Dietary L-arginine supplementation enhances intestinal development and expression of vascular endothelial growth factor in weanling piglets

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Abstract

Oral administration of L-arginine has been reported to prevent gut disease in human infants. However, little is known about the effects of dietary arginine supplementation on intestinal development of weaned piglets. In the present study, twenty 21-d-old castrated piglets with 5·3 (SEM 0·13) kg body weight (BW) were weaned from sows, individually housed and randomly assigned to one of the two maize- and soyabean meal-based diets supplemented with 0 or 1 % L-arginine. After consuming the diets for 7 d, six pigs were randomly selected from each group to obtain various tissues. Compared with control pigs, dietary supplementation with 1 % L-arginine did not affect feed intake but enhanced ($P<0·05$) the relative weight of the small intestine (+33 %), daily BW gain (+38 %) and feed efficiency (+28 %). The villus height of the duodenum, jejunum and ileum in arginine-supplemented piglets was 21, 28 and 25 % greater ($P<0·05$) than in the non-supplemented control group. Arginine supplementation increased ($P<0·05$) protein levels for vascular endothelial growth factor (VEGF) in duodenal, jejunal and ileal mucosae by 14, 39 and 35 %, respectively. Compared with the control group, dietary supplementation with 1 % L-arginine increased ($P<0·05$) plasma concentrations of arginine and insulin (+36 %), and decreased ($P<0·05$) plasma concentrations of cortisol (−33 %), NH_3 (−21 %) and urea (−19 %). These results indicate that arginine supplementation enhances intestinal growth, development and expression of VEGF in early-weaned pigs fed a maize- and soyabean meal-based diet. The findings may have important implications for neonatal pigs under stressful or diseased conditions.

Key words: Arginine: Pigs: Growth: Intestine: Vascular endothelial growth factor

Arginine participates in multiple pathways with enormous nutritional and physiological importance, including the synthesis of protein, NO, creatine, proline, glutamate, polyamines and agmatine as well as the secretion of hormones[1–6]. Increasing evidence shows that L-arginine is an essential amino acid for young mammals, particularly under conditions of severe stress[7,8]. For instance, our previous studies have shown that dietary supplementation with arginine, which is 50–95 % greater than National Research Council-recommended intake, stimulates muscle growth[1,9], enhances the immune status[5] and improves microvascular development[10] in neonatal pigs weaned at 7–21 d of age. Particularly, NO (a metabolite of

L-arginine) is a major vasodilator that regulates vascular tone and haemodynamics. Proline (another product of arginine catabolism) is a key component of extracellular matrix collagen that is crucial for angiogenesis and vascular remodelling[11,12]. Systemic administration of L-arginine has been proposed as a safe and effective method to enhance the synthesis of NO, proline and polyamines in animals, therefore improving wound healing and microcirculation[13–15].

Weaning stress is associated with reductions in food consumption, weight gain and growth, as well as increases in the incidence of diarrhoea and disease, intestinal dysfunction and atrophy, and deaths in piglets[16–22]. In response

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to weaning, the digestive system of piglets must adapt to a dry diet, which is often based primarily on plant sources of ingredients (e.g. maize and soyabean meal)[23,24]. In the piglet small intestine, microvessels are present mainly in the mucosa and submucosa[25,26], and the optimal development of villi depends on an adequate supply of nutrients from both blood via the intestinal microvessel and enteral feeding[27]. Changes in gut morphology may result in microvessel injury and disorders of microcirculation[28,29]. Microvascular endothelial dysfunction may be a major factor contributing to impaired absorption and transport of nutrients in animals[30].

Of particular interest, weanling piglets have a particularly high requirement for dietary arginine[31]. Due to depressed feed intake in the first week post-weaning, low arginine intake may be one of the reasons for increased intestinal epithelial damage in early-weaned pigs. Adverse effects of an L-arginine deficiency may also include abnormal gene expression in the vasculature[10,11]. Therefore, we hypothesised that dietary L-arginine supplementation might prevent or alleviate intestinal atrophy and microcirculation disorder by increasing the expression of vascular endothelial growth factor (VEGF) in the small intestine of weanling pigs. The present study was conducted to test this hypothesis using 21- to 28-d-old piglets.

Materials and methods

Animals and feeding

We conducted the experiment in accordance with the Chinese guidelines for animal welfare, and it was approved by the Animal Welfare Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences[32].

Basal diets were formulated to meet National Research Council-recommended nutrient requirements for weanling piglets[33]. Two diets were formulated by supplementing the basal diet with 0 and 1% L-arginine (free base). The diets were made isonitrogenous by the addition of L-alanine, as described by Kim & Wu[34].

A total of twenty large White × Landrace castrated piglets from five litters were weaned at 21 d of age (5·3 (SEM 0·13) kg) and assigned randomly to one of the two treatment groups (ten pigs/group), representing dietary supplementation with 0·0% control or 1% L-arginine (Table 1). Piglets were individually housed in 1·3 × 1·2 m pens with slotted stainless steel floors[35]. Each pen was equipped with a feeder and a nipple waterer. This facility allows pigs to have free access to feed and water. The room temperature was maintained at 25–27°C[36]. All the piglets were fed four times per day at 07.00, 11.00, 15.00 and 19.00 hours. The feed intake was measured daily. Fresh manure samples were collected daily for the determination of moisture. Samples with moisture content higher than 70% were considered

Table 1. Composition of the basal diet (as-fed basis)

	Content (%)
Dietary ingredients	
Maize	50·00
Soyabean meal	20·00
Whey	10·00
Fish meal	6·00
Soyabean oil	2·50
Lactose	2·50
Sucrose	2·00
Glucose	1·80
L-Lys.HCl	1·30
Salt	0·30
Limestone	0·80
CaHPO$_4$	1·80
Premix*	1·00
Nutrient levels in the diet†	
Digestible energy (MJ/kg)	14·44
Crude protein (%)	23·50
Total Arg (%)	1·14
Total Lys (%)	1·35
Total Ca (%)	0·80
Total P (%)	0·65

* Supplied per kg diet: Cu, 10 mg; Fe, 100 mg; Na, 0·30 mg; Zn, 100 mg; Mn, 10 mg; cholecalciferol, 386 IU; retinyl acetate, 3086 IU; all-*rac*-α-tocopheryl acetate, 15·4 IU; menadione, 2·3 mg; riboflavin, 3·9 mg; D-pantothenic acid, 15·4 mg; niacin, 23 mg; choline, 77 mg; cyanocobalamin, 15·4 μg.
† Analysed values.

as diarrhoea. The incidence of diarrhoea for the piglets was calculated as[18]:

Incidence of diarrhoea =

((number of piglets with diarrhoea

× number of days of diarrhoea)/(total number of piglets

× number of days of experiment)) × 100.

Sample collection

Following a 7 d period of arginine supplementation, all piglets were weighed, and at 1 h after the last meal, jugular venous blood samples were obtained from heparinised tubes for the analysis of hormones and metabolites, whereas six pigs from each group were randomly selected and humanely killed by a lethal intraperitoneal injection of sodium pentobarbital[4]. Samples of duodenum, jejunum, ileum, liver, kidney, heart, spleen and lung were obtained immediately after the abdomen was opened[37].

Analysis of metabolites and hormones

Amino acids in plasma were analysed using a HPLC method involving precolumn derivatisation with ortho-phthaldialdehyde[38]. L-Norvaline was used as an internal standard. An automated biochemistry analyzer (Synchron CX Pro; Beckman Coulter, Fullerton, CA, USA) was used to determine concentrations of urea, NH$_3$, glucose, total protein and immune globulins in plasma, according to

commercial kits and the manufacturer's instructions[39,40]. Cortisol, insulin, growth hormone, insulin-like growth factor-1, triiodothyronine and thyroxine in plasma were determined by RIA according to reagent kits and the manufacturer's instructions (China Institute of Atomic Energy, Beijing, China)[41–43].

Histological analysis

Intestinal tissue samples were fixed as described previously[11]. Briefly, samples were placed in 10% neutral buffered formalin and embedded in paraffin for subsequent histological measurement. Six cross-sections were obtained from each formalin-fixed segment and processed for histological examination using the standard haematoxylin and eosin method[11]. Villus height and crypt depth were measured according to Wu et al.[44,45]. The histological analysis was performed by an investigator who was unaware of the origin of tissue sections.

Immunohistochemistry

Protein levels for VEGF were measured as described previously[11]. Briefly, sections of formalin-fixed paraffin-embedded tissues were digested with 3% H_2O_2 for 20 min at room temperature and incubated sequentially with 10% normal rabbit serum for 20 min after microwave antigen recovery, with VEGF (1:50) at 4°C overnight and then with corresponding biotinylated secondary antibodies against rabbit and streptavidin peroxidase. Subsequently, binding of the primary antibody was detected with diaminobenzidine. Sections were counterstained with haematoxylin. In the negative control, the antibodies were substituted by PBS. Immunochemical staining sections were photographed using a Leica DFC 320 digital camera (Leica Microsystems, Cambridge, UK). The optical density for tissues was integrated by computer-assisted image analysis (Image-Pro Plus; Media Cybernetics, Bethesda, MD, USA) in each 400× magnified field[46]. Eight microscopic fields for each section were quantified.

Statistical analysis

Results are expressed as means with pooled SEM. Data were analysed statistically using the General Linear Model procedure of the Statistical Analysis System (version 2000; SAS Institute, Cary, NC, USA) for one-way ANOVA. Differences between the groups were determined by the Student–Newman–Keuls multiple comparison test. Relationship between intestinal villus height and VEGF protein levels was evaluated by the Pearson correlation analysis (SAS Institute). Probability values ≤0·05 were taken to indicate statistical significance.

Table 2. Effects of dietary L-arginine supplementation for 7 d on the growth performance and diarrhoea incidence of weaned pigs

(Mean values with their pooled standard errors, n 10)

Variable	Control	1% Arg	Pooled SEM
Initial body wt (kg)	5·27	5·33	0·11
Final body wt (kg)	6·17[a]	6·72[b]	0·19
ADG (g)	120[a]	187[b]	2·6
ADFI (g/d)	183	187	13
Feed:gain ratio	1·56[a]	1·12[b]	0·03
Diarrhoea incidence (%)	3·8	3·2	0·52

ADG, average daily gain; ADFI, average daily feed intake.
[a,b] Mean values within a row with unlike superscript letters were significantly different (P<0·05).

Results

Feed intake and growth performance

Daily food intake did not differ between control and arginine-supplemented piglets during a 7 d experimental period (Table 2). The initial body weight (BW) at 21 d of age did not differ between the two groups (Table 2). However, compared with the control group, dietary supplementation with L-arginine increased (P<0·05) the final BW by 9% and enhanced daily weight gain of piglets by 56%, respectively. Moreover, 1% L-arginine supplementation decreased (P<0·05) the ratio of DM intake:BW gain (g/g; an indicator of feed efficiency) by 28%. Diarrhoea incidence did not differ between the control and arginine-supplemented groups.

Plasma amino acids

Dietary supplementation with 1% L-arginine increased (P<0·05) plasma concentrations of arginine and ornithine by 33 and 30%, respectively, but had no effect on plasma concentrations of citrulline or lysine (Table 3).

Table 3. Effects of dietary L-arginine supplementation for 7 d on plasma metabolites and hormones in weaned piglets

(Mean values with their pooled standard errors, n 6)

Variable	Control	1% Arg	Pooled SEM
Arg (μmol/l)	284[a]	377[b]	13
Ornithine (μmol/l)	102[a]	133[b]	5·9
Citrulline (μmol/l)	65·3	70·8	6·5
Lys (μmol/l)	407	373	32·3
Urea (mmol/l)	3·19[a]	2·60[b]	0·53
NH_3 (μmol/l)	86·7[a]	71·6[b]	3·1
Glucose (mmol/l)	8·46	8·30	0·79
Total protein (g/l)	49·7	52·4	3·2
Total albumin (g/l)	27·5	29·1	2·0
Cortisol (μg/l)	132[a]	88·7[b]	8·1
Insulin (U/ml)	14·2[a]	18·1[b]	1·2
GH (U/ml)	0·14	0·19	0·05
IGF-1 (μg/l)	246	257	7·3
T3 (μg/l)	0·84	1·14	0·16
T4 (μg/l)	71·7	78·9	10

GH, growth hormone; IGF-1, insulin-like growth factor-1; T3, triiodothyronine; T4, thyroxine.
[a,b] Mean values within a row with unlike superscript letters were significantly different (P<0·05).

Plasma concentrations of histidine, asparagine, aspartate, isoleucine, leucine, methionine, phenylalanine, serine, taurine, threonine, tryptophan, tyrosine or valine did not differ between the control and arginine-supplemented groups (data not shown).

Plasma urea, ammonia and hormones

Dietary supplementation with 1% L-arginine decreased ($P<0.05$) plasma concentrations of NH_3 and urea by 17 and 18%, respectively (Table 3). Plasma concentrations of glucose, total protein and total albumin were not affected by L-arginine supplementation (Table 3). Compared with control pigs, dietary supplementation with 1% L-arginine increased ($P<0.05$) plasma concentrations of insulin by 27% and decreased ($P<0.05$) plasma concentrations of cortisol by 33% (Table 3). Dietary supplementation with 1% L-arginine did not affect plasma concentrations of growth hormone, insulin-like growth factor-1, triiodothyronine or thyroxine (Table 3).

Relative weight of internal organs

The relative weights (g/kg BW) of kidney, heart, spleen and lung were not affected by L-arginine supplementation (data not shown). The relative weight of the small intestine was 33% heavier ($P<0.05$) in arginine-supplemented pigs compared with that of the control group (73·1 *v.* 54·9 g/kg BW, pooled SEM = 4·4 g/kg BW).
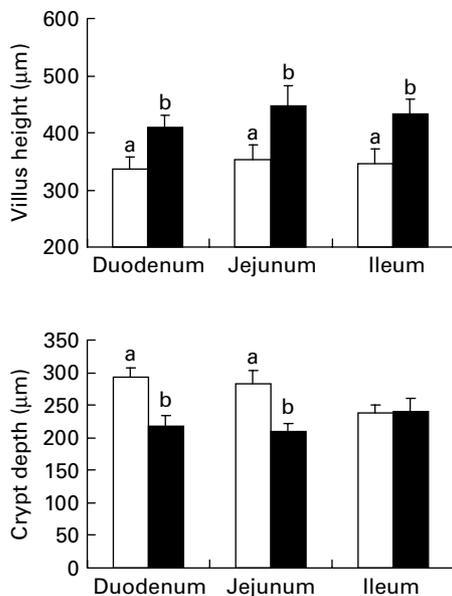

Fig. 1. Effects of dietary L-arginine supplementation for 7 d on the morphology of the small intestine in early-weaned piglets. Values are means with pooled SEM (*n* 6). [a,b] Mean values with unlike letters were significantly different ($P<0.05$). ■, Control; □, 1% L-arginine.

Small-intestinal morphology and vascular endothelial growth factor immunoreactive expression

To determine the effect of L-arginine supplementation on intestinal development, the piglet small intestine was collected at the end of the experiment, and the villus height and crypt depth were examined (Fig. 1). Supplementation with 1% L-arginine increased ($P<0.05$) the villus height throughout the small intestine, compared with the control group. Crypt depth was greater ($P<0.05$) in the duodenum and jejunum of the control group than that of the arginine-supplemented pigs.

The immunoreactive VEGF protein was readily detected in the piglet small intestine (Fig. 2). Quantitatively, dietary supplementation with 1% L-arginine increased ($P<0.05$) the integrated optical density in the duodenal submucosa, middle jejunal mucosa and submucosa, and the ileal mucosa by 14, 39 and 54%, respectively (Table 4 and Fig. 2). Correlation coefficients (R^2) between villus height and VEGF protein intensity were 0·954, 0·958 and 0·956 ($P<0.01$), respectively, for the duodenum, jejunum and ileum.

Discussion

Intestinal development of the piglet was greatly suppressed in the first week after weaning in association with intestinal dysfunction and atrophy[22], which is the major cause of reductions in nutrient absorption and utilisation as well
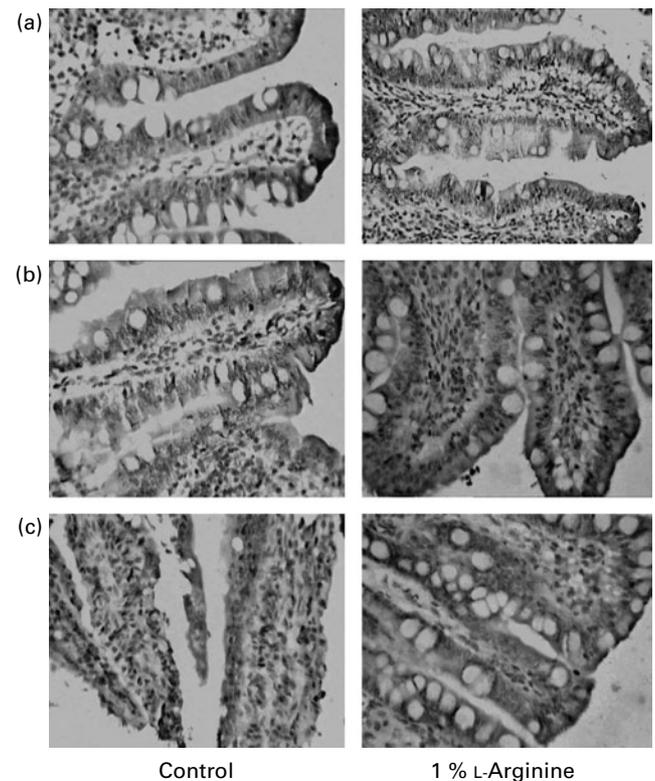

Fig. 2. Immunostaining of the vascular endothelial growth factor protein in the duodenum (a), jejunum (b) and ileum (c) of early-weaned piglets.

Table 4. Effects of dietary L-arginine supplementation for 7 d on vascular endothelial growth factor protein levels in the small intestine of weaned piglets

(Mean values (arbitrary unit) with their pooled standard errors, n 6)

Intestine	Control	1 % L-Arg	Pooled SEM
Duodenum	981[a]	1121[b]	53
Jejunum	893[a]	1244[b]	76
Ileum	715[a]	1098[b]	85

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

as increases in diarrhoea and deaths. Available evidence shows that VEGF enhances intestinal vascular development, endothelial function and epithelial cell migration while inhibiting endothelin-1 release as well as platelet aggregation and adhesion[47–50]. Results of the present study indicated that dietary supplementation with 1 % L-arginine had a positive effect on increasing villus height (Fig. 1) and the expression of the VEGF protein (Table 4 and Fig. 2). Notably, increases in both relative weight and villus height of the small intestine were positively correlated with the augmented expression of VEGF (see the Results section). The findings indicate that dietary supplementation with 1 % arginine can improve the vascular development of the small intestine in weanling pigs by stimulating VEGF expression.

Elevated levels of cortisol in plasma are considered to be biomarkers for weaning stress[34]. Interestingly, in the present study, we found that dietary supplementation with 1 % L-arginine decreased the circulating level of cortisol, but underlying mechanisms are unknown. Cortisol is the major glucocorticoid in pigs[51]. It is synthesised in the adrenal cortex and released into the circulation in response to external and internal factors acting on the hypothalamus and pituitary glands[52]. It is possible that NO or L-arginine itself attenuates the release of the adrenocorticotropic hormone from corticotroph cells and/or interferes with adrenocorticotropic hormone's actions on the adrenal cortex via specific receptors (e.g. type 2 melanocortin receptors). Further research is warranted to test this novel hypothesis.

Arginine plays important roles in both growth and metabolic function in piglets[53]. In rodents and human subjects, L-arginine is a nutritionally essential amino acid under conditions associated with increased utilisation relative to endogenous synthesis, including growth, inflammation and tissue repair[44]. In such cases, dietary supply can become rate limiting for the arginine-metabolising pathways[54]. As previously reported[39], arginine is deficient in weanling piglets (Table 3). Interestingly, intestinal synthesis of citrulline and arginine from glutamine and glutamate decreases by 70–73 % in 7-d-old suckling pigs in comparison with newborn pigs and declines further in 14- to 21-d-old pigs[55]. Thus, dietary supplementation with 0·2 and 0·4 % arginine to 7- to 21-d-old pigs (artificially reared on a milk feeding system) dose dependently

enhanced plasma arginine concentrations (30 and 61 %) and reduced plasma NH_3 levels (20 and 35 %)[56].

Insulin is a major regulatory hormone in glucose and fat metabolism, vascular function, inflammation, tissue remodelling and the somatotropic axis of growth[40,41]. Arginine is a potent stimulator of the secretion of insulin by pancreatic β-cells and of growth hormone by the anterior pituitary gland in mammals[45], including young pigs[46]. This is consistent with our finding that dietary supplementation with 1 % L-arginine increased plasma levels of insulin. Through an increase in arginine availability and the concurrent increases in plasma concentrations of anabolic hormones (Table 3), dietary arginine supplementation improved the efficiency of nutrient utilisation for enhancing tissue protein synthesis and growth performance. In support of this view, plasma concentrations of urea (the major nitrogenous product of protein and amino acid catabolism) were markedly reduced in arginine-supplemented pigs compared with control pigs (Table 3). Growth hormone is another important hormone in growth regulation, which plays a role in the secretion of thyroid hormone (mainly produced in the liver). However, L-arginine supplementation did not affect circulating levels of growth hormone or thyroid hormones (Table 3).

In keeping with the previous report[11], dietary supplementation with 1 % arginine markedly enhanced daily weight gain in weaning piglets (Table 2), indicating that arginine deficiency is a major factor limiting their maximum growth performance. It is noteworthy that experimental conditions differed greatly between the present study and the work of Zhan et al.[10]. For instance, the initial age (21 d) and initial mean BW (5·3 kg) of piglets in the present study were greater than those of the piglets used by Zhan et al. (14 d and 5·0 kg, respectively). Additionally, food intake of piglets was lower in the Zhan et al.'s experiment (140 g/d) compared with the present one (185 g/d) (Table 2). Furthermore, the doses of supplemental arginine (0·7 v. 1 %) differed between the two investigations. Regardless of the low feed intake, 0·7 % arginine supplementation may not be sufficient to meet the requirement of weanling piglets for maximal growth, whereas 1·2 % arginine supplementation caused severe diarrhoea[11]. Clearly, proper supplementation of arginine is critical for experiment design.

Another significant finding from the present study is that dietary supplementation with 1 % L-arginine increased the weight of the small intestine (see the Results section), which is consistent with the observation that arginine can increase protein synthesis[57], inhibit protein degradation and enhance the proliferation of intestinal epithelial cells[58]. It is now known that arginine activates the mammalian target of rapamycin signalling in the intestine[59], therefore promoting the initiation of polypeptide formation. These findings provide a molecular basis for our observation that L-arginine supplementation can improve the feed efficiency and growth performance of weaning

piglets. Because preterm infants are deficient in arginine and exhibit intestinal dysfunction[48], the present results may have important implications for managing this compromised population of neonates.

In conclusion, dietary supplementation with 1% L-arginine stimulates intestinal VEGF expression and development in weanling piglets, thereby contributing to improved vascular function and growth performance. Adequate provision of L-arginine in the weanling diet may be an effective means of ameliorating microvessel injury and enhancing the absorption of nutrients in the small intestine.

Acknowledgements

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