Amino Acids (2009) 37:643–651
DOI 10.1007/s00726-008-0184-9

ORIGINAL ARTICLE

Dietary L-arginine supplementation alleviates immunosuppression induced by cyclophosphamide in weaned pigs

J. Han · Y. L. Liu · W. Fan · J. Chao · Y. Q. Hou · Y. L. Yin · H. L. Zhu · G. Q. Meng · Z. Q. Che

Received: 30 June 2008 / Accepted: 12 September 2008 / Published online: 28 September 2008
© Springer-Verlag 2008

Abstract A study was conducted to investigate the effects of L-arginine (Arg) on performance and immune function in cyclophosphamide (CY) immunosuppressed weaned pigs. The weaned pigs were allotted randomly into one of three treatments, including: (1) non-challenged control; (2) CY-challenged group; and (3) CY + 0.5% Arg. On days 14 and 21 of the trial, pigs were injected with CY or sterile saline. Blood samples were obtained on days 21 and 28 of the trial for further analysis. On day 28, delayed-type hypersensitivity reaction was evaluated. Arg alleviated the decrease of average daily gain ($P < 0.05$) induced by CY challenge from days 21 to 28. Arg mitigated the CY-induced decrease of total white blood cell numbers ($P < 0.05$) on day 28 and improved the lymphocyte percentage on day 21 ($P < 0.05$). Arg increased the delayed-type hypersensitivity reaction ($P < 0.05$), and attenuated the decrease of bovine serum albumin antibody level caused by CY treatment ($P < 0.05$) on day 28. In addition, Arg elevated the levels of serum interleukin-2 and interferon-γ ($P < 0.05$) on day 28, and mitigated the decrease of serum interferon-γ level on day 21 ($P < 0.05$). These results indicate that Arg supplementation has beneficial effects in attenuating the immunosuppressive effects of CY challenge, therefore improving growth performance of young pigs.

Keywords Arginine · Cyclophosphamide · Immunosuppression · Weaned pigs

Abbreviations

ADFI	Average daily feed intake
ADG	Average daily gain
Arg	Arginine
BW	Body weight
PBS	Phosphate buffered saline
CY	Cyclophosphamide
PHA	Phytohemagglutinin
BSA	Bovine serum albumin
ConA	Concanavalin A
IgG	Immunoglobulin G
EDTA	Ethylene diaminetetra-acetate
SEM	Standard error of the mean

J. Han · Y. L. Liu (✉) · W. Fan · J. Chao · Y. Q. Hou · Y. L. Yin · H. L. Zhu · G. Q. Meng · Z. Q. Che
Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Changqing Garden, 430023 Wuhan, Hubei, People's Republic of China
e-mail: yulanflower@126.com

Y. L. Liu
State Key Laboratory of Animal Nutrition, China Agricultural University, 100094 Beijing, China

Y. L. Yin
Institute of Subtropical Agriculture, The Chinese Academy of Sciences, 410125 Changsha, China

Introduction

In swine production, many factors such as infection, stress and early-weaning can result in immunosuppression (El-Abasy et al. 2004), which may decrease growth performance and increase disease susceptibility and mortality (He et al. 2007; Tayade et al. 2006). Thus, immunosuppression can result in a great deal of economic loss, and nutritional modulation of the immune system is very important to the compromised pigs, especially the weaned pigs with immature immune function.

L-Arginine (Arg), a basic amino acid, is traditionally thought of as a nutritionally nonessential amino acid (Wu and Morris 1998). However, in the last two decades, Arg has been shown to be an essential amino acid for birds, carnivores and young mammals and a conditionally essential amino acid for adults (Wu et al. 2004, 2007a). Of particular interest, Arg is the nitrogenous precursor for the synthesis of nitric oxide, a key mediator of the immune response (Popovic et al. 2007) and neurological function (Orlando et al. 2008; Suenaga et al. 2008). Numerous experiments have shown a modulatory role of dietary Arg on immune function (Li et al. 2007; Popovic et al. 2007). Sharma et al. (2004) have reported that Arg supplementation in stressed rats and mice resulted in enhancement of humoral and cellular immune responses. Tayade et al. (2006) demonstrated that Arg supplementation in chickens immunized with intermediate plus strain of infectious bursal disease vaccine improved the humoral immune response. Similarly, dietary Arg supplementation enhanced the immune status of pregnant sows, neonatal pigs and weaned pigs, thereby reducing morbidity and mortality in response to infectious pathogens (Li et al. 2007; Tan et al. 2008a). In addition, Arg is a potent secretagogue for insulin, growth hormone, prolactin and insulin-like growth factor-I (Flynn et al. 2002; Newsholme et al. 2005), and these hormones can mediate an NO-independent effect of Arg on immune function (Li et al. 2007). However, few studies have been conducted to evaluate these effects in immunosuppressed weaned piglets.

In the current study, we used a well documented model to induce immunosuppression in weaned pigs by injecting cyclophosphamide (CY) (Abruzzo et al. 2000; El-Abasy et al. 2004). Our purpose was to investigate the effects of dietary Arg supplementation on performance and immune function in the immunosuppressed weaned pigs.

Materials and methods

Animal care and diets

The animal protocol for this research was approved by the Animal Care and Use Committee of Hubei Province. Twenty-four crossbred pigs (Duroc 'Large White' Landrace) weaned at 28 ± 3 days of age were used in this experiment. After 7-day adaptation, the pigs (35 ± 3 days of age, average body weight of 9.32 ± 0.24 kg) were randomly allotted into three treatments by body weight. The pigs were housed in 1.20 × 1.10 m^2 pens with eight replicate pens per treatment and one pig per pen. Each pen was equipped with plastic slotted floor, a feeder and a nipple waterer to allow pigs ad libitum access to feed and water. The basal diet (Table 1) was formulated to meet or exceed NRC (1998) requirements for all nutrients. Crude protein, calcium and phosphorus of diets were analyzed according to the procedures of the Association of Official Analytical Chemists (AOAC 1990). Room temperature was maintained at 25–27°C by air conditioning.

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

Item	%
Ingredient	
Corn	56.47
Soybean meal (44% CP)	22.00
Wheat middling	6.00
Fish meal	6.00
Soy oil	1.20
Milk replacer	4.00
Glycine[a]	0.86
Cornstarch[a]	0.14
Limestone	0.65
Dicalcium phosphate	1.00
NaCl	0.31
L-Lysine. HCl (78.8% Lysine)	0.32
Butylated hydroquinone	0.05
Vitamin and mineral premix[b]	1.00
Nutrient composition	
Digestible energy[c] (MJ/kg)	13.60
Crude protein[d]	20.00
Calcium[d]	0.80
Total phosphorus[d]	0.70
Total lysine[c]	1.35
Total methionine + cystine[c]	0.65
Total arginine[c]	1.28

[a] In the 0.5% Arg diet, 0.86% glycine and 0.14% cornstarch were replaced by 0.5% Arg and 0.5% cornstarch. The basal and 0.5% Arg diets were isonitrogenous

[b] Provided the following amounts of vitamins and trace minerals per kilogram of complete diet: retinol acetate, 2700 μg; cholecalciferol, 62.5 μg; dl-α-tocopheryl acetate, 20 mg; menadione, 3 mg; vitamin B_{12}, 18 μg; riboflavin, 4 mg; niacin, 40 mg; pantothenic acid, 15 mg; choline chloride, 400 mg; folic acid, 700 μg; thiamin,1.5 mg; pyridoxine, 3 mg; biotin, 100 μg; Zn, 80 mg ($ZnSO_4 \cdot 7H_2O$); Mn, 20 mg ($MnSO_4 \cdot 5H_2O$); Fe, 83 mg ($FeSO_4 \cdot H_2O$); Cu, 25 mg ($CuSO_4 \cdot 5H_2O$); I, 0.48 mg (KI); Se, 0.36 mg ($Na_2SeO_3 \cdot 5H_2O$)

[c] The composition of other essential amino acids in the basal diet (calculated values) was: histidine, 0.48%; isoleucine, 0.83%; leucine, 1.80%; phenylalanine, 0.99%; threonine, 0.84%; tryptophan, 0.38%; tyrosine, 0.45%; valine, 1.05%

[d] Analyzed

Experimental design

Treatments included: (1) non-challenged control (CONTR, pigs fed a control diet and injected with sterile saline); (2) CY-challenged group (CY, pigs fed the same control diet

and challenged with CY); and (3) CY + 0.5% Arg treatment (pigs fed a 0.5% Arg diet and challenged with CY). The dose of 0.5% Arg (L-Arg; purity > 99%; Ajinomoto, Japan) was chosen according to our previous study (Liu et al. 2008). We supplemented 0.86 and 0% glycine (purity > 99%; Ajinomoto, Japan) to the control and 0.5% Arg diets to obtain isonitrogenous diets, respectively. On days 14 and 21 of the trial, the CY and CY + 0.5% Arg groups were administrated intraperitoneally with CY at 50 mg/kg BW, and the CONTR group was administrated an equivalent amount of 0.9% (w/v) NaCl solution. The CY (Suzhou Unite Pharmaceutiical Co., Jiangsu, China) was dissolved in sterile 0.9% NaCl solution. Pigs were individually weighed and feed disappearance was measured on days 0, 14, 21 and 28 throughout the 28-day trial. Feed/gain (F/G) ratio for each period was calculated.

Blood sample collections

On days 21 and 28 of the trial, blood samples (six pigs per treatment) were collected into 10 mL uncoated vacuum tubes (Becton Dickinson Vacutainer System, Franklin Lake, NJ, USA) after 12-h fasting, and centrifuged (3,500×*g* for 10 min) to separate serum. Sera were stored at −80°C until analysis. A second 10 mL sample was collected into a heparinized vacuum tube (Becton Dickinson Vacutainer System, Franklin Lake, NJ, USA) from the same pig for the lymphocyte proliferation study. A third 2 mL sample was collected into an EDTA-K3 anticoagulative vacuum tube (Becton Dickinson Vacutainer System) from the same pig for total and differential leukocyte counts. All the assays were performed in duplicate.

Total and differential leukocyte counts

Total and differential leukocytes counts were assessed by an automated heamatology analyzer Sysmex K4500 (TOA Medical Electronics Co., Kobe, Japan). The differential leukocyte percentage was calculated as the ratio of leukocyte number to total leukocyte number.

Assessment of cellular mediated immune response

Lymphocyte proliferation was measured by using a colorimetric assay with 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Inc., St Louis, USA) in cultures of purified peripheral blood mononuclear cells according to the method of Liu et al. (2003). Briefly, mononuclear cells were isolated by gradient centrifugation from the peripheral blood obtained on days 21 and 28 of the trial, at 3,000×g for 30 min. The cells were washed three times, and then suspended in RPMI-1640 culture medium containing 10% (vol/vol) heat-inactivated fetal calf serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin and 25 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethane-sulfonic acid. After a final wash, cell activity was detected by trypan blue dye exclusion, and the cells were counted to adjust to $2' \times 10^6$ cells/mL culture medium. Then 100 μL of cell suspension was added into 96-well microtiter plates with a total culture volume of 200 μL.

Lymphocyte mitogen concanavalin A (ConA; C-2010, Sigma Chemical Inc.) was added at a final concentration of 8 μg/mL culture medium, and then the plates were incubated at 37°C in a 5% CO_2 incubator for 66 h. After incubation, 10 μL MTT solution (5 mg MTT/mL in 1/15 M phosphate-buffered saline, pH 7.6) was added to each well and the plates were incubated at 37°C for another 6 h. Following incubation, 100 μL of a 10% sodium dodecyl sulfate in 0.04 M HCl solution was added to lyse the cells and solubilize the MTT crystals. Plates were read at 570 nm using an automated microplate reader (Bio-Rad, Model 550, Hercules, CA, USA). Lymphocyte proliferation was expressed as a stimulation index, calculated as the absorbance of wells incubated with ConA divided by the absorbance of wells incubated without ConA.

Delayed-type hypersensitivity reaction was carried out according to the method described by Kornegay et al. (1989). In this experiment, phytohemagglutinin (PHA; Sigma Chemical Inc.) was diluted (150 μg/0.1 mL) in sterile PBS (pH 7.2). On day 28, following blood sample collection, 0.1 mL PHA was injected intradermally on the left flank of the pig about 5 cm posterior to the last nipple and 5 cm from the midline. In the same way, 0.1 mL sample of sterile 0.9% NaCl solution was injected on the right flank of the pig as a control. Skinfold thickness was measured using micrometric calipers 24 h after injection of PHA.

Assessment of humoral immune response

IgG concentration was determined using a radial immunodiffusion test kit according to the method of Hicks et al. (1998). Five microliters of standard solutions and diluted serum sample were added to a separately identified well of the test plates. The plate was securely covered and placed in a 37°C, humidified incubator for 48–72 h. Following incubation, plates were removed and placed over a source of illumination to clearly see precipitin rings. The external diameters of the rings were measured to the nearest 0.1 mm using the scale provided. A reference curve was plotted using the diameters measured from standard solutions. From the reference curve, the IgG concentration of each diluted test sample was calculated by multiplying the concentration read from the curve by the dilution factor to obtain the actual concentration.

On day 14 of the trial, six pigs per treatment were injected intramuscularly with 1 mg/kg BW bovine serum albumin (BSA) to determine BSA specific antibody titers. The BSA (Roche Diagnostic Corporation, IN, USA) was dissolved in sterile 0.9% NaCl solution. Blood samples were collected on days 21 and 28 of the trial. Antibody titers to BSA were determined by enzyme linked immunosorbent assay (ELISA) which was described by He et al. (2007) with slight modifications. Briefly, 96-well plates (Corning Inc., USA) were coated with 100 μL of a solution containing 10 μg BSA in 1 mL of carbonate buffer (0.06 M, pH 9.6; 1.59 g sodium carbonate and 2.93 g sodium bicarbonate were dissolved in 1 L distilled water) and left overnight at 4°C. Plates were then washed three times with 0.01 M phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 (Sigma Chemical Inc., St. Louis, USA). Then plates were sealed with 200 μL of 0.01 M phosphate-buffered saline (pH 7.2) containing 0.03% chicken ovalbumin (Sigma Chemical Inc.) and incubated at 37°C for 2 h. Plates were then washed three times with 0.01 M phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20. Serum samples were diluted with 0.01 M phosphate-buffered saline (pH 7.2) at a dilution of 1:80. Then 100 μL of the diluted serum samples were added to the plates and incubated at 37°C for 1 h. Plates were washed three times with 0.01 M phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20. After washing, a 100 μL solution of rabbit anti-pig IgG conjugated to horseradish peroxidase (Sigma Chemical Co.) was added to each well. After incubation at 37°C for 1 h, the plates were washed three times and 100 μL of substrate, which contained 10 mL of citric acid-phosphate-buffered saline (pH 5.0), 1 mg of 3,3′,5,5′-tetramethylbenzidine (Beijing Biosynthesis Biotechnology Co., Beijing, China) in 0.5 mL anhydrous ethanol and 32 μL of 0.75% H_2O_2, was added to the wells. After incubation for 15 min at room temperature, the reaction was stopped with 1.25 M sulphuric acid, and the plates were read at an absorbance of 405 nm using an ELISA plate reader (Bio-Rad, Model 550, Hercules, CA, USA) against the negative control (PBS replaced the diluted serum).

Serum cytokines

Serum interferon-γ (IFN-γ) was analyzed using a commercially available porcine ELISA kit (R&D Systems, Inc., Minneapolis, MN). The detection limit of porcine IFN-γ was 6.1 pg/mL. The intra- and inter-assay CV were 3.3 and 8.0%. The average recovery of IFN-γ from porcine serum is 98%. Serum interleukin-2 (IL-2) was analyzed using a commercially available swine IL-2 ELISA kit (Uscnlife Science and Technology Co., MO, USA). The detection limit of porcine IL-2 was 1.95 pg/mL.

Statistical analysis

All data were analyzed by ANOVA using the general linear model procedures of SAS (SAS Inst. Inc., Cary, NC, USA) appropriate for randomized complete block design. Differences between means were determined using the Duncan's multiple range tests. Differences were considered as significant when $P < 0.05$.

Results

Growth performance

The growth performance data are presented in Table 2. During days 0–14, 14–21, and 0–28 of the trial, there was no difference in average daily gain (ADG), average daily feed intake (ADFI), and F/G among treatments. During

Table 2 Effects of dietary supplementation of Arg on the growth performance of weaned pigs during pre- and post-challenge periods

Item[1]	CONTR	CY	CY + 0.5% Arg	SEM	*P* value
0–14 days (35–49 days of age)					
ADG (g)	408	427	409	44	0.883
ADFI (g)	664	725	718	89	0.758
F/G	1.63	1.70	1.77	0.17	0.724
14–21 days (49–56 days of age)					
ADG (g)	435	431	470	67	0.814
ADFI (g)	850	861	945	93	0.553
F/G	1.98	2.10	2.02	0.22	0.853
21–28 days (56–63 days of age)					
ADG (g)	607^{c}	280^{a}	402^{b}	56	<0.001
ADFI (g)	1170^{b}	705^{a}	893^{a}	100	0.004
F/G	1.93^{a}	2.56^{b}	2.24^{ab}	0.18	0.020
0–28 days (35–63 days of age)					
ADG (g)	463	393	424	41	0.254
ADFI (g)	835	787	830	76	0.793
F/G	1.80	2.02	1.96	0.10	0.140

CY was injected intraperitoneally to the CY and CY + 0.5% Arg groups on days 14 and 21 of the trial. CONTR (non-challenged control) = pigs fed a control diet and injected with sterile saline; CY (CY challenged control) = pigs fed the same control diet and challenged with CY; CY + 0.5% Arg = pig fed a 0.5% Arg diet and challenged with CY

SEM standard error of the mean, *ADG* average daily gain, *ADFI* average daily feed in take, *F/G* feed/gain, *CY* cyclophosphamide

[1] The pigs were weaned at 28 ± 3 days of age, and the trial began after 1-week adaptation (35 days of age). Pigs were individually weighed and feed disappearance was measured on days 0, 14, 21 and 28 of the trial (the pigs were 35, 49, 56 and 63 days of age, respectively)

[a, b, c] Values within a row with different letters differ ($P < 0.05$); $n = 8$

Table 3 Effects of dietary supplementation of Arg on total and differential leukocyte counts in weaned pigs after repeated cyclophosphamide (CY) challenge

Item	CONTR	CY	CY + 0.5%Arg	SEM	*P* value
21 days					
White blood cell (10^6/mL)	20.20	16.15	15.93	2.59	0.285
Lymphocyte (10^6/mL)	11.67	10.10	10.65	1.59	0.617
Monocyte (10^6/mL)	0.73	0.65	0.87	0.26	0.714
Neutrophil (10^6/mL)	7.80^{b}	5.40^{ab}	4.42^{a}	1.29	0.052
Lymphocyte (%)	58.13^{a}	61.57^{a}	68.42^{b}	3.18	0.017
Monocyte (%)	3.68	4.67	5.02	1.14	0.496
Neutrophil (%)	38.18^{b}	33.78^{b}	26.57^{a}	2.86	0.004
28 days					
White blood cell (10^6/mL)	20.08^{c}	8.08^{a}	10.77^{b}	1.05	<0.001
Lymphocyte (10^6/mL)	13.75^{b}	5.40^{a}	8.05^{a}	1.40	<0.001
Monocyte (10^6/mL)	0.68^{b}	0.30^{a}	0.42^{ab}	0.16	0.083
Neutrophil (10^6/mL)	5.65^{b}	2.38^{a}	2.30^{a}	1.24	0.026
Lymphocyte (%)	66.66	72.58	69.68	4.73	0.474
Monocyte (%)	3.80	4.47	4.17	0.83	0.726
Neutrophil (%)	29.54	22.98	26.15	4.39	0.353

CY was injected intraperitoneally to the CY and CY + 0.5% Arg groups on days 14 and 21 of the trial. Blood samples were obtained on days 21 and 28 of the trial. CONTR (non-challenged control) = pigs fed a control diet and injected with sterile saline; CY (CY challenged control) = pigs fed the same control diet and challenged with CY; CY + 0.5% Arg = pig fed a 0.5% Arg diet and challenged with CY

SEM standard error of the mean

[a, b, c] Values within a row with different letters differ ($P < 0.05$); $n = 6$

days 21–28 of the trial, CY challenge resulted in a 53.9% reduction of ADG, a 39.7% reduction of ADFI and a 32.6% increase of F/G compared to the CONTR pigs ($P < 0.05$). Compared to the CY pigs, pigs fed Arg had a 43.6% higher ADG ($P < 0.05$). However, the CY + 0.5% Arg pigs still showed a lower ADG ($P < 0.05$) compared to CONTR pigs during days 21–28. In addition, the F/G ratio did not differ between the CONTR and the CY + 0.5% Arg groups during days 21–28 ($P > 0.05$).

Total and differential leukocyte counts

Compared to the CONTR pigs, the CY pigs had lower ($P < 0.05$) concentrations of white blood cells, lymphocytes, monocytes and neutrophils on day 28 (Table 3). Compared to the CY pigs, dietary Arg supplementation increased the lymphocyte percentage on day 21 and the white blood cell concentration on day 28 ($P < 0.05$). However, the CY + 0.5% Arg pigs still had a lower concentration of white blood cells on day 28 in comparison with the CONTR pigs ($P < 0.05$). In addition, the CY + 0.5% Arg group of pigs had a higher lymphocytes percentage on day 21 ($P < 0.05$) but a similar monocyte concentration on day 28 ($P > 0.05$) compared to the CONTR pigs. Arg supplementation decreased the neutrophil percentage on day 21 compared to the CONTR pigs and the CY pigs ($P < 0.05$).

Cellular immune mediated response

The data for lymphocyte proliferation and delayed type hypersensitivity reaction are summarized in Table 4. The CY treatment had no effect on lymphocyte proliferation and skinfold thickness indicative of hypersensitivity reaction. In addition, 0.5% Arg supplementation had no effect

Table 4 Effects of dietary supplementation of Arg on cellular mediated immune response in weaned pigs after repeated cyclophosphamide (CY) challenge

Item	CONTR	CY	CY + 0.5%Arg	SEM	*P* value
Skinfold thickness(mm)	0.39^{a}	0.36^{a}	0.63^{b}	0.010	0.050
Lymphocyte proliferation					
21 days	1.226	1.217	1.222	0.070	0.992
28 days	1.222	1.214	1.369	0.075	0.099

CY was injected intraperitoneally to the CY and CY + 0.5% Arg groups on days 14 and 21 of the trial. Blood samples were obtained on days 21 and 28 of the trial. CONTR (non-challenged control) = pigs fed a control diet and injected with sterile saline; CY (CY challenged control) = pigs fed the same control diet and challenged with CY; CY + 0.5% Arg = pig fed a 0.5% Arg diet and challenged with CY

SEM standard error of the mean

[a, b, c] Values within a row with different letters differ ($P < 0.05$); $n = 6$

on lymphocyte proliferation. However, higher skin thickness was detected in the CY + 0.5% Arg pigs compared to the CY pigs and the CONTR pigs ($P < 0.05$).

Humoral immune response

The humoral immune response was measured by the specific antibody response to BSA and IgG. As shown in Table 5, the CY treatment or Arg supplementation had no effect on antibody response to BSA on day 21. The CY pigs had a lower level of the BSA antibody compared to the CONTR pigs ($P < 0.05$). The CY + 0.5% Arg pigs had a higher level of the BSA antibody on day 28 compared to the CY pigs and the CONTR pigs ($P < 0.05$). The CY challenge and Arg supplementation did not affect the concentration of IgG in serum.

Serum cytokines

The data of serum cytokines (IL-2 and IFN-γ) are presented in Table 6. Compared to the CONTR pigs, the CY treatment had no effect on serum IL-2 levels on days 21 and 28. Arg supplementation increased IL-2 levels on day 21, compared to the CY pigs ($P < 0.05$). The CY challenge decreased the serum level of IFN-γ on day 21 compared to the CONTR pigs ($P < 0.05$). Dietary supplementation of Arg enhanced serum IFN-γ levels on days 21 and 28, compared to the CY pigs ($P < 0.05$). Moreover, compared to the CONTR pigs, the CY + 0.5% Arg group of pigs had a higher level of IFN-γ in serum on days 21 and 28 ($P < 0.05$).

Table 5 Effects of dietary supplementation of Arg on humoral immune response in weaned pigs after repeated cyclophosphamide (CY) challenge

Item	CONTR	CY	CY + 0.5%Arg	SEM	*P* value
IgG (mg/mL)					
21 days	2.50	2.72	2.98	0.242	0.180
28 days	2.09	2.22	2.16	0.104	0.453
BSA antibody[1]					
21 days	0.429	0.430	0.438	0.032	0.951
28 days	0.571[b]	0.439[a]	0.661[c]	0.041	<0.001

CY was injected intraperitoneally to the CY and CY + 0.5% Arg groups on days 14 and 21 of the trial. Blood samples were obtained on days 21 and 28 of the trial. CONTR (non-challenged control) = pigs fed a control diet and injected with sterile saline; CY (CY challenged control) = pigs fed the same control diet and challenged with CY; CY + 0.5% Arg = pig fed a 0.5% Arg diet and challenged with CY

SEM standard error of the mean, *IgG* Immunoglobulin G, *BSA* bovine serum albumin

[1] BSA was injected intramuscually to six pigs per treatment on day 14. Specific antibody production was measured by ELISA at days 21 and 28. The values were expressed as absorbance at 450 nm

[a, b, c] Values within a row with different letters differ ($P < 0.05$); $n = 6$

Table 6 Effects of dietary supplementation of Arg on serum cytokines in weaned pigs after repeated cyclophosphamide (CY) challenge

Item	CONTR	CY	CY + 0.5%Arg	SEM	*P* value
IL-2 (pg/mL)					
21 days	9.67[ab]	8.29[a]	10.73[b]	0.90	0.049
28 days	11.58	12.21	11.67	1.55	0.907
IFN-γ (pg/mL)					
21 days	25.66[b]	15.39[a]	44.19[c]	4.42	<0.001
28 days	31.68[a]	31.79[a]	52.67[b]	7.40	0.018

CY was injected intraperitoneally to the CY and CY + 0.5% Arg groups on days 14 and 21 of the trial. Blood samples were obtained on days 21 and 28 of the trial. CONTR (non-challenged control) = pigs fed a control diet and injected with sterile saline; CY (CY challenged control) = pigs fed the same control diet and challenged with CY; CY + 0.5% Arg = pig fed a 0.5% Arg diet and challenged with CY

SEM standard error of the mean, *IL-2* interleukin-2, *IFN-γ* interferon-γ

[a, b, c] Values within a row with different letters differ ($P < 0.05$); $n = 6$

Discussion

Immunosuppression can result in reduced feed intake, body weight gain, feed conversion ratio, infection resistance, antibody synthesis and cellular immunity (El-Abasy et al. 2004; Sharma et al. 2004). To evaluate whether Arg supplementation could improve the immune function of immunosuppressed weaned pigs, we used a well documented CY-induced immunosuppressive model for weaned pigs (He et al. 2007; Ninomiyal et al. 2005). CY, widely used in organ transplantation and treatment of various autoimmune disorders (El-Abasy et al. 2004), is a potent immunosuppressive agent. In the present study, the CY challenge decreased performance of weaned pigs during the second CY challenge, which is line with the findings of El-Abasy et al. (2004) and He et al. (2007). Additionally, the weaned pigs treated with CY had lower concentrations of total white blood cells and differential leukocytes, the serum BSA antibody and IFN-γ compared to the CONTR pigs, which indicates that CY suppressed immune function in weaned pigs. However, in vitro lymphocyte proliferation and in vivo delayed-type hypersensitivity reaction were not affected by CY administration. It has been reported that CY affects primarily antibody-mediated immunity, and causes depletion of B-lymphocytes and suppresses humoral immunity (Corrier et al. 1991). However, reports on the effect of CY on T-cell activity are not consistent. For instance, CY has been reported to have no effect (Lefkovits et al. 1974) or an inhibitory effect (Hou et al. 2007) on

T-cell function. In our current study, CY decreased lymphocyte concentration but not lymphocyte proliferation.

Results of the present study indicate that prior to CY challenge, Arg supplementation had no effect on growth performance of weaned pigs. However, pigs supplemented with 0.5% Arg enhanced weight gain during the second CY challenge period compared to the CY pigs, which suggests that Arg attenuated the growth-suppressive effects of CY treatment. Similarly, Liu et al. (2008) reported that 0.5% Arg supplementation alleviated the weight loss induced by *E. coli* lipopolysaccharide challenge in weaned pigs. Although Arg can be synthesized from glutamine, glutamate, and proline by young pigs via the intestinal-renal axis (Wu and Knabe 1995; Wu 1997), the endogenous synthesis of Arg is inadequate for their maximal growth and optimal metabolic function (Kim and Wu 2004; Wu et al. 2004, 2007a). Accordingly, Yao et al. (2008) reported that supplementing 0.6% Arg to the diet for neonatal pigs increased daily gain, plasma insulin concentration, and protein synthesis in skeletal muscle. Similarly, Arg acts as a metabolic regulator to increase protein synthesis and decrease protein catabolism under infection and stress conditions (Frank et al. 2007), by stimulating the secretion of insulin, growth hormone and glucagons (Wu et al. 2000) and cellular signaling mechanisms (Jobgen et al. 2006). Therefore, it is possible that 0.5% Arg supplementation attenuated the growth-suppressive effects of the CY challenge partially by decreasing protein catabolism and increasing protein synthesis in skeletal muscle. Notably, an anabolic effect of Arg to favor whole-body protein deposition has also been reported for finishing pigs (Tan et al. 2008b).

Total and differential white blood cells are commonly measured indices for immune status in pigs (Tan et al. 2008a). In the present study, 0.5% Arg supplementation increased the numbers of white blood cells on day 28 and the percentage of lymphocytes on day 21 compared to the CY pigs. In agreement with our findings, van der Schueren et al. (2001) reported that preoperative and postoperative supplementation of Arg to neck cancer patients increased the number of leukocytes. Stechmiller et al. (2004) also found that Arg stimulated the production and activity of leukocytes in critically ill patients. It is well-known that adrenal hormones affect blood leukocyte distribution (Geffner et al. 1995). Thus, an increase in plasma corticosterone level was associated with a decrease in the numbers of total white blood cells and lymphocytes (Cunnick et al. 1990). Additionally, Arg could decrease corticosterone biosynthesis in rats (Cymeryng et al. 1999) and mice (Repetto et al. 2006). Therefore, it is possible that supplementing Arg to the CY-challenged pigs increased the number of white blood cells and lymphocytes partially by decreasing plasma corticosterone level.

In vitro lymphocyte proliferation and in vivo delayed-type hypersensitivity reaction are widely used to evaluate cell-mediated immune function in experimental animals (Li et al. 2007). In the present study, dietary supplementation of 0.5% Arg had no effect on lymphocyte proliferation compared to the CY pigs. Our findings are consistent with the results of Torre et al. (1993) who reported that adding Arg to the drinking water (7.5 g/L) did not affect lymphocyte proliferation in rats with lipopolysaccharide-induced inflammation. Langkamp-Henken et al. (2000) also reported that Arg supplementation did not enhance lymphocyte proliferation in nursing home residents with pressure ulcers. However, several experiments have shown that Arg supplementation stimulated lymphocyte proliferation (Liu et al. 2005; Tayade et al. 2006). The reason for the discrepancy might be associated with the amount of Arg supplemented to the experimental diets (Kong et al. 2008), the regulatory effect of dietary amino acids (Galli 2007; Hu et al. 2008), and the species of experimental animals (Tujioka et al. 2007; Yin et al. 2008). The supplemental dose of Arg is not expected to cause an amino acid imbalance in pigs (Wu et al. 2007b). Our results of delayed-type hypersensitivity showed an elevated cell-mediated immune response in vivo after Arg supplementation. This result indicates that Arg modulates T cell-mediated hypersensitivity reaction to mitogens. Similarly, enhanced delayed type hypersensitivity reactions were observed in the immunosuppressed chickens induced by the infectious bursal disease virus (Tayade et al. 2006) and in athymic nude mice (Kirk et al. 1992).

The BSA antibody is used to evaluate humoral immune function in experimental animals. In the current study, Arg supplementation resulted in a higher level of the BSA antibody on day 28 compared to the CY pigs and the CONTR pigs. In agreement with our observations, many studies have reported that Arg supplementation enhanced the specific antibody production in stress animals (Sharma et al. 2004). Additionally, Arg plays a major role in B-cell maturation (De Jonge et al. 2002). It is possible that feeding pigs an Arg-rich diet promoted the production of the BSA antibody partially by stimulating the differentiation and maturation of B cells associating with the humoral immune response.

To future explore the effect of Arg on modulating immune function in weaned pigs under the immunosuppressive status, the serum concentrations of IL-2 and INF-γ were measured in the present study. IL-2 and IFN-γ are involved in immune regulation and host defense (Li et al. 2007). IL-2, produced primarily by T helper 1 (TH1), regulates the differentiation and activation of T cells, B cells, natural and lymphocyte-activated killer cells, monocytes and macrophages that are involved in cellular and humoral immune responses. IFN-γ is a potent immunomodulatory cytokine primarily produced by T-helper 1

and NK cells. IFN-γ participates in a variety of immune responses, including T cell proliferation and differentiation, pro-inflammatory cytokine production and macrophage activation by up-regulating major histocompatibility complex class II molecules, and induces effector molecules such as reactive nitrogen intermediates (Allam et al. 2007). In the present study, Arg supplementation increased IL-2 concentration in serum on day 28, and IFN-γ concentration on days 21 and 28, compared to the CY pigs. Similarly, Angele et al. (1999) reported that L-Arg increased IL-2 release in rats following trauma-hemorrhage and Yeh et al. (2003) found that dietary Arg supplementation increased IFN-γ concentration in serum of burned mice. Additionally, we observed the CY + 0.5% Arg group of pigs had a 70% higher IFN-γ concentration in serum compared to the CONTR pigs on days 21 and 28. Because the CY + 0.5% Arg group of pigs had better growth performance during days 21–28 compared to the CY pigs. Therefore, it is likely that a higher IFN-γ concentration had no negative effect in Arg-supplemented pigs.

TH1 lymphocytes secrete predominantly IL-2 and INF-γ, whereas T helper 2 (TH2) lymphocytes secrete IL-4, IL-5, IL-6 and IL-10 (Raymond and Wilkie 2004). TH1 lymphocytes play a role in cell-mediated immune functions, such as delayed-type hypersensitivity and activation of cytotoxic T lymphocytes and inflammatory macrophages, while TH2 lymphocytes are responsible for B lymphocyte, mast cell and eosinophil activation (Angele et al. 1999). In the present study, we observed that IL-2 and INF-γ levels in serum were increased and delayed type hypersensitivity reaction were enhanced simultaneously in Arg-supplemented pigs after CY challenge. Thus, we suggest that the alleviating effects of Arg supplementation on the suppressive cell-mediated immune response of CY treated pigs were associated with increasing release of IL-2 and INF-γ. Furthermore, Arg supplementation increased the BSA antibody level in serum. However, we did not determine the cytokines secreted by TH2 lymphocytes.

In the current work, glycine was used as the isonitrogenous control. Because this amino acid is known to influence immune function (Li et al. 2007), some of the effects of Arg may have been masked in the present study. In addition, the small sample was a limitation for identifying modest differences among treatment groups. Further studies are needed to evaluate the effects of glycine and Arg on these measurements in pigs.

In conclusion, dietary supplementation of Arg exerts beneficial effects in attenuating the immunosuppressive status of the CY-challenged weaned pigs. The alleviating effects of Arg supplementation on suppressive immune function are associated with increasing levels of IL-2 and INF-γ in piglet serum. Under stress conditions, Arg is beneficial for young pigs.

Acknowledgements This work was supported by the National Natural Science Foundation of China (30500362), the National Basic Research Program of China (2004CB117504), the Hubei Provincial Department of Education (D200718003), and the State Key Laboratory of Animal Nutrition (2007KLAN002).

References

Abruzzo GK, Gill CJ, Flattery AM, Li K, Leighton C, Smith JG, Pikounis VB, Bartizal K, Rosen H (2000) Efficacy of the echinocandin caspofungin against disseminated aspergillosis and candidiasis in cyclophosphamide-induced immunosuppressed mice. Antimicrob Agents Chemother 44:2310–2318

Allam M, Julien N, Zacharie B, Penney C, Gagnon L (2007) Enhancement of Th1 type cytokine production and primary T cell activation by PBI-1393. Clin Immunol 125:318–327

Angele MK, Mall N, Knoferl MW, Ayala A, Cioffi WG, Chaudry IH (1999) L-Arginine restores splenocyte functions after trauma and hemorrhage potentially by improving splenic blood flow. Am J Physiol Cell Physiol 276:C145–C151

AOAC (1990) Official methods of analysis, 15th edn. Association of Official Analytical Chemists, Washington, DC

Corrier DE, Elissald MH, Ziprin RL, Deloach JR (1991) Effect of immunosuppression with cyclophosphamide, cyclosporin, or dexamethazone on Salmonella colonization of broiler chickens. Avian Dis 35:40–45

Cunnick JE, Lysle DT, Kucinski BJ, Rabin BS (1990) Evidence that shock-induced immune suppression is mediated by adrenal hormones and peripheral beta-adrenergic receptors. Pharmacol Biochem Behav 36:645–651

Cymeryng CB, Dada LA, Colonna C, Mendez CF, Podesta EJ (1999) Effects of l-arginine in rat adrenal cells: involvement of nitric oxide synthase. Endocrinology 140:2962–2967

De Jonge WJ, Kwikkers KL, te Velde AA, van Deventer SJ, Nolte MA, Mebius RE, Ruijter MJ, Lamers WH (2002) Arginine deficiency affects early B cell maturation and lymphoid organ development in transgenic mice. J Clin Invest 110:1539–1548

El-Abasy M, Motobu M, Nakamura K, Koge K, Onodera T, Vainio O, Toivanen P, Hirota Y (2004) Preventive and therapeutic effects of sugar cane extract on cyclophosphamide-induced immunosuppression in chickens. Int Immunopharmacol 4:983–990

Flynn NE, Meininger CJ, Haynes TE, Wu G (2002) The metabolic basis of arginine nutrition and pharmacotherapy. Biomed Pharmacother 56:427–438

Frank JW, Escobar J, Nguyen HV, Jobgen SC, Jobgen WS, Davis TA, Wu G (2007) Oral N-carbamylglutamate supplementation increases protein synthesis in skeletal muscle of piglets. J Nutr 137:315–319

Galli F (2007) Amino acid and protein modification by oxygen and nitrogen species. Amino Acids 32:497–499

Geffner JR, Trevani AS, de D'ElIa I, Diament M, Klein D, Giordano M (1995) Involvement of nitric oxide in the regulation of peripheral blood leukocyte counts. J Leukoc Biol 58:391–394

He X, Yang XJ, Guo YM (2007) Effects of different dietary oil sources on immune function in cyclophosphamide immunosuppressed chickens. Anim Feed Sci Tech 139:186–200

Hicks TA, McGlone JJ, Scott Whisnant C, Kattesh HG, Norman RL (1998) Behavioral, endocrine, immune, and performance measures for pigs exposed to acute stress. J Anim Sci 76:474–483

Hou FX, Yang HF, Yao T, Chen W (2007) The immunosuppressive effects of 10 mg/kg cyclophosphamide in Wistar rats. Environ Toxicol Pharmacol 24:30–36

Hu CA, Khalil S, Zhaorigetu S, Liu Z, Tyler M, Wan G, Valle D (2008) Human Δ1-pyrroline-5-carboxylate synthase: function and regulation. Amino Acids. doi: 10.1007/S00726-008-0075-0

Jobgen WS, Fried SK, Fu WJ, Meininger CJ, Wu G (2006) Regulatory role for the arginine-nitric oxide pathway in metabolism of energy substrates. J Nutr Biochem 17:571–588

Kim SW, Wu G (2004) Dietary arginine supplementation enhances the growth of milk-fed young pigs. J Nutr 134:625–630

Kirk SJ, Regan MC, Wasserkrug HL, Sodeyama M, Barbul A (1992) Arginine enhance T-cell responses in athymic nude mice. J Parenter Enteral Nutr 16:429–432

Kong XF, Yin YL, He QH, Yin FG, Liu HJ, Li TJ, Huang RL, Geng MM, Ruan Z, Deng ZY, Xie MY, Wu G (2008) Dietary supplementation with Chinese herbal powder enhances ileal digestibilities and serum concentrations of amino acids in young pigs. Amino Acids. doi: 10.1007/s00726–008-0176-9

Kornegay ET, van Heugten PHG, Lindemann MD, Blodgett DJ (1989) Effects of biotin and high copper levels on performance and immune response of weanling pigs. J Anim Sci 67:1471–1477

Langkamp-Henken B, Herrlinger-Garcia KA, Stechmiller JK, Nickerson-Troy JA, Lewis B, Moffatt L (2000) Arginine supplementation is well tolerated but does not enhance mitogen-induced lymphocyte proliferation in elderly nursing home residents with pressure ulcers. J Parenter Enteral Nutr 24:280–287

Lefkovits H, Reber K, Lefkovits I (1974) An in vitro analysis of the immune capabilities of mice treated with immunosuppressive drugs. Int Arch Allergy Appl Immunol 46:689–694

Li P, Yin YL, Li DF, Kim SW, Wu GY (2007) Amino acids and immune function. Brit J Nutr 98:237–252

Liu YL, Li DF, Gong LM, Feng ZY, Yi GF, Gaines AM, Carroll JA (2003) Effects of fish oil supplementation on performance as well as immunological, adrenal and somatotropic responses of weaned pigs after *Escherichia coli* lipopolysaccharide challenge. J Anim Sci 81:2758–2765

Liu CT, Chen KM, Lee SH, Tsai LJ (2005) Effect of supplemental L-arginine on the function of T lymphocytes and the formation of advanced glycosylated end products in rats with streptozotocin-induced diabetes. Nutrition 21:615–623

Liu YL, Huang JJ, Hou YQ, Zhu HL, Zhao SJ, Ding BY, Yin YL, Yi GF, Shi JX, Fan W (2008) Dietary arginine supplementation alleviates intestinal mucosal disruption induced by *Escherichia coli* lipopolysaccharide in weaned pigs. Br J Nutr 100:552–560

Newsholme P, Brennnan L, Rubi B, Maechler P (2005) New insights into amino acid metabolism, beta-cell function and diabetes. Clin Sci 108:185–194

Ninomiyal M, Mikamo H, Tanaka K, Watanabe K, Tamayal T (2005) Efficacy of micafungin against deep-seated candidiasis in cyclophosphamide-induced immunosuppressed mice. J Antimicrob Chemother 55:587–590

NRC (1998) Nutrient Requirements of Swine, 10th edn. National Academic Press, Washington, DC

Orlando GF, Wolf G, Engelmann M (2008) Role of neuronal nitric oxide synthase in the regulation of the neuroendocrine stress response in rodents: insights from mutant mice. Amino Acids 35:17–27

Popovic PJ, Zeh HJIII, Ochoa JB (2007) Arginine and immunity. J Nutr 137:1681S–1686S

Raymond CR, Wilkie BN (2004) Th-1/Th-2 type cytokine profiles of pig T-cells cultured with antigen-treated monocyte-derived dendritic cells. Vaccine 22:1016–1023

Repetto EM, Pannunzio V, Astort F, Calejman CM, Moreno MB, Pignataro OP, Cymeryng CB (2006) Characterization of L-arginine transport in adrenal cells: effect of ACTH. Am J Physiol Endocrinol Metab 291:291–297

Sharma KK, Mediratta PK, Reeta KH, Mahajan P (2004) Effect of L-arginine on restraint stress induced modulation of immune responses in rats and mice. Pharmacol Res 49:455–460

Stechmiller JK, Childress B, Porter T (2004) Arginine immunonutrition in critically ill patients: a clinical dilemma. Am J Crit Care 13:17–23

Suenaga R, Tomonaga S, Yamane H, Kurauchi I, Tsuneyoshi Y, Sato H, Denbow DM, Furuse M (2008) Intracerebroventricular injection of L-arginine induces sedative and hypnotic effects under an acute stress in neonatal chicks. Amino Acids 35:139–146

Tan BE, Li XG, Kong XF, Huang RL, Ruan Z, Deng ZY, Xie MY, Shinzato I, Yin YL, Wu G (2008a) Dietary L-arginine supplementation enhances the immune status in early-weaned piglets. Amino Acids. doi:10.1007/s00726–008-0155-1

Tan BE, Yin YL, Liu ZQ, Li XG, Xu HJ, Kong XF, Huang RL, Tang WJ, Shinzato I, Smith SB, Wu G (2008b) Dietary L-arginine supplementation increases muscle gain and reduces body fat mass in growing-finishing pigs. Amino Acids. doi:10.1007/s00726-008-0148-0

Tayade C, Jaiswal TN, Mishra SC, Koti M (2006) L-Arginine stimulates immune response in chickens immunized with intermediate plus strain of infectious bursal disease vaccine. Vaccine 24:552–560

Torre PM, Ronnenberg AG, Hartman WJ, Prior RL (1993) Oral arginine supplementation does not affectlymphocyte proliferation during endotoxin-induced inflammation in rats. J Nutr 123:481–488

Tujioka K, Okuyama S, Yokogoshi H, Fukaya Y, Hayase K, Horie K, Kim M (2007) Dietaryγ-aminobutyric acid affects the brain protein synthesis rate in young rats. Amino Acids 32:255–260

van der Schueren MAE, Quak JJ, van der Flier BME, Kuik DJ, Langendoen SI, Snow GB, Green CJ, van Leeuwen PAM (2001) Effect of perioperative nutrition, with and without arginine supplementation, on nutritional status, immune function, postoperative morbidity, and survival in severely malnourished head and neck cancer patients. Am J Clin Nutr 73:323–332

Wu G (1997) Synthesis of citrulline and arginine from proline in enterocytes of postnatal pigs. Am J Physiol 272:G1382–G1390

Wu G, Knabe DA (1995) Arginine synthesis in enterocytes of neonatal pigs. Am J Physiol Regulatory Integrative Comp Physiol 269:R621–R629

Wu G, Morris SM (1998) Arginine metabolism: nitric oxide and beyond. Biochem J 336:1–17

Wu G, Meininger CJ, Knabe DA, Bazer FW, Rhoads JM (2000) Arginine nutrition in development, health and disease. Curr Opin Clin Nutr Metab Care 3:59–66

Wu G, Knabe DA, Kim SW (2004) Arginine nutrition in neonatal pigs. J Nutr 134:2783S–2790S

Wu G, Bazer FW, Davis TA, Jaeger LA, Johnson GA, Kim SW, Knabe DA, Meininger CJ, Spencer TE, Yin YL (2007a) Important roles for the arginine family of amino acids in swine nutrition and production. Livest Sci 112:8–22

Wu G, Bazer FW, Cudd TA, Jobgen WS, Kim SW, Lassala A, Li P, Matis JH, Meininger CJ, Spencer TE (2007b) Pharmacokinetics and safety of arginine supplementation in animals. J Nutr 137:1673S–1680S

Yao K, Yin YL, Chu WY, Liu ZQ, Deng D, Li TJ, Huang RL, Zhang JS, Tan B, Wang WC, Wu GY (2008) Dietary arginine supplementation increases mTOR signaling activity in skeletal muscle of neonatal pigs. J Nutr 138:867–872

Yeh SL, Tsai HJ, Chiu WC, Shang HF (2003) Effects of dietary arginine supplementation on nutrient metabolism and survival rate in burned mice. Nutr Res 23:331–341

Yin FG, Liu YL, Yin YL, Kong XF, Huang RL, Li TJ, Wu GY, Hou YQ (2008) Dietary supplementation with *Astragalus* polysaccharide enhances ileal digestibilities and serum concentrations of amino acids in early weaned piglets. Amino Acids. doi: 10.1007/s00726–008-0142-6