Regulatory role of L-proline in fetal pig growth and intestinal epithelial cell proliferation

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Abstract

L-proline (Pro) is a precursor of ornithine, which is converted into polyamines via ornithine decarboxylase (ODC). Polyamines play a key role in the proliferation of intestinal epithelial cells. The study investigated the effect of Pro on polyamine metabolism and cell proliferation on porcine enterocytes in vivo and in vitro. Twenty-four Huanjiang mini-pigs were randomly assigned into 1 of 3 groups and fed a basal diet that contained 0.77% alanine (Ala, iso-nitrogenous control), 1% Pro or 1% Pro with 1% Pro increased fetal weight; the protein and DNA concentrations of the fetal small intestine; and ODC protein abundances and polyamine concentrations in the fetal intestines and IPEC-J2 cells (P < 0.05). Supplementing Pro to either gilts or IPEC-J2 cells increased ODC protein abundances and polyamine concentrations in the fetal intestines and IPEC-J2 cells (P < 0.05). In comparison with the Pro group, the combined administration of Pro and DFMO reduced the expression of ODC protein and spermine concentration in the fetal intestine, as well as the concentrations of putrescine, spermidine and spermine in IPEC-J2 cells (P < 0.05). Meanwhile, the percentage of cells in the S-phase and the mRNA levels of proto-oncogenes c-fos and c-myc were increased in response to Pro supplementation, whereas depletion of cellular polyamines with DFMO increased tumor protein p53 (p53) mRNA levels (P < 0.05). Taken together, dietary supplementation with Pro improved fetal pig growth and intestinal epithelial cell proliferation via enhancing polyamine synthesis.

1. Introduction

Although arginine had been assumed to be the main precursor of ornithine in mammalian cells, there is no arginase expression in porcine placentas at all stages of gestation and in the enterocytes of neonatal pigs (Wu et al., 2005, 2013). Of particular note, Wu et al. (2000a, b, 2005, 2008) discovered that L-proline (Pro) is extensively catabolized into ornithine and polyamine via L-proline oxidase and ornithine decarboxylase (ODC) in the enterocytes of neonatal pigs and in the porcine placenta. Ornithine is
decarboxylated to putrescine by ODC, the rate-controlling enzyme for polyamine–biosynthesis in cells (Pietila et al., 1997; Seiler and Raul, 2007). Ornithine decarboxylase is rapidly responsive to conditions that change cell replication and differentiation (Slotkin et al., 2000), and its irreversible inhibitor is alpha-difluoromethylornithine (DFMO) (Bitonti et al., 1985).

The fetal pig intestine undergoes rapid growth and development during gestation (Grosse et al., 2011). Although the development of the gastrointestinal tract is stimulated by the transition from parenteral nutrition before birth (via the maternal placenta) to exclusively enteral nutrition after birth, maternal nutrition plays an important role in fetal intestinal growth (Sangild et al., 2002a, 2002b; Wu et al., 2006a,b). The supply of nutrients from mother to fetus is closely related to maternal nutrition and placental development. There are reports that dietary supplementation with Pro to gestating mice enhances fetal polyamine synthesis and embryonic/fetal survival (Liu et al., 2019a, b). Polyamines are necessary for the integrity of the intestinal mucosal barrier (Wang et al., 2015) and are important regulators of early mammalian embryogenesis, placental trophoblast growth, and embryonic development (Wu et al., 2004). Polyamine synthesis and concentrations in the porcine placenta are dynamic during gestation, with the highest polyamine concentration occurring at d 40 of gestation, declining between d 40 and 90 of gestation, and remaining at the lower levels through d 110 of gestation (Wu et al., 2005). However, the fetal intestinal mass and the activities of brush-border enzymes increase significantly from d 70 to 110 of gestation (McPherson et al., 2004), and the placental development of pigs is maximal by d 70 of gestation (Knight et al., 1977). Accumulation of polyamines during early gestation may promote the development of the fetal intestine during the late gestation (Kwon et al., 2003; Herring et al., 2018). Notably, d 70 of gestation is the starting time-point for fetal intestinal rapid development, and the maximal placental development at d 70 of gestation also indicated the optimal nutrients supply from maternal to fetal blood (Wu et al., 2004; Woods et al., 2018). Pro could promote the maturation of the mucosa and maintain intestinal integrity in early-weaned piglets (Wang et al., 2015), and similar results have been reported for polyamines (Liu et al., 2019b). However, whether Pro has a positive effect on promoting fetal intestinal development through supplementation to maternal diets is unknown. If Pro supplementation in early gestation has any benefit to fetal intestinal development at d 70 of gestation, it will be helpful to lay a foundation for the rapid intestinal growth in the late gestation. The Huanjiang mini-pig is one of the famous indigenous pig breeds in China, which is mainly distributed in the southern China (Zhao, 2018). The Huanjiang mini-pig is increasingly used as a suitable experimental model, because it has similar anatomical, physiological and metabolic characteristics to human (Yang et al., 2012). Many researchers have studied nutritional interventions, microbial metabolite composition, and amino acid profiles in pregnant Huanjiang mini-pig (Yang et al., 2012; Kong et al., 2016; Ji et al., 2017). Therefore, the present study was conducted with the Huanjiang mini-pig, as well as the intestinal porcine epithelial (IPEC-J2) cells, to test the hypothesis that Pro is beneficial for improving the intestinal and whole-body growth of fetal pigs.

2. Materials and methods

All animals used in this study were managed according to the Chinese Guidelines for Animal Welfare. The experimental protocol was approved by the Animal Care and Use Committee of the Chinese Academy of Sciences (Beijing, China).

2.1. Animals and experimental design

The study was conducted at the Research Center of Mini-pig, Huanjiang Observation and Research Station for Karst Ecosystems, Huanjiang, Guangxi. Thirty-six primiparous Huanjiang mini-pigs were fed the basal diet for the first 15 d after mating (mating twice in 1 d, with 12 h interval; the day of mating was considered as d 0 of gestation), and were randomly assigned into 3 groups (12 pigs per group), representing supplementation with 0.77% alanine (Ala, iso-nitrogenous control), 1% Pro, or 1% Pro + 0.0167% DFMO to the basal diet from d 15 to 70 of gestation. According to NRC (1998), the requirement of Pro for gestating sow is 0.67%. Here, 1% Pro was extra added to the Huanjiang mini-pig’s diet. Sows that were not pregnant (4 pigs per group) were culled to ensure that all pigs sacrificed on d 70 of gestation were pregnant. The basal diet was formulated to meet the nutrient requirements for gestating sows according to the breeding standards for Chinese local pigs (Table 1). The amount of added Pro was based on the results reported by Kirchgesser et al. (1995) and Wu et al. (2011). The dosage of DFMO was based on our preliminary study with gestating rats. Ala and Pro were obtained from Ajinomoto Inc. (Tokyo, Japan). DFMO was from Wuhan Dahua Pharmaceutical Co., Ltd (Wuhan, China). All animals were housed individually in 2 m × 3 m pens with scarified-cement flooring. Each pen was equipped with a feeder and a nipple drinker. The temperature of housing facilities was maintained from 22 to 28 °C. All pigs had free access to drinking water, and were fed their respective diets (0.85 kg/d from d 15 to 45 of gestation; 1.03 kg/d from d 46 to 70 of gestation) twice daily (09:00 and 18:00). Dietary supplementation was not initiated until d 15 of gestation, because adding 1% arginine-HCl to the basal diet (12% CP) on d 0 of gestation reduced maternal progesterone concentration and embryonic survival in gilts (Li et al., 2010) but such an adverse effect of arginine supplementation did not occur when it was initiated on d 14 of gestation (Li et al., 2014). Wu et al. (2013) suggested that the increased production of nitric oxide (NO) through arginine supplementation may reduce the number of follicles that ovary and the concentrations of progesterone in maternal plasma. In current study, extra supplementation of 1% Pro may promote arginine de novo synthesis and, thereby increase NO production by arginine.

| Item | Content |
|------|---------|
| Ingredients | |
| Corn | 54.0 |
| Soybean meal | 12.0 |
| Rice bran | 30.0 |
| Premix1 | 4.0 |
| Total | 100.0 |
| Chemical composition2 | |
| Digestible energy3, kcal/kg | 3,202 |
| Crude protein | 12.04 |
| Calcium | 0.78 |
| Phosphorus | 0.62 |
| Lysine | 0.53 |
| Arginine | 0.65 |
| i-proline | 0.67 |

1 Premix provided for 1 kg of complete diet: vitamin A, 10,200 IU; vitamin D, 1,600 IU; vitamin E, 75 IU; vitamin K3, 75 mg; vitamin B1, 3 mg; vitamin B2, 16 mg; vitamin B6, 3 mg; vitamin B12, 0.8 mg; niacin, 69 mg; p-pantothenic acid, 42 mg; folic acid, 4 mg; biotin, 1 mg; choline, 900 mg; Fe (FeSO4), 150 mg; Cu (CuSO4), 11.2 mg; Zn (ZnO), 63 mg; Mn (MnO), 32 mg; I, 1.5 mg; Co (CoSO4), 0.3 mg; Se (Na2SeO3), 0.25 mg; Ca, 200 mg; P, 20 mg.
2 Analyzed unless indicated otherwise.
3 Calculated.
catabolism. Gestating sows were fed based on 3% of their average BW (28.4, 34.05 and 43.2 kg on d 15, 45 and 70 of gestation, respectively).

On d 70 ± 1.78 (mean ± SD) of gestation, 24 pregnant sows (8 pigs per group) were electrically stunned and sacrificed. The fetal weight and number of fetuses per litter were determined. The whole small intestine and large intestines (the contents were not removed) from fetuses were washed in ice-cold physiological saline then collected, snap-frozen in liquid nitrogen, and stored at −80 °C for polyamine determination, RNA extraction, and Western blot analysis.

2.2. Fetal intestinal DNA and protein assay

About 0.5-g small or large intestine was homogenized with 5-mL DNA20L (for DNA assay) or dd H2O (for protein assay), and the supernatant fluid was obtained following centrifugation (10,000 × g 10 min for DNA assay; 8,000 × g, 10 min for protein assay). The fetal intestinal DNA content was determined according to the description of Anwar et al. (1988). Standards of calf thymus DNA was supplied by Sigma (Sigma–Aldrich Co., St Louis, MO). Protein concentration was measured using the bicinchoninic acid assay method with bovine serum albumin (BSA) as the standard (Beyotime Institute of Biotechnology, Shanghai, China). Data are expressed as milligram per gram wet weight of organ.

2.3. Cell culture and treatment

The sources of the reagents used for cell culture were the same as those we described previously (Tan et al., 2010). Fetal bovine serum (FBS), high glucose (25 mmol/L) Dulbecco’s modified Eagle medium–high glucose (DMEM-H), and antibiotics were purchased from Thermo Scientific (Waltham, MA, USA). Plastic culture plates were manufactured by Corning Inc. (Corning, NY, USA). Unless indicated, all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

IPEC-J2 cells were obtained from Guang Zhou Jennio Biotech Co., Ltd (Guangzhou, China). IPEC-J2 cells were seeded and cultured with DMEM-H medium containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a 5% CO2 incubator. After overnight incubation, the cells were cultured in a basal medium (DMEM-H medium with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin) containing 0 µmol/L Pro, 400 µmol/L Pro, or 400 µmol/L Pro + 10 mmol/L DFMO for 4 d, and the medium was changed every 2 d. The optimum time and the dosages of Pro and DFMO for cell growth were chosen on the basis of cell viability (Appendix Fig. 1).

2.4. Cell viability and cycle assay

About 0.8 × 104 cells per well of IPEC-J2 cells were seeded in 96-well plates and grown as usual (Tan et al., 2010). After culture with 200, 300, 400, 500, or 600 µmol/L Pro, or with 2, 5, 8, 10, or 12 mmol/L DFMO for 2 or 4 d, the cells were washed by phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA) and a fresh basal medium was replaced. Cell Counting Kit–8 (CCK-8, Dojindo, Beijing, China) was added to each well, incubated for 2 h, and read on the spectrophotometer at 450 nm; the measured absorbance was proportional to the number of viable cells.

IPEC-J2 cells (8 × 106 per well) were seeded in 6-well cell culture plates for determination of cell-cycle progression. IPEC-J2 cells were cultured as described above and dissociated by trypsin–EDTA–ethylenediaminetetraacetic acid (EDTA, Invitrogen, Grand Island, NY, USA) separately. About 1 × 106 cells were pelleted by centrifuging at 1,000 × g for 5 min. The supernatant fluid was removed and the cell pellet was washed with ice-cold PBS twice, and 1 mL of 70% cold ethanol was slowly added during vigorous mixing. Samples were stored at 4 °C. To assess cell cycle, harvested cells were stained with propidium iodide (PI; Key GEN, Nanjing, China) according to the manufacturer’s instructions. Cell cycle arrest was analyzed by flow cytometry (BD FACScalibur, USA). Fluorescence of PI was monitored at 617 nm.

2.5. Determination of polyamines in the intestine and IPEC-J2 cells

Polyamines were quantified according to a modified method described by Xu et al. (2014). Briefly, 0.01 g samples of intestine were acidified with 2 mL of 0.4 mol/L HClO4, and the supernatant fluid was obtained following centrifugation (8,000 × g for 10 min). IPEC-J2 cells (15 × 104 per dish) were seeded in 10-cm dishes, and after a 4-d period of culture in a DMEM-H medium containing 0 µmol/L Pro, 400 µmol/L Pro, or 400 µmol/L Pro + 10 mmol/L DFMO, cells were collected with 700 µL of 0.4 mol/L HClO4 separately. The supernatant fluid was obtained following centrifugation (8,000 × g for 10 min). Derivatization of polyamines was performed with 1 mL of NaOH, saturated Na2CO3 buffer (pH 10.6) and 1 mL of dansyl chloride (10 mg/mL), avoiding a light reaction in a 40 °C water bath for 30 min. Dansylated derivatives were then extracted with 1 mL of ammonia (5%; vol/vol) and 3 mL of absolute ether, and subsequently dried through refrigerated centrifugation (CR22 GII, HITACHI, Japan) before being dissolved in 600-µL methyl alcohol (HPLC grade). Polyamines were quantified by high-performance liquid chromatography using a reverse-phase column (Agilent 1260, Agilent, USA). Polyamine standards (including putrescine, spermidine, and spermine) were purchased from Sigma Chemical Co. (USA).

2.6. Real-time quantitative PCR

The expressions of ODC, potassium voltage-gated channel, shaker-related subfamily, member 1 (Kv1.1) and member 5 (Kv1.5) mRNA in the small and large intestines of fetuses and the expression of tumor protein p53 (p53), and proto-oncogenes c-fos and c-myc mRNA in the IPEC-J2 cells were determined by real-time quantitative PCR according to the procedure described by Wang et al. (2015). Primers are shown in Table 2 and β-actin was used as a housekeeping gene to normalize target gene transcript levels. The comparative Ct value method was used to quantify expression levels of target genes relative to those of β-actin.

Table 2

| Gene            | Accession no. | Primers                   |
|-----------------|---------------|---------------------------|
| β-Actin         | XM_0031242803 | F: 5′-GGATGCGACAGGGAGATCGACG-3′<br>R: 5′-ACTCTGCTGAAATTGGTACAG-3′ |
| ODC             | NM_0011229883 | F: 5′-TGTACATGCTTCAAGCGCTGCTG-3′<br>R: 5′-AGTACATTACGCTGAACAGCAGCT-3′ |
| Kv1.1           | XM_003135537.1 | F: 5′-TITTTAGACACTTGGTTAGACG-3′<br>R: 5′-ATGGACACCTGAGAAGATCG-3′ |
| Kv1.5           | NY655585.1    | F: 5′-GCCGTGTTCAACTTCTCTTCTC-3′<br>R: 5′-CTGTGCTTCTGTTGTTGTTG-3′ |
| p53             | NM_213824.3   | F: 5′-CTGGCTTACTGCAAACACAC-3′<br>R: 5′-GGTCTGTTATGCTAAATAC-3′ |
| c-fos           | NM_00112311.1 | F: 5′-GCAGCTACTGACACCTCA-3′<br>R: 5′-GCCATGTCACTGCTCAAGAC-3′ |
| c-myc           | NM_001005154.1| F: 5′-CCGACACCGAGGAGAATGC-3′<br>R: 5′-CTGGCTTTTACCTGGTTTC-3′ |

ODC – ornithine decarboxylase; Kv1.1 – potassium voltage-gated channel, shaker-related subfamily, member 1; Kv1.5 – potassium voltage-gated channel, shaker-related subfamily, member 5; p53 – tumor protein p53; c-fos and c-myc are proto-oncogenes; F = forward; R = reverse.
2.7. Western blot analysis

The abundances of ODC protein in the IPEC-J2 cells and the small and large intestine of fetuses were determined by Western blot analysis as described by Wang et al. (2016). The following antibodies were used for protein quantification: ornithine decarboxylase (ODC; 1:500; Bioss Inc., Beijing, China), and β-actin (1:1,000; Cell Signaling Technology, USA) as well as secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit IgG; 1:5,000; Boster Biological Technology, Wuhan, China). The images were detected by chemiluminescence (Millipore, Billerica, MA). Western blots were quantified by measuring the intensity of correctly sized bands using software (Alpha Imager 2200 Software; Alpha Innotech Corporation, San Leandro, CA, USA) and protein measurement was normalized to β-actin.

2.8. Statistical analysis

All statistical analyses were conducted by one-way ANOVA using IBM SPSS Statistics version 23. Tukey–Kramer multiple comparison procedure was used for Post-hoc comparisons. The Kruskal–Wallis test was used when data was not normally distributed. The Pearson correlation coefficients were evaluated to describe the relationships between the cell percentage in 5 phage and polyamine concentrations in IPEC-J2 cells. Differences were declared as statistically significant at $P < 0.05$. Data are expressed as means ± SEM.

3. Results

3.1. Fetal number and weight

Maternal dietary Pro supplementation enhanced ($P < 0.05$) the average fetal weight and total fetal weight per litter at d 70 of gestation, compared with those in the Ala-supplemented group. There was no difference in the number of fetuses per litter among the 3 treatment groups (Table 3).

3.2. DNA and protein contents in the fetal intestine

As shown in Table 4, maternal dietary Pro supplementation increased ($P < 0.05$) DNA and protein concentrations in the fetal small intestine, but had no effect on DNA or protein concentrations in the fetal large intestine.

3.3. Kv1.1, Kv1.5 and ODC mRNA levels in the fetal intestine

Kv1.1, Kv1.5 and ODC mRNA expression levels in the fetal intestine are shown in Fig. 1. Compared with the Ala group, Pro addition enhanced ($P < 0.05$) Kv1.1 mRNA expression level in the small and large intestines, whereas the combined addition of Pro and DFMO decreased ($P < 0.05$) Kv 1.1 mRNA levels in the fetal small intestine. There was no difference in Kv1.5 or ODC mRNA abundances in the small and large intestines among the Ala, Pro, or Pro + DFMO groups.

3.4. Polyamine metabolism in the fetal intestine

ODC is a rate-controlling enzyme in the biosynthesis of polyamines, and its sole function is to catalyze the decarboxylation of ornithine to putrescine (Igarashi and Kashiwagi, 2010). To further explore polyamine synthesis in the fetal intestine, we determined ODC protein expression as well as putrescine, spermidine, and spermine concentrations in the fetal small and large intestines. The relative abundances of ODC protein in the small and large intestines are shown in Fig. 2. Dietary DFMO supplementation reduced ($P < 0.05$) ODC protein levels in the fetal small and large intestines, compared with the Pro group. The ODC protein abundance in the fetal small intestine of Pro-supplemented pigs was higher ($P < 0.05$) than that in the Ala group. Polyamine concentrations in the fetal intestine are presented in Table 5. Maternal dietary supplementation with Pro during pregnancy markedly increased ($P < 0.05$) the concentrations of putrescine in the small and large intestines, as well as spermidine in the large intestine, compared to the Ala group, whereas Pro + DFMO treatment decreased ($P < 0.05$) spermine concentrations in the fetal small intestine.

3.5. Cell viability of IPEC-J2 cells

The viability of IPEC-J2 cells treated with different concentrations of DMFO for 2 or 4 d is illustrated in Appendix figure A. Addition of 2, 5, 8 mmol/L DMFO to culture medium did not affect IPEC-J2 cell viability, but 10 or 12 mmol/L DMFO decreased cell viability on d 2 ($P < 0.05$). On d 4, DMFO reduced cell viability in a dose-dependent manner (from 5 to 12 mmol/L) ($P < 0.05$). Pro addition has no effect on cell viability on d 2, whereas enhancing cell viability on d 4 in a dose-dependent manner, with 400 μmol/L Pro showing the best promotion effect ($P < 0.05$) (Appendix figure B).

3.6. Polyamine metabolism in the IPEC-J2 cells

The protein level of ODC and polyamine concentrations in IPEC-J2 cells are shown in Fig. 3 and Table 6, respectively. Pro at 400 μmol/L increased ($P < 0.05$) ODC protein expression in IPEC-J2 cells. Combined addition of 400 μmol/L Pro and 10 mmol/L DFMO remarkably inhibited ($P < 0.05$) ODC protein expression, but 5 mmol/L or 8 mmol/L DFMO supplementation did not affect ODC expression in IPEC-J2 cells, compared with the 0 and 400 μmol/L Pro groups. According to results on cell viability and ODC protein

| Table 3 | The number and weight of fetal pigs (n = 8 litters). |
|---------|-------------------------|
| Item    | Treatment | SEM | P-value |
|---------|-----------|-----|---------|
| 0.77% Ala | 1% Pro | 1% Pro + 0.0167% DFMO |         |
| Average fetal weight, g | 101.54b | 120.75a | 114.57ab | 2.825 | 0.013 |
| Fetal number per litter | 7.00 | 7.75 | 7.75 | 0.282 | 0.479 |
| Total fetal weight per litter, g | 400 | 425 | 414 | 33.483 | 0.020 |

Ala = alanine; Pro = l-proline; DFMO = α-difluoromethylornithine. a, b Values with different letters within the same row are significantly different ($P < 0.05$).
abundance, we selected 400 \(\mu\)mol/L Pro and 10 mmol/L DFMO to do the follow-up experiments. As shown in Table 6, the spermidine and spermine concentrations in IPEC-J2 cells were increased by 400 \(\mu\)mol/L Pro \((P < 0.05)\). However, the combined addition of 400 \(\mu\)mol/L Pro and 10 mmol/L DFMO decreased putrescine, spermidine, and spermine concentrations in cells, compared with the other 2 groups \((P < 0.05)\) Table 7.

3.7. Cell cycle progression of IPEC-J2 cells

The results on cell cycle progression are presented in Fig. 4. Flow cytometry analysis showed that the percentage of cells in the S phase was increased in the 400 \(\mu\)mol/L Pro group \((P < 0.05)\), compared with the DFMO group \((P < 0.05)\). Meanwhile, correlation analysis showed that the S phase cell percentage had a high positive correlation with putrescine \((r = 0.0862, P < 0.05)\), spermidine \((r = 0.0962, P < 0.05)\) and spermine \((r = 0.0971, P < 0.05)\) levels in IPEC-J2 cells.

3.8. p53, c-fos, c-myc mRNA levels in IPEC-J2 cells

In Fig. 4C, Pro treatment remarkably increased \((P < 0.05)\) the relative mRNA levels of c-fos and c-myc in IPEC-J2 cells, compared with the other 2 groups. The p53 mRNA levels in the 400 \(\mu\)mol/L Pro + 10 mmol/L DFMO groups were higher \((P < 0.05)\) than those in the control group.

4. Discussion

Available evidence shows that a lower birth weight is associated with high rates of neonatal morbidity and mortality, low efficiency of food utilization, as well as permanent stunting effects on postnatal growth and development (Fowden et al., 2006; Wu et al., 2006a,b). Therefore, fetal growth and development during pregnancy is very important. In the current study, maternal dietary supplementation with Pro enhanced the average fetal weight and total fetal weight per
litter at d 70 of gestation compared with the control group. This result is consistent with the report by Wu et al. (2008). However, maternal Pro supplementation did not increase the number of fetuses, which may be due to: (1) sufficient Pro and arginine intake from the basal diet, (2) failure of development of conceptuses during the peri-implantation period, resulting in embryonic losses in early gestation (Wu et al., 2013), (3) the limited uterine capacity of Huanjiang mini-pigs, and (4) a delay in initiating Pro supplementation. Note that: (1) the feed intake of gestating sows in the present study, rather than d 14 of gestation (Li et al., 2014); the study was restricted (3% of sows’ body weight), but was about 80% greater than that reported for gilts used in the work of Li et al. (2014); and (2) Pro supplementation was started on d 15 of gestation in the present study, rather than d 14 of gestation (Li et al., 2014).

Mucosal growth is associated with cell proliferation and differentiation, which are characterized by the increased capacity for protein, DNA, and RNA synthesis in mucosal epithelia (Morisset, 1993). Dietary supplementation with Pro in pregnant sows increased protein and DNA concentrations in the small intestine of fetal pigs, in vitro cell culture, whereas IPEC-J2 cell viability was elevated by addition of 400 μmol/L Pro to culture medium but reduced by addition of 10 mmol/L DFMO. In young pigs, Pro promoted protein and DNA syntheses, as well as cell proliferation, in association with an increase in intestinal polyamine concentrations (Wu et al., 2010a, b). Peulen et al. (2000) reported that exogenous spermine could enter the epithelial cells of the small intestine and have a direct effect on stimulating their syntheses of protein, DNA and RNA. Similarly, the proliferation of intestinal epithelial cells is increased by polyamines but repressed by polyamine depletion in vivo and in vitro (Rathor et al., 2011; Wang, 2007). In our present study, either in vivo administration of Pro or in vitro addition with Pro increased polyamine concentrations and ODC protein abundance in enterocytes. Notably, adding 10 mmol/L DFMO to culture medium for IPEC-J2 cells depleted the cellular polyamines. Likewise, incubation of IEC-6 cells with DFMO for 4 d depleted the cells of putrescine and spermidine, and reduced intracellular spermine concentration by 60% (Pfeffer et al., 2001). Supplementation with DFMO to maternal diets decreased spermine concentration in the fetal small intestine compared with the Pro group, but did not affect intestinal putrescine and spermidine concentrations. It is possible that the dosage of DFMO supplementation is low. Many studies on DFMO intervention were conducted in mice (0.5% to 2% wt/vol DFMO; Ignatenko et al., 2008; Imamura et al., 2016; Kim et al., 2017), rats (500 mg/kg DFMO; Slotkin et al., 2000) or in vitro cell culture (0.5 to 20 mmol/L DFMO; Cui and Kim, 2005; Desforges et al., 2013; Lin et al., 2010). It is possible there are species differences in the response of animals to dietary DFMO supplementation because of variations in their metabolic patterns (Wu, 2018).

To our knowledge, this is the first time DFMO was supplemented to swine. An effect of DFMO on reducing the polyamine pool would have occurred if the dose of DFMO was increased. Polyamine homeostasis is regulated by feedback loops, including regulation of polyamine synthesis, uptake, release, and catabolism under physiological conditions (Seiler and Raul, 2007). When Pro was supplemented to gestating sows, ornithine is synthesized directly from Pro (Wu et al., 2008) and indirectly the conversion of Pro into arginine via interorgan metabolism of amino acids (Wu and Morris, 1998). The maternal tissues of sows can hydrolyze arginine into

| Item                                      | Treatment               | SEM   | P-value |
|-------------------------------------------|-------------------------|-------|---------|
| Small intestine, μg/g small intestine     | 0.77% Ala 1% Pro 1% Pro + 0.0167% DFMO |       |         |
| Putrescine                                | 3.17ab                  | 4.38a | 1.193 0.001 |
| Spermidine                                | 83.25                   | 86.13 | 10.701 0.690 |
| Spermine                                  | 94.77bc                 | 99.48a| 1.063 0.010 |
| Large intestine, μg/g large intestine     | 5.12c                   | 7.07a | 0.224 < 0.001 |
| Putrescine                                | 101.72ab                | 124.64a| 3.395 0.020 |
| Spermidine                                | 126.65                  | 135.38| 2.954 0.430 |

Ala = alanine; Pro = L-proline; DFMO = α-difluoromethylornithine.

a, b Values with different letters within the same row are significantly different (P < 0.05).

Fig. 3. Ornithine decarboxylase (ODC) protein abundances in IPEC-J2 cells as determined by Western blot analysis. (A) Representative relative ODC protein expression histogram of IPEC-J2 cells cultured in DMEM–H medium containing 400 μmol/L Pro, 400 μmol/L Pro + 5, 8 or 10 mmol/L DFMO for a 4-d period. (B) Lane 1, control group; Lane 2, 400 μmol/L Pro group; Lane 3-1, 400 μmol/L Pro + 5 mmol/L DFMO group; Lane 3-2, 400 μmol/L Pro + 8 mmol/L DFMO; Lane 3-3, 400 μmol/L Pro + 10 mmol/L DFMO. IPEC-J2 cells — intestinal porcine epithelial cells; Pro — L-proline; DFMO — α-difluoromethylornithine. Data, expressed as the relative values to those for cells in the control group, are means ± SEM, n = 6. a, b Bars sharing different letters differ at P < 0.05, as analyzed by one-way ANOVA. **, P < 0.05; ††, P < 0.01, as analyzed by independent T-test analysis between control and Pro groups.
ornithine via the action of arginases I and II, and the resulting ornithine is available for utilization by the conceptus (Wu et al., 2018). The ODC pathway has a key role in polyamine regulation and an increase in ODC activity is usually associated with a rapid accumulation of cellular polyamines (Lin et al., 1998). Collectively, these results support the view that Pro is a major source of polyamines in the porcine fetal intestine (Wang et al., 2016; Wu et al., 2008).

Polyamines not only promote the proliferation of intestinal cells but also stimulate early mucosal restitution in rats (Wang and Johnson, 1991) and cell migration in vitro cell models (Rao et al., 2007). Polyamines also regulate intestinal epithelia restitution and migration through Ca\(^{2+}\) signaling by activating K\(^+\) channels (Rao et al., 2012). We failed to detect a change in cytosolic free Ca\(^{2+}\) concentration in the porcine fetal intestine or IPEC-J2 cells, but analyzed the expression of Kv channels of the fetal intestine. Supplementation with exogenous spermine or overexpression of ODC could stimulate the activity of Kv channels (Rao et al., 2001, 2012), and increasing the expression of Kv channels could elevate cytosolic free Ca\(^{2+}\) concentration by driving Ca\(^{2+}\) influx (Rao et al., 2012; Wang et al., 2000). We found in current study that the levels of Kv1.1 mRNA in the small and large intestines were increased in response to maternal dietary Pro supplementation, but the Kv1.1 mRNA was decreased in the DFMO-treated group. Rao et al. (2001, 2012) and Seiler and Raul (2007) reported the similar finding that polyamines regulate the expression of Kv channels, whereas an inhibition of polyamine synthesis by treatment with DFMO decreases Kv channel gene expression.

In addition, polyamines have a positive effect on procession through the cell cycle (Greene et al., 2013). Our results show that IPEC-J2 cells in the S-phase were increased in response to Pro supplementation and had a high positive correlation to the increased cellular polyamine concentrations, suggesting that Pro may stabilize DNA and promote protein synthesis through enhancing intracellular polyamine concentrations (Tan et al., 2015). A series of observations from previous studies (Liu et al., 2005; Patel

### Table 6

| Item       | Control | 400 µmol/L Pro | 400 µmol/L Pro + 10 mmol/L DFMO | SEM | P-value |
|------------|---------|----------------|-------------------------------|-----|---------|
| Putrescine | 4.66a   | 4.37a          | 0.51b                         | 0.68 | < 0.001 |
| Spermidine | 2.52b   | 3.20b          | 0.00c                         | 0.49 | < 0.001 |
| Spermine   | 4.98b   | 7.39b          | 2.04c                         | 0.79 | 0.002   |

IPEC-J2 cells = intestinal porcine epithelial cells; Pro = l-proline; DFMO = a-difluoromethylornithine. 
*Values with different letters within the same row are significantly different (P < 0.05).

### Table 7

Pearson correlation coefficients (r) between S phase cell percentage and polyamine concentrations in IPEC-J2 cells (n = 8).

| Item       | Putrescine | Spermidine | Spermine |
|------------|------------|------------|----------|
| r          | r          | r          | r        |
| S%         | 0.0868     | 0.0962     | 0.0971   |

IPEC-J2 cells = intestinal porcine epithelial cells; S% = S phase cell percentage of IPEC-J2 cells.

![Fig. 4](image_url)

**Fig. 4.** Cell cycle progression of IPEC-J2 cells analyzed using cell flow cytometry. (A) Representative flow-cytometry diagrams of cells treated with 400 µmol/L Pro, 400 µmol/L Pro + 10 mmol/L DFMO for a 4-d period. (B) The percentage of cell populations in each phase of the cell cycle. (C) Relative expressions of p53, c-fos and c-myc mRNA in IPEC-J2 cells determined by real-time quantitative PCR analysis. IPEC-J2 cells = intestinal porcine epithelial cells; Pro = l-proline; DFMO = a-difluoromethylornithine; Dip G1 = IPEC-J2 cells in G1 phase; Dip G2 = IPEC-J2 cells in G2 phase; Dip S = IPEC-J2 cells in S phase. Data are expressed as means ± SEM, n = 6. a,b Bars sharing different letters differ P < 0.05.
and Wang, 1997; Wang et al., 1993) demonstrate that polyamines stimulate cell proliferation at least partly through their ability to regulate proto-oncogene expression. In the current study, pro administration was shown to upregulate the mRNA expression of c-fos and c-myc, which was associated with the increased percentage of cells in the S phase. Wang et al. (1993, 1994) suggested that increased expression of proto-oncogenes is related to early modulation of mucosal growth and may mediate polyamine-stimulated increase in cell division and proliferation. Not of note, an inhibition of polyamine synthesis by DFMO induced an increase of p53 mRNA expression in IPEC-J2 cells. Expression of the p53 gene is highly regulated by the cell according to its state of growth, activation of the p53 gene expression induces growth arrest, apoptosis, or both (Appella and Anderson, 2001; Slei et al., 2004). There is evidence showing that polyamine negatively affects the gene expression of p53, and polyamine depletion significantly increases the p53 gene expression (Li et al., 1999, 2001; Wang, 2007).

5. Conclusion

Supplementing 1% Pro to the maternal diet of gestating Huanjiang mini-pigs enhanced fetal weight, DNA and protein concentrations, as well as K+ channel expressions in the fetal intestine. Our in vivo and in vitro results showed that Pro promoted polyamine synthesis by modulating ODC protein expression, and played an important role in cell-cycle progression and cell proliferation in enterocytes. Depletion of cellular polyamines by exposure to DFMO decreased small-intestinal Kv 1.1 mRNA levels and cell viability. Taken together, results of the current study indicated that Pro, as a functional amino acid for fetal animals, was beneficial in stimulating fetal growth and intestinal epithelial cell proliferation via modulating polyamine synthesis and availability.

Author contributions

Jing Wang: investigation, visualization, writing - original draft; Bi’e Tan: supervision; Jianjun Li: data curation; Xiangfeng Kong: conceptualization, methodology; Minjie Tan: resources; Guoyao Wu: writing - review & editing.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can appropriately influence our work. There is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgements

This study was supported by National Key R&D Program (2017YFD0500503), Postdoctoral Science Foundation (BX20180096), Hunan Province Science and Technology Projects (2017RS059) and Innovation Province Project (2019RS3021) in China, as well as Texas A&M AgriLife Research (H-8200) in USA.

Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2020.07.001.

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