

## Dietary L-arginine supplementation enhances the immune status in early-weaned piglets

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**Abstract** This study was conducted to test the hypothesis that dietary L-arginine supplementation enhances immunity in early weaned piglets. Seventy piglets weaned at 7 days of age were assigned to five groups (14 pigs/group), representing supplementation of 0.0, 0.2, 0.4, 0.6, and 0.8% L-arginine to a milk-based formula. On Day 7 after initiation of treatment, spleen weight in piglets supplemented with 0.2 and 0.8% arginine was heavier and

thymus size was larger in piglets supplemented with 0.6% arginine, whereas serum concentration of immunoglobulin (Ig) M was higher but that of IL-8 was lower in piglets supplemented with 0.6 and 0.8% arginine, compared with the control group. Dietary supplementation with 0.8% arginine increased the numbers of white blood cells and granulocytes, and gene expression of interleukin (IL)-8 in spleen. On Day 14, compared with control piglets, granulocyte numbers were greater but lymphocyte numbers were lower in piglets supplemented with 0.2 and 0.4% arginine, whereas splenic expression of IL-8 and tumor necrosis factor- $\alpha$  genes was increased in piglets supplemented with 0.8% arginine. Additionally, IgG and IgM concentrations in serum and growth performance were greater in piglets supplemented with 0.4–0.8% arginine, compared with unsupplemented piglets. Collectively, dietary supplementation with 0.4–0.8% L-arginine for 2 weeks enhances both cellular and humoral immunity in piglets by modulating the production of leukocytes, cytokines and antibodies. These results indicate that increasing L-arginine provision is beneficial for optimal immune responses in young pigs and also have important implications for designing the next generation of improved formula for human infants.

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### Abbreviations

Ig	Immunoglobulin
IL	Interleukin
NO	Nitric oxide
RT-PCR	Real-time polymerase-chain reaction
TNF	Tumor necrosis factor

## Introduction

The current swine industry strives to wean piglets at an early age to prevent sow-originated infectious diseases and maximize sows' production potential (Dong and Pluske 2007; Kim et al. 2007). This practice presents a tremendous challenge to neonatal pigs, whose immune system is not well developed in the first 4 weeks of life (Yang and Schultz 1986). Nutritional means, including dietary supplementation with glutamine (Johnson et al. 2006), zinc (Ou et al. 2007), or herbs (Kong et al. 2007), have been used to improve the immunity of early-weaned piglets. However, due to low feed intake in the first week post-weaning, these approaches cannot provide piglets with sufficient arginine, an amino acid crucial for optimal immune responses (Li et al. 2007).

Arginine, which is an abundant amino acid in tissue proteins (Wu et al. 1999), is utilized by multiple pathways, including the synthesis of protein, nitric oxide (NO), polyamines and creatine (Wu and Morris 1998). Through cGMP-dependent signaling pathways, NO regulates a variety of physiological and biochemical processes (Jobgen et al. 2006). Of particular interest, weanling piglets have a particularly high requirement for dietary arginine (Kim and Wu 2004; Wu et al. 2004). A large body of evidence from studies with rats and mice indicates that adequate provision of arginine is required for lymphocyte development and that dietary arginine supplementation enhances immune function in various models of immunological challenges (Li et al. 2007). However, little is known about the effect of dietary arginine supplementation on the immune status of early-weaned piglets. Therefore, the major objective of the present study was to determine an immunoregulatory role for arginine in young pigs.

## Materials and methods

### Animals and feeding

Seventy piglets (Landrace × Yordshire) weaned at 7 days of age were assigned randomly to five treatment groups (14 pigs/group), representing supplementation with 0.0% (control), 0.2, 0.4, 0.6 and 0.8% L-arginine (Ajinomoto, Tokyo, Japan) to a milk-based diet (Table 1). All diets were made isonitrogenous with addition of appropriate amounts of alanine at the expense of lactose and glucose, as described by Kim and Wu (2004). Diets were formulated to meet National Research Council-recommended nutrient requirements for weanling piglets (NRC 1998). The piglets were housed individually in an environmentally controlled nursery with hard plastic slatted flooring and fed their respective liquid diets (water/diet = 4:1) 4 times per day at 7:00, 11:00, 15:00 and 19:00. All animals had free access to drinking water.

### Sample collection

On Days 7 and 14 after initiation of treatment, 6 piglets in each treatment group were selected randomly for obtaining blood samples from the jugular vein at 2 h after feeding. Approximately 1 mL of the blood was placed in ethylenediamine-tetraacetic acid-coated tubes for the determination of total and differential counts of leucocytes. In addition, 8 mL blood samples were contained in heparinized tubes, followed immediately by centrifugation at 3,000×g for 10 min. The resultant sera were transferred to polystyrene tubes and stored at −80°C for analysis of immunoglobulins and cytokines. When blood sampling was completed, piglets were anesthetized with sodium pentobarbital and killed by jugular puncture (Kong et al. 2007). Thymus and spleen were obtained and weighed. Spleens were immediately snap-frozen in liquid nitrogen and stored at −80°C for the extraction of total RNA. This study was carried out in accordance with the Chinese guidelines for animal welfare and was approved by the Animal Care and Use Committee of the Chinese Academy of Sciences.

### Determination of total and differential counts of blood leucocytes

Total and differential numbers of blood leucocytes were determined using an Auto-Hemocytometer (Cell Dyn BT-2100, Abbott Diagnostics, Abbott Park, IL, USA) within 2 h after blood samples were obtained. The procedures

**Table 1** Composition of the basal diet

Ingredients (g/kg)	Nutrient content (g/kg)		
Whey protein concentrate	600	Digestible energy (MJ/kg)	14.4
Cream powder	260	Crude protein	260.1
α-Casein	62	Calcium	9
Lactose	36	Total phosphorus	7
Glucose	16.5	Lysine	15.8
Calcium lactate	10	Methionine	5.3
Vitamin premix <sup>a</sup>	1	Arginine	5.8
CaH <sub>2</sub> PO <sub>4</sub>	10		
Trace mineral premix <sup>b</sup>	2		
L-Lysine	1		
L-Methionine	1		
Terramycin-HCl	0.5		

<sup>a</sup> Supplied per kg diet: vitamin A, 2,200 IU; vitamin D<sub>3</sub>, 220 IU; vitamin E, 16 IU; vitamin K, 0.5 mg; vitamin B<sub>12</sub>, 0.02 mg; riboflavin, 4 mg; niacin, 20 mg; pantothenic acid, 12 mg; choline chloride, 0.6 mg; folic acid, 0.3 mg; thiamin, 1.5 mg; pyridoxine, 2 mg; biotin, 0.08 mg

<sup>b</sup> Supplied per kg diet: Zn ([C<sub>5</sub>H<sub>11</sub>NO<sub>2</sub>S]<sub>2</sub>Zn), 100 mg; Mn ([C<sub>2</sub>H<sub>4</sub>N<sub>2</sub>O<sub>2</sub>]<sub>2</sub>Mn), 5 mg; Fe ([C<sub>2</sub>H<sub>4</sub>O<sub>2</sub>N]<sub>2</sub>Fe), 100 mg; Cu ([C<sub>2</sub>H<sub>5</sub>NO<sub>2</sub>]<sub>2</sub>Cu), 10 mg; I (KI), 0.2 mg; Se (Na<sub>2</sub>SeO<sub>3</sub>), 0.3 mg

were performed according to the manufacturer's instructions (Kong et al. 2007).

#### Determination of serum IgG, IgM and cytokines

Serum concentrations of IgG and IgM were measured using immunoassay kits from Triple J Farms (Bellingham, WA, USA) according to the manufacturer's instructions. Serum concentrations of interleukin (IL)-1 $\beta$ , IL-2, IL-6, IL-8, and tumor necrosis factor (TNF)- $\alpha$  were determined by radio-immunoassays using kits provided by Beijing Chemclin Biotech (Beijing, China).

#### Analysis of IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ gene expression in spleen

Total RNA was extracted from spleen using the Trizol Reagent (Invitrogen) and concentration was measured by optical density at 260 nm (Deng et al. 2007). The cDNA was reverse-transcribed from 0.2  $\mu$ g of eluted RNA using the First Strand cDNA Synthesis Kit (MBI Fermentas, Newington, NH, USA) based on the manufacturer's instruction manual. The real-time PCR for IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ , as well as  $\beta$ -actin as internal control, was performed using their respective primer pairs (Table 2). The amplification mixture contained 5  $\mu$ L cDNA, 0.5  $\mu$ L

of each primer, and components of the iQ SYBR Green Supermix (Bio-Rad, Richmond, CA, USA) in a final volume of 25  $\mu$ L. The PCR analysis was performed at 94°C for 10 s, followed by 35 cycles of denaturation at 94°C for 10 s, annealing at 54.7°C for 30 s, and extension at 72°C for 15 s. The relative quantification of gene amplification by RT-PCR was performed using cycle threshold ( $C_t$ ) values (Deng et al. 2007). The comparative  $C_t$  value method was employed to quantitate expression levels for cytokine genes relative to those for  $\beta$ -actin, as described by Fu et al. (2006).

#### Calculation and statistical analysis

The relative weights of lymphoid organs were calculated as the organ weights divided by body weights (g/kg). All data, expressed as mean  $\pm$  SEM, were subjected to ANOVA analysis using the SPSS 13.0 Programme (Chicago, IL, USA). The differences among group means were compared using the Duncan multiple comparison test. Probability values  $<0.05$  were taken to indicate statistical significance.

## Results

#### Effects of dietary arginine supplementation on growth performance of piglets

Feed intake did not differ ( $P > 0.05$ ) among all the groups of piglets ( $63.5 \pm 2.4$  g dry matter/kg body weight per day) during the 14-day-period of experiment. On Days 7 and 14 after initiation of treatment, piglets supplemented with 0.6 and 0.8% arginine had heavier body weights ( $P < 0.05$ ) than piglets in the other groups (Table 3). On Day 14, dietary supplementation with 0.4% arginine increased ( $P < 0.05$ ) the body weight of piglets by 8%, compared with the control group. Over the entire 2-week of study, the daily weight gain of piglets supplemented with 0.4, 0.6 and 0.8% arginine was 20, 49 and 48% greater ( $P < 0.05$ ) than that for the control group, respectively (Table 3).

**Table 2** Sequences of PCR primers

Gene	Primer	
$\beta$ -actin	Forward	GGATGCAGAAGGAGATCACG
	Reverse	ATCTGCTGGAAGGTGGACAG
IL-1 $\beta$	Forward	CAAAGCCGCAAGATATAA
	Reverse	GAAATTCAGGCAGCAACCAT
IL-6	Forward	AAGGTGATGCCACCTCAGAC
	Reverse	TCTGCCAGTACCTCCTTGCT
IL-8	Forward	AAACTGGCTGTTGCCTTCTTG
	Reverse	CCAGGCAGACCTCTTTTCCAT
TNF- $\alpha$	Forward	ATGGATGGGTGGATGAGAAA
	Reverse	TGGAAGCTGTTGGGGAGAAG

**Table 3** Effects of dietary arginine supplementation on the growth performance of early-weaned piglets

Data are mean  $\pm$  SEM,  $n = 14$ , 14, and 8 on Days 0, 7, and 14, respectively  
<sup>a-c</sup> Mean values sharing different superscripts within a row differ ( $P < 0.05$ )

Items	0.0% Arginine	0.2% Arginine	0.4% Arginine	0.6% Arginine	0.8% Arginine
Body weight (kg)					
Day 0	2.73 $\pm$ 0.03	2.73 $\pm$ 0.03	2.76 $\pm$ 0.02	2.75 $\pm$ 0.02	2.72 $\pm$ 0.02
Day 7	3.38 $\pm$ 0.03 <sup>b</sup>	3.41 $\pm$ 0.05 <sup>b</sup>	3.51 $\pm$ 0.24 <sup>b</sup>	3.79 $\pm$ 0.13 <sup>a</sup>	3.75 $\pm$ 0.27 <sup>a</sup>
Day 14	4.47 $\pm$ 0.06 <sup>c</sup>	4.65 $\pm$ 0.26 <sup>bc</sup>	4.81 $\pm$ 0.39 <sup>b</sup>	5.30 $\pm$ 0.49 <sup>a</sup>	5.27 $\pm$ 0.38 <sup>a</sup>
Average daily gain (g/day)					
Days 0–7	92.8 $\pm$ 4.93 <sup>b</sup>	98.0 $\pm$ 6.69 <sup>b</sup>	107.3 $\pm$ 5.26 <sup>b</sup>	147.7 $\pm$ 4.78 <sup>a</sup>	146.9 $\pm$ 9.00 <sup>a</sup>
Days 7–14	156.1 $\pm$ 9.20 <sup>b</sup>	175.5 $\pm$ 13.3 <sup>b</sup>	185.5 $\pm$ 9.17 <sup>ab</sup>	209.8 $\pm$ 11.3 <sup>a</sup>	217.9 $\pm$ 12.5 <sup>a</sup>
Days 0–14	122.1 $\pm$ 5.01 <sup>c</sup>	134.1 $\pm$ 5.69 <sup>bc</sup>	146.6 $\pm$ 5.95 <sup>b</sup>	181.6 $\pm$ 6.38 <sup>a</sup>	180.1 $\pm$ 5.71 <sup>a</sup>

### Effects of dietary arginine supplementation on relative organ weights of piglets

The relative weights of spleen in piglets supplemented with 0.2 and 0.8% arginine were 32 and 14% greater ( $P < 0.01$ ), respectively, on Day 7 after initiation of treatment, when compared with the control group (Fig. 1). In addition, dietary supplementation with 0.6% arginine increased ( $P < 0.05$ ) the relative weights of thymus by approximately 150% on Day 7, in comparison with the control and other groups of piglets, whereas a higher dose of arginine (0.8%) reduced ( $P < 0.05$ ) the relative weight of thymus by 24%, compared with the control group. On Day 7, dietary supplementation with 0.4 and 0.6% arginine had no effect ( $P > 0.05$ ) on the relative weight of spleen, and dietary supplementation with 0.2% and 0.4% arginine did not affect ( $P < 0.05$ ) thymus size. On Day 14, the relative weights of the spleen and thymus were similar ( $P > 0.05$ ) among all the treatment groups (Fig. 1).

### Effects of dietary arginine supplementation on total and differential counts of blood leucocytes

Dietary supplementation with 0.8% arginine increased ( $P < 0.05$ ) the numbers of white blood cells and

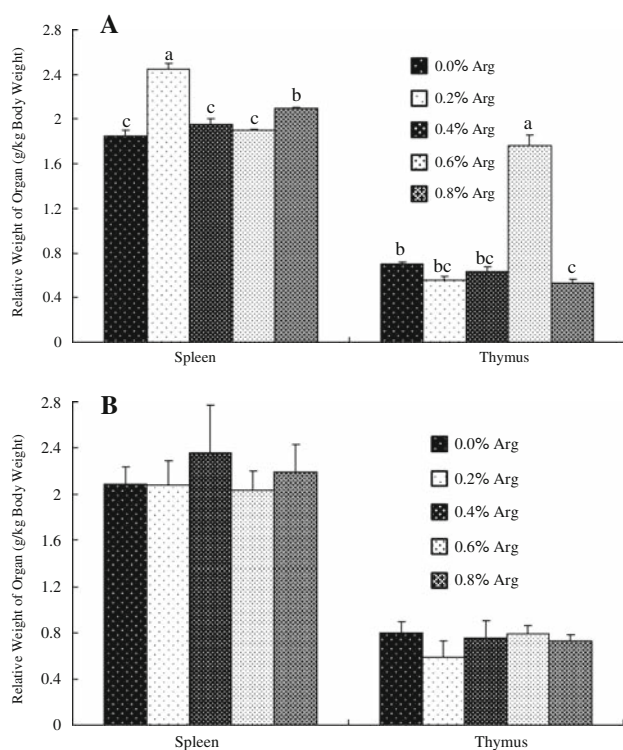
granulocytes, as well as the percentage of blood granulocytes by 60–80%, on Day 7 after initiation of treatment, in comparison with all other groups of piglets (Table 4). On Day 7, the numbers of white blood cells or granulocytes did not differ ( $P > 0.05$ ) among piglets supplemented with 0.0–0.6% arginine, and the number of lymphocytes and middle cells did not differ ( $P > 0.05$ ) among all groups of pigs. Dietary arginine supplementation had no effect ( $P > 0.05$ ) on the percentage of blood lymphocytes or middle cells on Day 7, except that the percentage of granulocytes was higher ( $P < 0.05$ ) in piglets supplemented with 0.8% arginine than that in the control group.

On Day 14, dietary supplementation with 0.4 and 0.6% arginine reduced ( $P < 0.05$ ) the number of white blood cells, compared with the other groups of piglets (Table 4). The number of total blood lymphocytes was lower ( $P < 0.05$ ) in piglets supplemented with 0.2–0.6% arginine, compared with piglets supplemented with 0.0 and 0.8% arginine. On Day 14, the percentage of lymphocytes were lower ( $P < 0.05$ ) but the number and percentage of granulocytes were higher ( $P < 0.05$ ) in piglets supplemented with 0.2 and 0.4% arginine, when compared with the other groups of piglets (Table 4). Dietary supplementation with 0.2–0.8% arginine had no effect ( $P > 0.05$ ) on the total number or percentage of blood middle cells.

### Effects of dietary arginine supplementation on serum concentrations of immunoglobulins and cytokines

On Day 7, dietary supplementation with 0.2–0.8% arginine did not affect ( $P > 0.05$ ) serum concentrations of IgG, but dietary supplementation with 0.6–0.8% arginine increased ( $P < 0.05$ ) serum concentrations of IgM by approximately 150–200%, compared with the other three groups (0.0, 0.2, and 0.4% arginine) (Table 5). In contrast, on Day 14, dietary supplementation with 0.2% arginine enhanced ( $P < 0.05$ ) serum concentrations of IgG by 12%, but a higher dose of arginine (0.4–0.8%) did not result in a further increase in this parameter. Supplementing 0.4, 0.6 and 0.8% arginine to the diet increased ( $P < 0.05$ ) serum concentrations of IgM by 62, 77 and 91%, respectively, in comparison with the control group.

Dietary supplementation with 0.2–0.8% arginine had no effect ( $P > 0.05$ ) on serum concentrations of IL-1 $\beta$  and TNF- $\alpha$  on Day 7 or IL-2 and IL-6 on either Day 7 or Day 14 (Table 5). However, on Day 14, dietary supplementation with 0.4% arginine increased ( $P < 0.05$ ) serum concentration of IL-1 $\beta$  by 32–50%, in comparison with all other groups of piglets. Serum concentration of TNF- $\alpha$  was highest ( $P < 0.05$ ) in piglets supplemented with 0.2% arginine among the treatment groups and was not affected ( $P > 0.05$ ) by dietary supplementation with higher doses of arginine (Table 5). Interestingly, IL-8 was the only



**Fig. 1** Effects of dietary L-arginine (Arg) supplementation on the relative organ weights in early-weaned piglets on Day 7 (a) and Day 14 (b). Data are mean  $\pm$  SEM,  $n = 6$ . <sup>a-c</sup> Mean values sharing different superscripts within each lymphoid organ differ ( $P < 0.05$ )

**Table 4** Effects of dietary arginine supplementation on total and differential counts of blood leucocytes in early-weaned piglets

Items	0.0% Arginine	0.2% Arginine	0.4% Arginine	0.6% Arginine	0.8% Arginine
White blood cells ( $\times 10^9/L$ )					
Day 7	10.1 $\pm$ 0.31 <sup>b</sup>	9.85 $\pm$ 0.89 <sup>b</sup>	8.83 $\pm$ 0.63 <sup>b</sup>	10.7 $\pm$ 2.29 <sup>b</sup>	16.3 $\pm$ 0.12 <sup>a</sup>
Day 14	16.8 $\pm$ 1.15 <sup>a</sup>	14.9 $\pm$ 0.91 <sup>a</sup>	9.50 $\pm$ 0.57 <sup>b</sup>	12.0 $\pm$ 0.42 <sup>b</sup>	17.3 $\pm$ 1.48 <sup>a</sup>
Lymphocytes ( $\times 10^9/L$ )					
Day 7	7.70 $\pm$ 0.87	8.15 $\pm$ 0.87	7.87 $\pm$ 0.72	8.05 $\pm$ 1.71	10.7 $\pm$ 1.84
Day 14	14.2 $\pm$ 0.79 <sup>a</sup>	10.7 $\pm$ 0.33 <sup>b</sup>	6.37 $\pm$ 0.19 <sup>c</sup>	10.3 $\pm$ 0.18 <sup>b</sup>	14.6 $\pm$ 1.26 <sup>a</sup>
Lymphocytes (% of total blood leukocytes)					
Day 7	78.3 $\pm$ 3.28	81.8 $\pm$ 3.47	87.8 $\pm$ 2.94	77.2 $\pm$ 3.27	73.5 $\pm$ 4.29
Day 14	84.7 $\pm$ 2.57 <sup>a</sup>	74.4 $\pm$ 3.73 <sup>bc</sup>	69.3 $\pm$ 2.24 <sup>c</sup>	86.9 $\pm$ 1.90 <sup>a</sup>	80.9 $\pm$ 2.66 <sup>ab</sup>
Middle cells ( $\times 10^9/L$ )					
Day 7	0.65 $\pm$ 0.06	0.63 $\pm$ 0.12	0.46 $\pm$ 0.09	1.13 $\pm$ 0.39	1.12 $\pm$ 0.32
Day 14	0.91 $\pm$ 0.14	1.07 $\pm$ 0.16	0.60 $\pm$ 0.08	0.73 $\pm$ 0.11	0.90 $\pm$ 0.28
Middle cells (% of total blood leukocytes)					
Day 7	6.30 $\pm$ 0.37	5.93 $\pm$ 0.63	5.19 $\pm$ 0.96	7.79 $\pm$ 1.20	6.17 $\pm$ 0.45
Day 14	5.43 $\pm$ 0.71	6.69 $\pm$ 0.76	6.40 $\pm$ 0.37	5.59 $\pm$ 0.73	5.39 $\pm$ 0.47
Granulocytes ( $\times 10^9/L$ )					
Day 7	1.75 $\pm$ 0.34 <sup>b</sup>	1.05 $\pm$ 0.20 <sup>b</sup>	0.62 $\pm$ 0.05 <sup>b</sup>	1.53 $\pm$ 0.28 <sup>b</sup>	3.13 $\pm$ 0.81 <sup>a</sup>
Day 14	1.68 $\pm$ 0.33 <sup>b</sup>	3.13 $\pm$ 0.19 <sup>a</sup>	2.43 $\pm$ 0.19 <sup>a</sup>	1.06 $\pm$ 0.18 <sup>b</sup>	1.61 $\pm$ 0.11 <sup>b</sup>
Granulocytes (% of total blood leukocytes)					
Day 7	12.0 $\pm$ 1.38 <sup>b</sup>	10.9 $\pm$ 1.08 <sup>b</sup>	8.26 $\pm$ 0.82 <sup>b</sup>	11.6 $\pm$ 0.94 <sup>b</sup>	16.4 $\pm$ 1.80 <sup>a</sup>
Day 14	10.6 $\pm$ 0.44 <sup>bc</sup>	23.6 $\pm$ 1.97 <sup>a</sup>	21.8 $\pm$ 1.52 <sup>a</sup>	7.38 $\pm$ 0.43 <sup>c</sup>	12.7 $\pm$ 1.08 <sup>b</sup>

Data are mean  $\pm$  SEM,  $n = 6$

<sup>a-c</sup> Mean values sharing different superscripts within a row differ ( $P < 0.05$ )

measured cytokine that exhibited a change in serum concentrations on both Days 7 and 14 in response to the arginine treatment. Particularly, compared with the control group, serum concentrations of IL-8 were 72 and 61% lower ( $P < 0.05$ ), respectively, in piglets supplemented with 0.6 and 0.8% arginine on Day 7, and were 36 and 40% lower ( $P < 0.05$ ), respectively, in piglets supplemented with 0.2 and 0.8% arginine on Day 14 (Table 5).

Effects of dietary arginine supplementation on cytokine gene expression in spleen

Dietary supplementation with 0.2–0.8% arginine did not affect ( $P > 0.05$ ) splenic expression of IL-1 $\beta$  and IL-6 genes on Days 7 and 14 or TNF- $\alpha$  on Day 7 (Fig. 2). Compared with the control group, splenic expression of the IL-8 gene was higher ( $P < 0.05$ ) in piglets supplemented with 0.4–0.8% arginine on Day 7 and in piglets supplemented with 0.8% arginine on Day 14. Dietary supplementation with 0.8% arginine enhanced ( $P < 0.05$ ) TNF- $\alpha$  gene expression in spleen, compared with piglets supplemented with 0.0, 0.2 and 0.4% arginine.

## Discussion

Studies with rodents and humans demonstrate that arginine is an important immunomodulatory nutrient (Li et al. 2007;

Popovic et al. 2007). Its immunostimulatory effect is most promising in immunocompromised hosts under such stress conditions as trauma, surgery and or viral infection (Daly et al. 1988; Kelly et al. 1995; Rodriguez et al. 2003). In laboratory animals, administration of arginine increased thymus size and cellularity, stimulated lymphocyte proliferation in response to mitogen and alloantigen, augmented macrophage and natural killer cell-mediated lysis of tumor cells, and enhanced IL-2 production by lymphocytes (Reynolds et al. 1990; Yeh et al. 2002). At present, there is a paucity of information in the literature regarding the effects of supplemental arginine on the immune status in neonates. Because the pig is an established animal model for studying human nutrition (Wu et al. 2004), findings from this work may have important implications for designing the next generation of improved formula for infants.

Arginine, which is synthesized from glutamine/glutamate and proline via the intestinal-renal axis (Wu 1997; Hu et al. 2008), is a nutritionally essential amino acid for neonatal pigs (Kim and Wu 2004). It also stimulates the secretion of growth hormone and insulin that beneficially modulate the immune response (Evoy et al. 1998; Flynn et al. 2002). Compelling evidence shows that the supply of arginine from the milk-based diet and endogenous synthesis is inadequate for supporting maximal growth of 7- to 21-day-old piglets (Frank et al. 2007; Wu et al. 2004; Yao et al. 2008). During the weaning period, the problem of low

**Table 5** Effects of dietary arginine supplementation on serum concentrations of immunoglobulins and cytokines in early-weaned piglets

Items	0.0% Arginine	0.2% Arginine	0.4% Arginine	0.6% Arginine	0.8% Arginine
IgG (mg/dL)					
Day 7	413.8 ± 14.2	441.7 ± 9.23	439.7 ± 15.6	467.6 ± 13.2	428.1 ± 11.7
Day 14	381.3 ± 24.0 <sup>b</sup>	426.9 ± 11.0 <sup>a</sup>	447.9 ± 5.62 <sup>a</sup>	431.3 ± 10.4 <sup>a</sup>	427.1 ± 9.26 <sup>a</sup>
IgM (mg/dL)					
Day 7	3.90 ± 0.20 <sup>c</sup>	5.05 ± 0.39 <sup>c</sup>	4.64 ± 0.54 <sup>c</sup>	10.5 ± 0.67 <sup>b</sup>	13.2 ± 0.68 <sup>a</sup>
Day 14	7.96 ± 0.99 <sup>c</sup>	9.53 ± 0.42 <sup>bc</sup>	12.9 ± 1.79 <sup>ab</sup>	14.1 ± 1.34 <sup>a</sup>	15.2 ± 1.48 <sup>a</sup>
IL-1 $\beta$ (ng/ml)					
Day 7	0.040 ± 0.007	0.041 ± 0.005	0.051 ± 0.007	0.037 ± 0.004	0.035 ± 0.004
Day 14	0.042 ± 0.003 <sup>b</sup>	0.047 ± 0.004 <sup>b</sup>	0.062 ± 0.002 <sup>a</sup>	0.041 ± 0.006 <sup>b</sup>	0.041 ± 0.002 <sup>b</sup>
IL-2 (ng/ml)					
Day 7	4.27 ± 0.24	3.22 ± 0.45	3.23 ± 0.19	4.02 ± 0.47	3.21 ± 0.22
Day 14	3.58 ± 0.27	3.17 ± 0.18	3.55 ± 0.37	3.22 ± 0.65	3.32 ± 0.25
IL-6 (pg/ml)					
Day 7	42.5 ± 1.59	55.8 ± 2.57	42.2 ± 1.87	56.5 ± 6.58	52.9 ± 7.42
Day 14	44.9 ± 4.15	48.1 ± 6.45	63.2 ± 8.38	60.6 ± 9.62	53.3 ± 6.24
IL-8 (ng/ml)					
Day 7	0.074 ± 0.007 <sup>a</sup>	0.058 ± 0.002 <sup>a</sup>	0.053 ± 0.013 <sup>ab</sup>	0.021 ± 0.005 <sup>c</sup>	0.029 ± 0.009 <sup>bc</sup>
Day 14	0.042 ± 0.005 <sup>a</sup>	0.027 ± 0.001 <sup>b</sup>	0.032 ± 0.002 <sup>ab</sup>	0.035 ± 0.002 <sup>ab</sup>	0.025 ± 0.006 <sup>b</sup>
TNF- $\alpha$ (ng/ml)					
Day 7	0.247 ± 0.017	0.365 ± 0.048	0.308 ± 0.092	0.416 ± 0.057	0.406 ± 0.073
Day 14	0.321 ± 0.067 <sup>b</sup>	0.608 ± 0.039 <sup>a</sup>	0.392 ± 0.065 <sup>b</sup>	0.373 ± 0.06 <sup>b</sup>	0.346 ± 0.074 <sup>b</sup>

Data are mean ± SEM,  $n = 6$

<sup>a-c</sup> Mean values sharing different superscripts within a row differ ( $P < 0.05$ )

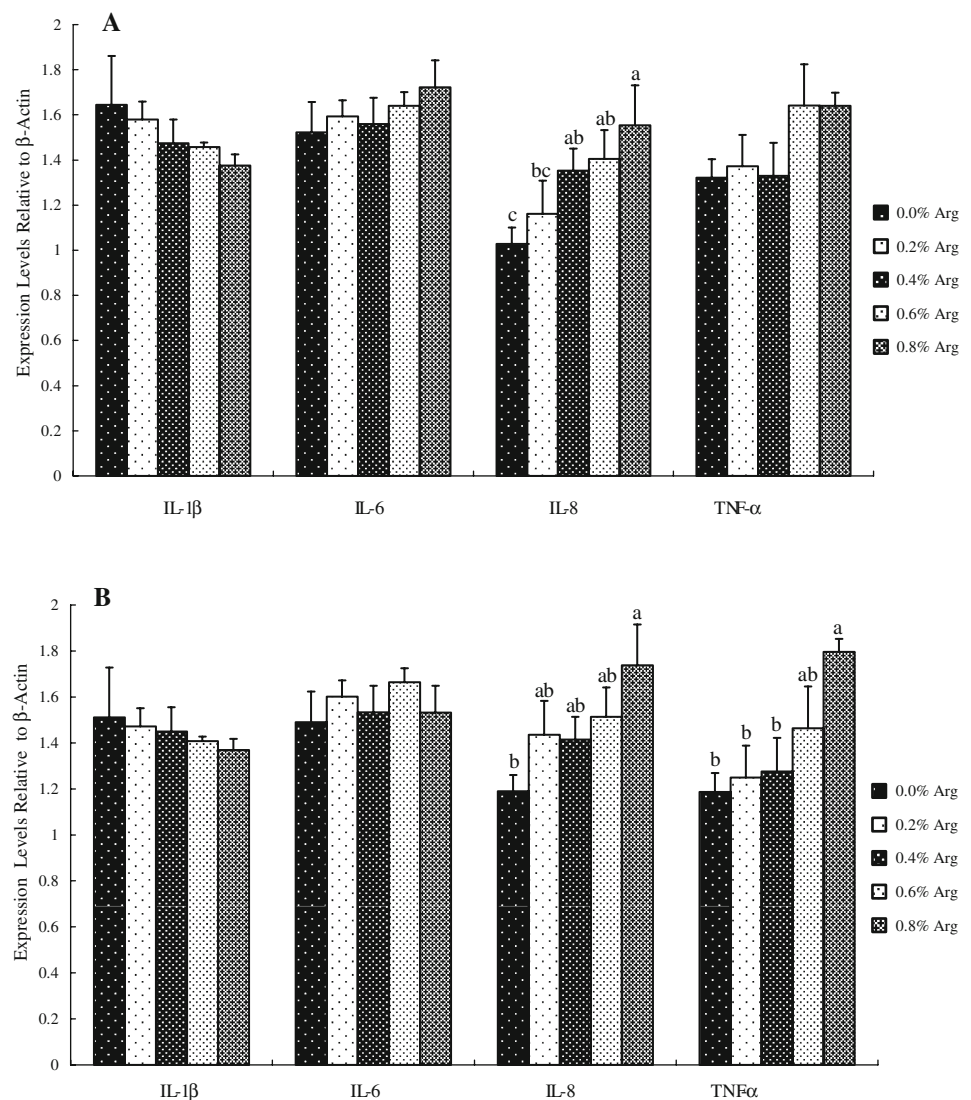
feed intake by piglets is exacerbated by high concentrations of cortisol (Wu et al. 2000). This stress hormone induces expression of arginase in multiple organs (including the small intestine) for the hydrolysis of arginine (Flynn et al. 1999; Morris 2002). The outcome is an arginine deficiency as indicated by elevated levels of ammonia and reduced levels of arginine in plasma (Wu et al. 1996). Thus, the weanling piglet is challenged by a variety of hormonal and environmental factors (such as pathogens and diet), which compromises its immune function (Lalles et al. 2007). Immune-enhancing diets, which can improve development of the immune system in young animals, may be an effective means to enhance the immune status (Li et al. 2007). Therefore, the early-weaned piglet provides an established animal model to test this hypothesis.

As key lymphoid organs, the thymus and spleen play an important role in the host immune response (Li et al. 2007). Kwak et al. (1999) reported that dietary supplementation with 0.2% arginine increased the weights of these two organs in chickens, but feeding an arginine-deficient diet impaired their growth and development. Similarly, extensive studies with mammals indicate that dietary arginine supplementation increased mitogenesis and functional reactivities of thymic and splenic lymphocytes (Barbul et al. 1980; Reynolds et al. 1990; Kelly et al. 1995). Consistent with these findings, dietary supplementation of 0.2 and 0.8% arginine to piglets increased the relative weight of spleen, whereas dietary supplementation of 0.6%

arginine increased the relative weight of thymus in the first week post weaning (Fig. 1), the most critical period for the survival of weanling piglets (Dong and Pluske 2007). Notably, the stimulatory effect of the arginine treatment on growth and development of lymphoid organs appears to depend on its dose, as either a low or a high dose of arginine was ineffective (Fig. 1). These results may be explained by a complex interaction between arginine and other dietary nutrients in regulating the synthesis of NO and polyamines (Wu and Meininger 2002; Flynn et al. 2002), whose biological actions are now known to vary with their cellular concentrations (Montanez et al. 2007; Nikolic et al. 2007).

The total and differential counts of leukocytes may indicate an inflammatory status of animals (Holtenius et al. 2004). Generally, a reduction in the number of blood leukocytes under inflammatory conditions may be an indicator of reduced infections in hosts (Evoy et al. 1998). In addition, both IgG and IgM play an important role in defending the host from infectious diseases (Li et al. 2007). Further, cytokines are crucial protein mediators in humoral immunity (Ma et al. 2007). Of particular interest, TNF- $\alpha$  regulates leukocyte recruitment through both upregulation of adhesion molecules on vascular endothelial cells and induction of cytokine and chemokine synthesis (Kips 2001). Furthermore, IL-6 plays an important role under inflammatory conditions, including bacterial infections (Song and Kellum 2005), whereas IL-8 is a potent

**Fig. 2** Effects of dietary L-arginine (Arg) supplementation on IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  gene expression levels in the spleen of early-weaned piglets on Day 7 (a) and Day 14 (b). Data are mean  $\pm$  SEM,  $n = 6$ . <sup>a-c</sup> Mean values sharing different superscripts within each cytokine differ ( $P < 0.05$ )



neutrophil chemotactic factor, which reinforces the recruitment of additional neutrophils to inflammatory sites (Evoy et al. 1998). As components of the immune system, these cytokines are crucial for the immune response (Escobar et al. 2004; Li et al. 2007).

Several lines of evidence from the present study supports the notion that dietary arginine supplementation enhances the immune status in early-weaned piglets. First, dietary supplementation with 0.8% arginine increased the relative weight of spleen on Day 7 (Fig. 1). Second, on Day 14, dietary supplementation with 0.4–0.6% arginine reduced the number of white blood cells and lymphocytes, while dietary supplementation with 0.6% arginine increased thymus size (Fig. 1) and the number of granulocytes (Table 4). Third, dietary supplementation with 0.6–0.8% arginine increased serum concentrations of IgM on Days 7 and 14, as well as IgG on Day 14 (Table 4). Fourth, on Day 14, dietary supplementation with 0.2 and 0.4%

arginine augmented serum concentrations of TNF- $\alpha$  and IL-1 $\beta$ , respectively (Table 5). Also, dietary supplementation with 0.8% arginine decreased serum concentrations of IL-8 on both Days 7 and 14 (Table 5), which may reflect a reduction in systemic infection (Evoy et al. 1998), while increasing splenic expression of the IL-8 gene (Fig. 2) as a possible mechanism for beneficially regulating cell proliferation and differentiation in the spleen (Broxmeyer et al. 1996). Finally, supplementing 0.4–0.8% arginine to the diet did not affect feed intake but markedly improved the growth performance of young pigs (Table 3). On the basis of changes in the measured parameters, dietary supplementation with 0.8% arginine appears to yield optimal immune responses in piglets.

Results of this study indicate that a gradual increase in the doses of supplemental arginine did not result in gradual response in most of the immune parameters measured. Rather, some changes were observed stochastically.

Additionally, in certain cases, only one dose yielded an influence, while both lower and higher levels did not have an effect on the same parameter. Clearly, there are complex immunological responses to dietary arginine supplementation, which likely depend on multiple factors (including developmental stage, target cells, metabolic pathways, balance among basic amino acids, levels of NO and polyamine production, and endocrine status in animals), as recently reported for muscle mTOR signaling (Yao et al. 2008), growth performance (Table 3), secretion of insulin and growth hormone (Kim and Wu 2004; Wu et al. 2007), reproduction (Mateo et al. 2007; Wu et al. 2008), and the digestive system (Zhan et al. 2008) in pigs. This underscores the need of the current work to establish an optimal dose of supplemental arginine for enhancing immune responses in neonates.

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