometry analyses and were calculated in 10 random fields. (n=10 per group). Scale bars: 20 μ m. Differences were considered significant at (*) $0.01 < p < 0.05$ and (**) $p < 0.01$.

Figure 3

Expression of CD3+ T cells, SIgA-positive cells, goblet cells, and secretory cells. a-c. Immunohistochemical staining of CD3+ T cells, SIgA-positive cells, and goblet cells. d. Immunofluorescent staining of secretory cells in pig small intestine. Positive cells were quantified using densitometry analyses and were calculated in 10 random fields. (n=10 per group). Scale bars: 100 μ m. Differences were considered significant at (*) $0.01 < p < 0.05$ and (**) $p < 0.01$.

Figure 4

Differences in mRNA expression of PRR genes in neonatal and weaned piglet small intestines. a-c. The mRNA levels of TLR1, TLR2, TLR 3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, MDA5, and Rig-I in the intestinal mucosa was determined in each animal using a SYBR-green qRT-PCR. All samples were tested in triplicate and the results are expressed as fold changes relative to the control animals. Differences were considered significant at (*) $p < 0.05$ and (**) $p < 0.01$.

Figure 5

Differences in the number of CD3+, CD4+, and CD8+ T cells between neonatal and weaned piglets. The frequencies of CD3+, CD4+, and CD8+ T cells in the peripheral blood-mononuclear cells of neonatal and weaned piglets were analyzed by flow cytometry.

Supplementary Files

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