ORIGINAL ARTICLE

Dietary L-glutamine supplementation improves pregnancy outcome in mice infected with type-2 porcine circovirus

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Abstract Porcine circovirus type 2 (PCV2) causes reproductive failure in swine. As glutamine can enhance immune function in animals, this study was conducted with mice to test the hypothesis that dietary glutamine supplementation will improve pregnancy outcome in PCV2-infected dams. Beginning on day 0 of gestation, mice were fed a standard diet supplemented with 1.0% L-glutamine or 1.22% L-alanine (isonitrogenous control). All mice were infected with PCV2 (2000 $TCID_{50}$) on day 10 of gestation. On day 17 of gestation, six mice from each group were euthanized to obtain maternal tissues and fetuses for hematology and histopathology tests. The remaining mice continued to receive their respective diets supplemented with 1.0% L-glutamine or 1.22% L-alanine through lactation. The PCV2 virus was present in maternal samples (serum and lung) of most mice in the control group but was not detected in the glutamine-supplemented mice. Dietary glutamine supplementation reduced abortion, decreased fetal deaths, and enhanced neonatal survival. The glutamine treatment also reduced concentrations of interleukin-6, while increasing concentrations of tumor necrosis factor-α and C-reactive protein, in the maternal serum of mice. Furthermore, glutamine supplementation attenuated microscopic lesions in maternal tissues (lung, spleen, and liver). Collectively, these results indicate that dietary glutamine supplementation is beneficial for ameliorating reproductive failure in virus-infected mice. The findings support the notion that gestating dams require adequate amounts of dietary glutamine for the optimal survival and growth of embryos, fetuses, and neonates, and have important implications for nutritional support of mammals (including swine and humans) during gestation and lactation.

Keywords Porcine circovirus type 2 · Pigs · Nutrition · Amino acids · Reproductive failure

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Abbreviations
CRP	C-Reactive protein
IL-1β	Interleukin-1β
IL-6	Interleukin-6
PCV2	Porcine circovirus type 2
qPCR	Quantitative polymerase chain reaction
TNF-α	Tumor necrosis factor-α

Introduction

Infection is the colonization of a host by pathogenic organisms (e.g., viruses, prions, bacteria, and fungi)

(Grau-Roma et al. 2011). Available evidence shows that infectious diseases are a major cause for the loss of pregnancy in both humans and livestock species (Vincent et al. 2010). Thus, prophylactic measures should be taken to protect gestating dams against the pathogens. Currently, the available methods include the use of antibiotics, the optimization of housing and management conditions, the development of vaccines, and improved breeding to increase resistance to disease. Unfortunately, the inclusion of antibiotics as dietary additives has been restricted because of concerns about the development of antimicrobial resistance and the transference of antibiotic resistance genes from animals to human microbiota (Monroe and Polk 2000). Also, housing and management conditions frequently cannot be optimized due to practical constraints in the system of swine production. Furthermore, vaccines may be associated with some disadvantages, including a prolonged period of time from research to marketing, efficacy, costs, and the risk of failure (Eamens et al. 2006). Thus, nutritional regulation, particularly the use of functional amino acids to modulate immune responses and enhance resistance to infectious diseases, may be an attractive solution (Li et al. 2007; Ren et al. 2011).

Porcine circovirus type 2 (PCV2) causes the post-weaning multi-systemic wasting syndrome in piglets (Allan et al. 1998). This disease is characterized by progressive weight loss, generalized enlargement of lymph nodes, as well as the symptoms of pallor, jaundice, and diarrhea (Stevenson et al. 2001). In addition to neonates, PCV2 can infect gestating pigs, resulting in reproductive failure, fetal deaths, and neonatal mortality (Ladekjaer-Mikkelsen et al. 2001). The PCV2 infection results in substantial economic losses to pork producers (Opriessnig et al. 2009). At present, effective methods for controlling PCV2 are limited.

Glutamine is an amino acid crucial for optimal immune responses (Li et al. 2007), fetal growth and survival (Wu et al. 2010a), and metabolic regulation (Bonetto et al. 2011; Cooksey and McClain 2011; Rogero et al. 2010). Specifically, glutamine plays a role in: (1) the metabolism of carbohydrates, fatty acids, and proteins, (2) proliferation and differentiation of fibroblasts, lymphocytes and enterocytes, (3) phagocytosis of pathogens by activated macrophages, (4) production of cytokines, and (5) intracellular redox status and antioxidative reactions (Li et al. 2007; Wells et al. 1999; Wu 2009; Xi et al. 2011). Thus, glutamine may be able to ameliorate the reproductive failure caused by PCV2. At present, little is known about the effects of glutamine on embryonic and fetal survival in animals or humans in response to infectious disease. We hypothesized that dietary glutamine supplementation may enhance the host resistance to PCV2 and pregnancy

outcome in mammals. The present study was conducted with mice to test this novel hypothesis.

Materials and methods

Preparation of PCV2 stock

A PCV2 infectious clone constructed by self-ligation of the PCV2 genome via a *SacII* enzyme site was used to generate the virus stock pools required for experimental infection (Ren et al. 2011). In brief, the continuous porcine kidney cell line PK-15 (Wen et al. 2008), free of PCV1 and PCV2, was cultured in the RPMI-1640 medium supplemented with 6% (vol/vol) fetal calf serum (FCS). The cell monolayer was dispersed by using trypsin–EDTA, and suspended in this RPMI-1640 medium. The porcine kidney PK-15 cells were simultaneously infected with the PCV2 clone. After a 72-h period of culture, the infected cells were frozen and thawed three times, and the cell extract was tested by the polymerase chain reaction (PCR) technique before being stored at $-20°C$. PCV2 stocks were titrated with the porcine kidney PK-15 cells (Wen et al. 2008).

Animals and experimental protocol

The experiment was conducted as described previously (Ren et al. 2011) with some modifications. In brief, 80 female primiparous KM mice were obtained from Laboratory Animal Center of Central South University (Hunan, China). The mice were housed individually in a pathogen-free mouse colony (room temperature = 20–30°C, relative humidity = 45–60%, lighting cycle = 12 h light and 12 h dark) and had free access to food and drinking water. The mice were randomly assigned to the L-glutamine group (supplementation of 1.0% L-glutamine to the basal diet, $n = 40$) or control (isonitrogenous) group (supplementation of 1.22% L-alanine to the basal diet, $n = 40$). Both glutamine and alanine were products of Ajinomoto Inc. (Tokyo, Japan). The content of amino acids in the basal diet was measured using an automatic amino acid analyzer (Kong et al. 2009) after acid hydrolysis (Li et al. 2011; Yin et al. 1993, 2008a). This basal diet contained 1.94% L-glutamate, 1.80% L-glutamine, and 0.91% L-alanine.

After 3 days of acclimation, female mice started to mate with fertile males of the same genetic background. Beginning on day 0 of gestation (the date of mating), the female mice started receiving dietary supplementation with either 1.0% L-glutamine or 1.22% L-alanine. On day 9 after copulation, 18 and 24 mice were found to be pregnant in the glutamine and control groups, respectively. All the mice were infected with PCV2 (2000 $TCID_{50}$) on day 10 of gestation and six mice from each group were euthanized on

day 17 of gestation to obtain maternal blood and organs as well as fetuses. The remaining mice continued to receive their respective diets supplemented with 1.0% ʟ-glutamine or 1.22% ʟ-alanine through lactation. Abortion rate is defined as the number of pregnant mice with abortion/the total number of pregnant mice. After parturition, neonates were reared by their mothers. Neonatal survival rate within the first 12 days after birth is defined as the number of live neonates/the total number of neonates. Daily weight gains of postnatal mice were recorded. This study was performed according to the guidelines of the Laboratory Animal Ethical Commission of the Chinese Academy of Sciences (the registry number was 011063506).

Analysis of cytokines in serum

Interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and C-reactive protein (CRP) in serum were measured using ELISA kits in accordance with the manufacturer's instructions (Cusabio Biotech Co., Ltd., China) as described by Yin et al. (2008b). An aliquot (100 µl) of serum or standard was added to duplicate wells of a microtiter plate, which had been pre-coated with a specific antibody. The buffer was used as a negative control. The plate was incubated for 2 h at 37°C. A 100 µl of a biotin-antibody was added to each well after the removal of the liquid from each well and incubated at 37°C for 1 h. The wells were washed three times with 200 µl of the washing buffer. A 100 µl quantity of horseradish peroxidase-conjugated avidin was then added to each well and incubated at 37°C for 1 h. After the final washing, an aliquot (90 µl) of the TMB substrate was added and incubated at 37°C for 30 min in the dark. The reaction was stopped with 50 µl of a terminating solution and absorbance measured at 450 nm.

Hematology tests for pregnant mice

Blood samples were prepared as we described previously (Kong et al. 2009; Yin et al. 2010a). Hematology tests were conducted at Huangxing Hospital (Changsha, China).

DNA extraction and quantitative PCR (qPCR) analysis of PCV2

DNA was extracted from maternal samples (5 mg spleen, 10 mg lung, and 100 µl serum) and 10 mg fetus, using tissue genomic DNA extraction kits (Betimes Biotechnology Co. Ltd., China) according to the manufacturer's instructions. DNA from the samples was eluted with 80 µl of elution buffer and stored at −20°C until analysis. DNA extracts were utilized for quantification of PCV2 genomes using the real-time PCR. Prior to the quantification of PCV2 genomes in the samples, a PCR standard for PCV2 was established. Briefly,

a PCV2 genome was cloned in the pMD®18-T Vector (Takara, China) after PCR amplification with the following primers: forward, 5′-CCGCGGGCTGGCTGAACTTTTG AAAG-3′ and reverse, 5′-CCGCGGAAATTTCTGACA AACGTTAC-3′ (GenBank accession number: EU095020), and transformed in TOP10 competent cells (Invitrogen, Grand Island, NY, USA). The plasmid was prepared using a PureLink™ HiPure Plasmid Midiprep Kit (Invitrogen). The PCV2 plasmid was mixed with the mouse DNA extracted from a PCV2 PCR-negative blood sample. Ten-fold dilutions of this mixture (from 10^{11} to 10^2 PCV2 copy numbers/µl) were used as a standard for PCV2 quantitation. The qPCR was performed using a SYBR Green detection kit (Takara, China), containing $MgCl_2$, dNTP, and Hotstar Taq polymerase. 1 µl of template solution was added to a total volume of 10 µL containing 5 µL SYBR Green mix, and 0.2 µL each of the forward and reverse primers (10 µM). We used the following protocol: (1) pre-denaturation (30 s at 95°C), (2) amplification and quantification, repeated for 40 cycles (5 s at 95°C, 34 s at 60°C), and (3) melting (60–99°C at a heating rate of 0.1°C/s and fluorescence measurement). Statistical analysis of qPCR data was performed as described by Fu et al. (2010).

Histopathology tests for pregnant mice

Maternal samples (spleen, liver and lung) of pregnant mice were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (5 mm thickness), and stained with hematoxylin and eosin for histopathologic examination. Microscopic lesions were evaluated in a blinded fashion by a veterinary pathologist using a previously described scoring system (Fenaux et al. 2002; Opriessnig et al. 2004). Lung sections were examined for the presence and severity of interstitial pneumonia, and scored on a scale from 0 (normal) to 6 (severe diffuse). Sections of the liver were evaluated for the presence of lymphohistiocytic inflammation and scored from 0 (none) to 3 (severe). Spleens were evaluated for the presence of lymphoid depletion and histiocytic inflammation ranging from 0 (normal) to 3 (severe).

Statistical analysis

Data are expressed as means ± the standard error of the mean (SEM). All statistical analyses were performed using the SPSS 16.0 software (Chicago, IL, USA). Data on the abortion rates of pregnant mice and the survival rates of neonates were analyzed by the χ^2 analysis (Li et al. 2010). Other data were analyzed by the student's t-test (Wei et al. 2011; Huang et al. 2005). Differences were considered significant at $P \leq 0.05$. P values of 0.05–0.10 were considered to represent a trend toward significance.

Results

Clinical observations

Food intake did not appear to differ between the glutamine-supplemented and control mice during gestation or lactation. After PCV2 infection, 4 of 18 pregnant mice in the glutamine group (22.2%) were aborted and the abortion rate was 50% in the control (alanine) group (Table 1). Although dietary L-glutamine supplementation did not affect litter birth weight, litter size (the number of newborns per liter) in the glutamine group was much higher ($P < 0.01$) than that in the control group (Table 1). Furthermore, the survival rate of neonates within the first 12 days after birth was higher in the glutamine group (82.2% vs. 59.4%, $P < 0.01$), compared to the control group (Table 2). No difference was observed in the daily weight gain of neonates in the first 12 days after birth between the glutamine and control groups (Table 2).

Serum cytokine profile and hematology of PCV2-infected mice

Concentrations of IL-1 β, IL-6, TNF-α and CRP in serum were measured on day 17 of gestation (7 days after infection with PCV2). As indicated in Table 3, dietary glutamine supplementation decreased ($P = 0.03$) concentrations of IL-6 in serum, while increasing ($P < 0.01$) concentrations of TNF-α in serum. Concentrations of CRP in serum tended to be higher ($P = 0.06$) in the glutamine group than those in the control group. In contrast, dietary glutamine supplementation did not affect serum IL-1β levels in the pregnant mice. No differences in hematology were detected between the glutamine and control groups,

including white blood cells, lymphocytes, neutrophilic granulocytes, red blood cells, hemoglobin, hematocrit, platelets, and thrombocytocrit (Table 4).

PCV2 virus load in maternal tissues and fetuses of PCV2-infected mice

To validate the ability of glutamine supplementation to clear PCV2 from the infected pregnant mice, we analyzed the PCV2 genome in maternal samples (serum, spleen and lung) and fetuses using qPCR. In the control group, PCV2 DNA was present in the maternal serum and lungs of most of the infected mice, absent from the maternal spleen, and detected in fetuses of 1 out of 6 mice (Table 5). The mean PCV2 $\log_{10}$ genomic copies per gram tissue or milliliter serum were 4.86 for the fetus, 6.44 for the maternal lung, and 7.81 for the maternal serum. In contrast, all maternal and fetal tissues were negative for the PCV2 DNA in the glutamine group.

Histopathologic analysis of maternal tissues in PCV2-infected mice

PCV2 infection resulted in interstitial pneumonia and alveolar wall thickening due to the infiltration of macrophages and lymphocytes in the maternal lung, lymphohistiocytic inflammation in the maternal liver, and lymphoid depletion and histiocytic inflammation in the maternal spleen of all pregnant mice (Fig. 1). However, the lesions in the maternal lung of glutamine-supplemented mice (Fig. 1a) were milder, compared with the control group (Fig. 1b). Glutamine-supplemented mice (Fig. 1c) also exhibited less lymphohistiocytic inflammation in the maternal liver, compared with the control group (Fig. 1d).

Table 1 Effects of dietary glutamine supplementation on the abortion rate and litter size in pregnant mice infected with PCV2

Variable	1.0% Gln	1.22% Ala	P value
Abortion rate of pregnant mice (%)	22.2 (18)	50.0 (22)	<0.01
Litter size at birth (n/litter)	10.9 ± 0.54	9.00 ± 0.26	<0.01
Birth weight of mice (g/mouse)	1.17 ± 0.05	1.26 ± 0.17	0.49

The rates of abortion were calculated on the basis of 18 and 22 pregnant mice, respectively, in the glutamine and alanine groups. 1.0% Gln: 1.0% glutamine + basal diet; 1.22% Ala: 1.22% alanine + basal diet

Table 2 Effects of dietary glutamine supplementation on neonatal survival and daily weight gains of neonates from mice infected with PCV2 during pregnancy

Variable	1.0% Gln	1.22% Ala	P value
Survival rate of neonates (%)	82.2	59.4	<0.01
Daily weight gain (g/mouse/day)	0.35 ± 0.04	0.33 ± 0.03	0.73

The survival rates of neonates were calculated on the basis of 45 and 32 newborn mice, respectively, in the glutamine and alanine groups *1.0% Gln* 1.0% glutamine + basal diet, *1.22% Ala* 1.22% alanine + basal diet

Table 3 Effects of dietary glutamine supplementation on the cytokine profile in the serum of pregnant mice infected with PCV2

Variable	1.0% Gln	1.22% Ala	P value
IL-1β (ng/ml)	48.9 ± 3.6	41.6 ± 5.2	0.33
IL-6 (pg/ml)	139 ± 20	357 ± 79	0.03
TNF-α (ng/ml)	1.32 ± 0.10	0.82 ± 0.04	<0.01
CRP (μg/ml)	90.3 ± 8.2	63.1 ± 8.3	0.06

IL-1β interleukin-1β, *IL-6* interleukin-6, *TNF-α* tumor necrosis factor-α, *CRP* C-reactive protein, *1.0% Gln* 1.0% glutamine + basal diet, *1.22% Ala* 1.22% alanine + basal diet

Table 4 Effects of dietary glutamine supplementation on hematology of pregnant mice infected with PCV2

Variable	1.0% Gln	1.22% Ala
White blood cells (10^9/L)	6.77 ± 0.72	6.78 ± 0.90
Lymphocytes (10^9/L)	5.28 ± 0.73	5.80 ± 0.75
Intermediate cells (10^9/L)	0.40 ± 0.08	0.28 ± 0.06
Neutrophilic granulocytes (10^9/L)	1.10 ± 0.15	0.72 ± 0.15
Red blood cells (10^{12}/L)	8.51 ± 0.24	7.68 ± 0.35
Hemoglobin (g/L)	134 ± 3.1	122 ± 5.6
Hematocrit	0.36 ± 0.01	0.34 ± 0.01
Mean corpuscular volume (fL)	43.0 ± 0.82	44.4 ± 1.03
MCH (pg)	15.7 ± 0.31	15.9 ± 0.32
MCHC (g/L)	366 ± 3.8	361 ± 3.2
RDW (%)	16.0 ± 0.79	16.2 ± 0.67
Platelets (10^9/L)	534 ± 88.0	523 ± 36
Mean platelet volume (fL)	5.63 ± 0.07	5.60 ± 0.13
Thrombocytocrit	0.30 ± 0.05	0.29 ± 0.02
PDW (%)	37.4 ± 2.7	33.7 ± 4.2

No difference in any variable was detected ($P > 0.10$) between the glutamine and alanine groups ($P > 0.10$). Maternal blood samples were obtained from mice on day 17 of gestation

MCH mean corpuscular hemoglobin (the average mass of hemoglobin per red blood cell), *MCHC* mean corpuscular hemoglobin concentration (a measure of the concentration of hemoglobin in a given volume of packed red blood cells), *PDW* platelet distribution width (a measure of variation in platelet size), *RDW* red cell distribution width (a measure of variation in red blood cell width), *1.0% Gln* 1.0% glutamine + basal diet, *1.22% Ala* 1.22% alanine + basal diet

Additionally, dietary glutamine supplementation decreased microscopic lesions in the maternal spleen (Fig. 1e), in comparison with the control group (Fig. 1f). Microscopic lesion scores in the collected maternal tissues are summarized in Table 6. Overall, mice in the glutamine group had lower scores of microscopic lesions in the lung ($P < 0.01$) and spleen ($P = 0.02$), compared with the control group. The maternal livers of glutamine-supplemented mice tended to have a lower score of microscopic lesions than the control group ($P = 0.06$).

Discussion

PCV2 was a widespread, circular, and single-stranded DNA virus. It was originally identified in piglets with multi-systemic wasting syndrome (Allan et al. 1998). Now, all the disorders resulting from PCV2 infection are termed as porcine circovirus-associated disease or porcine circovirus disease (Opriessnig et al. 2009). These disorders are manifest as dermatitis (Rosell et al. 2000), nephropathy syndrome (Rosell et al. 2000), reproductive disorders (West et al. 1999), enteritis (Kim et al. 2004), proliferative and necrotizing pneumonia (Grau-Roma and Segales 2007), and respiratory disease complex (Kim et al. 2003). Besides its highly pathogenic property, the virus exists ubiquitously in swine and its outbreak can cause great economical losses to the pork industry. Thus, many methods have been proposed to prevent and treat PCV2 infection, such as containment, sound husbandry, herd management, good sanitation, immunization and antibiotic therapy (Jung et al. 2010; Grau-Roma et al. 2011). Unfortunately, these methods have met limited success for various reasons under the practical conditions of swine production. Thus, nutritional regulation for increasing the immune responses of the host appears to be the first choice of prophylactic measures against PCV2 infection. Using the mouse model, we recently reported that dietary L-arginine supplementation had beneficial effects on the cytokine profile in the serum of PCV2-infected mice and could delay PCV2 replication and clear PCV2 from the maternal tissues, thereby partially reversing the reproductive failure in PCV2-infected pregnant mice (Ren et al. 2011). The current study, which focused on glutamine, is an extension of our long-standing work on nutrition and immunity (Li et al. 2007; Tan et al. 2010; Wu et al. 1991, 2010b). The findings support the notion that gestating dams require sufficient amounts of dietary glutamine for the optimal survival and growth of embryos, fetuses, and neonates, particularly in litter-bearing mammals (Wu et al. 2011a).

Several studies reported fetal deaths, mummification, and late-term abortion in PCV2-infected pregnant sows (Ladekjaer-Mikkelsen et al. 2001). Subsequent experiments confirmed the pathogenic capacity of PCV2 in the conceptus using intrafetal inoculation with PCV2 at the different stages of fetal development (Yoon et al. 2004). Substantial abortion also occurs in sows in response to intranasal inoculation (Park et al. 2005). All of these findings indicate that PCV2 has unfavorable effects on pregnant swine. The deleterious effect of PCV2 on pregnancy outcome was also observed in mice (Ren et al. 2011). Importantly, dietary glutamine supplementation decreased the abortion rate, while increasing fetal survival and litter size as well as the survival of neonates (Table 2).

Table 5 Effects of dietary glutamine supplementation on PCV2 virus loads in pregnant mice infected with PCV2 during pregnancy

Group	Maternal serum	Maternal lung	Maternal spleen	Fetus
1.0% Gln	0/6	0/6	0/6	0/6
1.22% Ala	4/6 (7.81 ± 1.97)*	5/6 (6.44 ± 1.45)*	0/6	1/6 (4.86 ± 0.00)

Mice were infected with PCV2 virus on Day 10 of gestation and then euthanized on day 17 of gestation to obtain maternal tissues and fetuses. Data are the number of positive mice/the number of tested mice, with the values in parentheses being the $\log_{10}$ genomic copies of PCV2 per g tissue

1.0% Gln 1.0% glutamine + basal diet, *1.22% Ala* 1.22% alanine + basal diet
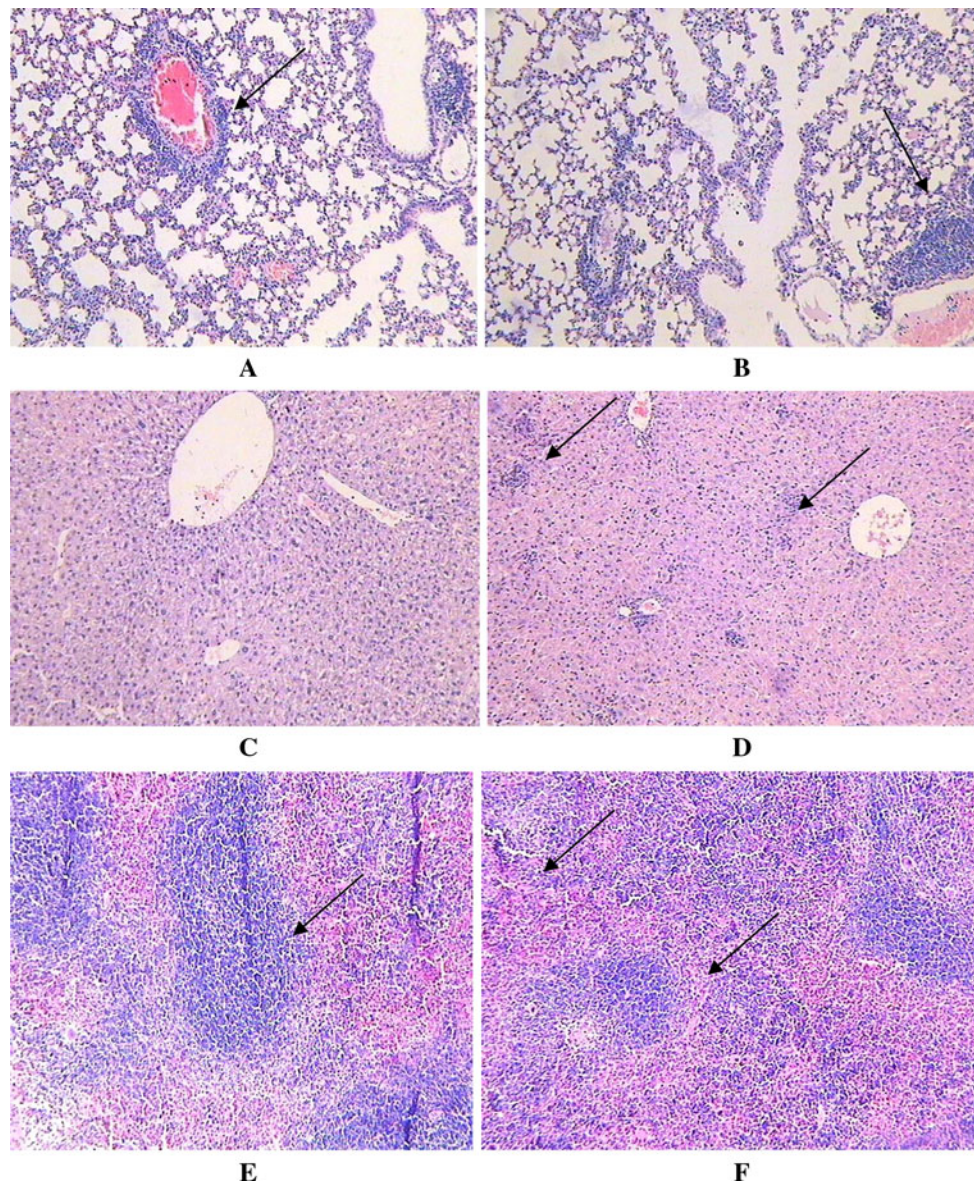
* $P < 0.01$ vs the Ala group

Fig. 1 Histopathological features of the maternal lung, liver and spleen (×100) in pregnant PCV2-infected mice fed a diet supplemented with 1.0% glutamine or 1.22% alanine (isonitrogenous control). Milder interstitial pneumonia was evident in the maternal lung in the glutamine group (**a**), compared with the control group (**b**). Notably, pregnant mice in the glutamine group (**c**) exhibited less lymphohistiocytic inflammation in the maternal liver compared with the control group (**d**). Additionally, the glutamine group had less severe lymphoid depletion (**e**) in the maternal spleen, compared with the control group (**f**)

Table 6 Microscopic lesion scores of maternal tissues in pregnant mice infected with PCV2

Tissue	1.0% Gln	1.22% Ala	P value
Lung	1.40 ± 0.24	2.80 ± 0.20	<0.01
Liver	0.60 ± 0.40	1.80 ± 0.37	0.06
Spleen	1.40 ± 0.25	2.40 ± 0.24	0.02

1.0% Gln 1.0% glutamine + basal diet, *1.22% Ala* 1.22% alanine + basal diet

A possible explanation for these observations is that dietary supplementation with the functional amino acids can enhance the responses of both the innate and adaptive immune systems (Li et al. 2007) and, therefore, the ability of the host to clear PCV2.

Like arginine (Blachier et al. 2011; Wu and Morris 1998), glutamine has vital and versatile functions, and is considered as a conditionally essential amino acid for swine (Wu et al. 2011a) and other mammals (Xi et al. 2011). Glutamine is a major energy substrate for lymphocytes, macrophages and other immune cells, and thus plays an important role in immune function (Li et al. 2007). In support of this notion, augmenting the amount of glutamine in the diet increases the survival of mice challenged by pathogenic bacteria (Suzuki et al. 1993), attenuates the growth reduction of endotoxin-treated neonatal piglets (Haynes et al. 2009), and reduces the growth of implanted tumors in rats (Shewchuk et al. 1997). Furthermore, Inoue et al. (1993) reported that only three of 38 glutamine-supplemented rats died (a mortality rate of 8%), compared with a mortality rate of 45% in the control group, in response to *Escherichia coli*-induced peritonitis. Additionally, glutamine is used as a precursor of glutamate for glutathione synthesis, extending the role of glutamine in modulating intracellular redox status and antioxidative responses (Wu et al. 2004). Oxidative stress is regarded as a causative factor in pregnancy-related disorders, such as embryonic resorption, recurrent pregnancy loss and fetal death (Wu et al. 2006). Thus, dietary glutamine supplementation can stimulate glutathione synthesis in animal cells (Haynes et al. 2009) to protect the host from inflammation and infection (Fang et al. 2002), thereby decreasing the incidence of abortion and neonatal mortality under diseased and stressful conditions.

To corroborate the data on pregnancy outcome with immune responses, we determined serum cytokine profiles and hematology in PCV2-infected mice. IL-1β is secreted by many kinds of cells, such as monocytes, macrophages (March et al. 1985). It enhances the function of T cells, B cells, natural killer cells and neutrophils, promotes the chemotaxis of monocytes, neutrophils and granulocytes to the infected areas, and increases the synthesis of acute phase proteins (Dinarello 1997). IL-6 also plays a very complex role in biological events, including cell-mediated immune responses, hematopoiesis, and regulation of endocrine and nervous systems (Naugler and Karin 2008). TNF-α is secreted by both macrophages and monocytes and crucial for immune regulation, including enhancements of lymphoid development, cell proliferation, and differentiation, as well as the activity of immunocytes (Chen et al. 2005). Additionally, CRP plays a role in host defense against bacterial pathogens, protection from lethal bacterial infection and endotoxemia, activation of complement, opsonization, and induction of phagocytosis (Szalai et al. 2000). In keeping with the clinical observations, concentrations of TNF-α and CRP were higher in the serum of the glutamine-supplemented mice than in the control group (Table 3).

Our finding that dietary glutamine supplementation improves pregnancy outcome is PCV2-infected mice is novel and important. Consistent with these data are several previous reports. First, an increase in dietary glutamine supply enhanced the ability of macrophages to produce TNF-α, IL-1β and IL-6 (Wells et al. 1999). Second, the production of TNF-α, IL-1 and IL-6 by cultured macrophages and monocytes was increased by elevated levels of glutamine in the cell culture medium (Wallace and Keast 1992). In this study, dietary glutamine supplementation decreased the concentration of IL-6 in the maternal serum (Table 3). Similar results were observed in mice on the fourth day after infection with *Erysipelothrix rhusiopathiae*. A possible reason for the apparently different findings between our and others' studies is the experimental design, namely cell culture vs whole-animal models. Indeed, large amounts of data indicate that circulating levels of IL-6 are elevated in PCV2-infected pigs exhibiting the natural multi-systemic wasting syndrome or dermatitis and nephropathy syndrome (Shi et al. 2010; Sipos et al. 2005). Thus, a reduced level of IL-6 in serum may be indicative of a reduced load of PCV2 in glutamine-supplemented mice.

Of particular interest, we found that all the maternal sera, spleens, lungs as well as the fetuses from the glutamine group were negative for the PCV2 DNA, whereas the PCV2 $\log_{10}$ genomic copies were relatively high in the control group (Table 5). Furthermore, microscopic lesions in the maternal spleen, liver and lung from the control group were more severe than those in the glutamine group. These results indicate that dietary L-glutamine supplementation may increase the clearance of PCV2 from the infected mice. The underlying mechanisms are unknown at present and should be explored in future studies. Glutamine and glutamate, along with proline, arginine and ornithine, constitute the glutamate family of amino acids (Dai et al. 2011; Flynn and Wu 1996; Wu et al. 1996). These amino acids play importance roles in the synthesis of nucleotides,

glucose, amino sugars, glutathione, and protein, as well as the growth of fibroblasts, lymphocytes and enterocytes, and the function of immune system (Castell et al. 2004; Wu et al. 1991). Generally, these amino acids have been classified as nutritionally non-essential based on the traditional definition (Wu 2010), but they are now recognized to be conditionally essential in pregnancy and neonatal growth as well as under stressful conditions (e.g., weaning and infection) (Wu et al. 2010a, 2011a, b). As noted above, glutamine plays a key role in the host defense against infections caused by bacteria, fungi, parasites, and virus (Li et al. 2007; Wells et al. 1999; Yoo et al. 1997). Additionally, α-ketoglutarate, a metabolite of glutamine (Wu 2009), is now known to have immunomodulatory and metabolic regulatory functions (Hou et al. 2010, 2011; Mühling et al. 2010; Yao et al. 2011).

In conclusion, our results indicate that dietary glutamine supplementation confers a positive effect on improving pregnancy outcomes in PCV2-infected mice through enhancing the immune response and the ability to clear PCV2. Moreover, the glutamine treatment decreases lesions in maternal tissues and increases neonatal survival. Finally, glutamine supplementation during the lactation period enhances the survival of neonates from mice infected with PCV2 during gestation. These results support the notion that gestating dams require adequate amounts of dietary glutamine for the optimal survival and growth of embryos, fetuses, and neonates. The findings have important implications for nutritional support of mammals (including swine and humans) during gestation and lactation.

References

Allan GM, McNeilly F, Kennedy S et al (1998) Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J Vet Diagn Invest 10:3–10

Blachier F, Davila AM, Benamouzig R et al (2011) Channelling of arginine in NO and polyamine pathways in colonocytes and consequences. Front Biosci 16:1331–1343

Bonetto A, Penna F, Minero VG et al (2011) Glutamine prevents myostatin hyperexpression and protein hypercatabolism induced in C2C12 myotubes by tumor necrosis factor-α. Amino Acids 40:585–594

Castell L, Vance C, Abbott R et al (2004) Granule localization of glutaminase in human neutrophils and the consequence of glutamine utilization for neutrophil activity. J Biol Chem 279:13305–13310

Chen PF, Xu XG, Liu XS et al (2005) Characterisation of monoclonal antibodies to the TNF and TNF receptor families. Cell Immunol 236:78–85

Cooksey RC, McClain DA (2011) Increased hexosamine pathway flux and high fat feeding are not additive in inducing insulin resistance: evidence for a shared pathway. Amino Acids 40:841–846

Dai ZL, Li XL, Xi PB et al (2011) Metabolism of select amino acids in bacteria from the pig small intestine. Amino Acids. doi: 10.1007/s00726-011-0846-x

Dinarello CA (1997) Interleukin-1. Cytokine Growth Factor Rev 8:253–265

Eamens GJ, Forbes WA, Djordjevic SP (2006) Characterisation of Erysipelothrix rhusiopathiae isolates from pigs associated with vaccine breakdowns. Vet Microbiol 115:329–338

Fang YZ, Yang S, Wu G (2002) Free radicals, antioxidants, and nutrition. Nutrition 18:872–879

Fenaux M, Halbur PG, Haqshenas G et al (2002) Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathologic lesions. J Virol 76:541–551

Flynn NE, Wu G (1996) An important role for endogenous synthesis of arginine in maintaining arginine homeostasis in neonatal pigs. Am J Physiol Regul Integr Comp Physiol 271:R1149–R1155

Fu WJ, Stromberg AJ, Viele K et al (2010) Statistics and bioinformatics in nutritional sciences: analysis of complex data in the era of systems biology. J Nutr Biochem 21:561–572

Grau-Roma L, Segales J (2007) Detection of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, swine influenza virus and Aujeszky's disease virus in cases of porcine proliferative and necrotizing pneumonia (PNP) in Spain. Vet Microbiol 119:144–151

Grau-Roma L, Fraile L, Segales J (2011) Recent advances in the epidemiology, diagnosis and control of diseases caused by porcine circovirus type 2. Vet J 187:23–32

Haynes TE, Li P, Li XL et al (2009) L-Glutamine or L-alanyl-L-glutamine prevents oxidant- or endotoxin-induced death of neonatal enterocytes. Amino Acids 37:131–142

Hou YQ, Wang L, Ding BY et al (2010) Dietary α-ketoglutarate supplementation ameliorates intestinal injury in lipopolysaccharide-challenged piglets. Amino Acids 39:555–564

Hou YQ, Wang L, Ding BY et al (2011) Alpha-ketoglutarate and intestinal function. Front Biosci 16:1186–1196

Huang RL, Yin YL, Wu GY et al (2005) Effect of dietary oligochitosan supplementation on ileal digestibility of nutrients and performance in broilers. Poul Sci 84:1383–1388

Inoue Y, Grant JP, Snyder PJ (1993) Effect of glutamine-supplemented intravenous nutrition on survival after Escherichia coli-induced peritonitis. J Parenter Enteral Nutr 17:41–46

Jung BG, Toan NT, Cho SJ et al (2010) Dietary aluminosilicate supplement enhances immune activity in mice and reinforces clearance of porcine circovirus type 2 in experimentally infected pigs. Vet Microbiol 143:117–125

Kim J, Chung HK, Chae C (2003) Association of porcine circovirus 2 with porcine respiratory disease complex. Vet J 166:251–256

Kim J, Ha Y, Jung K et al (2004) Enteritis associated with porcine circovirus 2 in pigs. Can J Vet Res 68:218–221

Kong XF, Zhang YZ, Yin YL et al (2009) Chinese Yam polysaccharide enhances growth performance and cellular immune response in weanling rats. J Sci Food Agric 89:2039–2044

Ladekjaer-Mikkelsen AS, Nielsen J, Storgaard T et al (2001) Transplacental infection with PCV-2 associated with reproductive failure in a gilt. Vet Rec 148:759–760

Li P, Yin YL, Li D et al (2007) Amino acids and immune function. Br J Nutr 98:237–252

Li XL, Bazer FW, Johnson GA et al (2010) Dietary supplementation with 0.8% ʟ-arginine between days 0 and 25 of gestation reduces litter size in gilts. J Nutr 140:1111–1116

Li XL, Rezaei R, Li P et al (2011) Composition of amino acids in feed ingredients for animal diets. Amino Acids 40:1159–1168

March CJ, MosleyB LarsenA et al (1985) Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature 315:641–647

Monroe S, Polk R (2000) Antimicrobial use and bacterial resistance. Curr Opin Microbiol 3:496–501

Mühling J, Tussing F, Nickolaus KA et al (2010) Effects of α-ketoglutarate on neutrophil intracellular amino and α-keto acid profiles and ROS production. Amino Acids 38:167–177

Naugler WE, Karin M (2008) The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. Trends Mol Med 14:109–119

Opriessnig T, Thacker EL, Yu S et al (2004) Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with Mycoplasma hyopneumoniae and porcine circovirus type 2. Vet Pathol 41:624–640

Opriessnig T, Patterson AR, Meng XJ et al (2009) Porcine circovirus type 2 in muscle and bone marrow is infectious and transmissible to naive pigs by oral consumption. Vet Microbiol 133:54–64

Park JS, Kim J, Ha Y et al (2005) Birth abnormalities in pregnant sows infected intranasally with porcine circovirus 2. J Comp Pathol 132:139–144

Ren W, Yin YL, Liu G et al (2011) Effect of dietary arginine supplementation on reproductive performance of mice with porcine circovirus type 2 infection. Amino Acids. doi: 10.1007/s00726-011-0942-y

Rogero MM, Borelli P, Fock RA et al (2010) Effects of glutamine on the nuclear factor-kappaB signaling pathway of murine peritoneal macrophages. Amino Acids 39:435–441

Rosell C, Segales J, Ramos-Vara JA et al (2000) Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. Vet Rec 146:40–43

Shewchuk LD, Baracos VE, Field CJ (1997) Dietary ʟ-glutamine supplementation reduces the growth of the Morris Hepatoma 7777 in exercise-trained and sedentary rats. J Nutr 127:158–166

Shi KC, Guo X, Ge XN et al (2010) Cytokine mRNA expression profiles in peripheral blood mononuclear cells from piglets experimentally co-infected with porcine reproductive and respiratory syndrome virus and porcine circovirus type 2. Vet Microbiol 140:155–160

Sipos W, Duvigneau JC, Pietschmann P et al (2005) Porcine dermatitis and nephropathy syndrome (PDNS) is associated with a systemic cytokine expression profile indicative of proinflammation and a Th1 bias. Vet Immunol Immunopathol 107:303–313

Stevenson GW, Kiupel M, Mittal SK et al (2001) Tissue distribution and genetic typing of porcine circoviruses in pigs with naturally occurring congenital tremors. J Vet Diagn Invest 13:57–62

Suzuki I, Matsumoto Y, Adjei AA et al (1993) Effect of a glutamine-supplemented diet on response to methicillin-resistant Staphylococcus aureus infection in mice. J Nutr Sci Vitaminol (Tokyo) 39:405–410

Szalai AJ, Van Cott JL, McGhee JR et al (2000) Human C-reactive protein is protective against fatal Salmonella enterica serovar typhimurium infection in transgenic mice. Infect Immun 68:5652–5656

Tan BE, Yin YL, Kong XF et al (2010) ʟ-Arginine stimulates proliferation and prevents endotoxin-induced death of intestinal cells. Amino Acids 38:1227–1235

Vincent AL, Ciacci-Zanella JR, Lorusso A et al (2010) Efficacy of inactivated swine influenza virus vaccines against the 2009 A/H1N1 influenza virus in pigs. Vaccine 28:2782–2787

Wallace C, Keast D (1992) Glutamine and macrophage function. Metabolism 41:1016–1020

Wei JW, Carroll RJ, Harden KK et al (2011) Comparisons of treatment means when factors do not interact in two-factorial studies. Amino Acids. doi:10.1007/s00726-011-0924-0

Wells SM, Kew S, Yaqoob P et al (1999) Dietary glutamine enhances cytokine production by murine macrophages. Nutrition 15:881–884

Wen L, He K, Yang H et al (2008) Complete nucleotide sequence of a novel porcine circovirus-like agent and its infectivity in vitro. Science in China C Life Sci 51:453–458

West KH, Bystrom JM, Wojnarowicz C et al (1999) Myocarditis and abortion associated with intrauterine infection of sows with porcine circovirus 2. J Vet Diagn Invest 11:530–532

Wu G (2009) Amino acids: metabolism, functions, and nutrition. Amino Acids 37:1–17

Wu G (2010) Functional amino acids in growth, reproduction and health. Adv Nutr 1:31–37

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

Wu G, Field CJ, Marliss EB (1991) Glutamine and glucose metabolism in rat splenocytes and mesenteric lymph node lymphocytes. Am J Physiol Endocrinol Metab 260:E141–E147

Wu G, Knabe DA, Flynn NE et al (1996) Arginine degradation in developing porcine enterocytes. Am J Physiol Gastrointest Liver Physiol 271:G913–G919

Wu G, Fang YZ, Yang S et al (2004) Glutathione metabolism and its implications for health. J Nutr 134:489–492

Wu G, Bazer FW, Wallace JM et al (2006) Intrauterine growth retardation: Implications for the animal sciences. J Anim Sci 84:2316–2337

Wu G, Bazer FW, Burghardt RC et al (2010a) Impacts of amino acid nutrition on pregnancy outcome in pigs: mechanisms and implications for swine production. J Anim Sci 88:E195–E204

Wu X, Ruan Z, Gao YL et al (2010b) Dietary supplementation with ʟ-arginine or N-carbamylglutamate enhances intestinal growth and heat shock protein-70 expression in weanling pigs fed a corn- and soybean meal-based diet. Amino Acids 39:831–839

Wu G, Bazer FW, Johnson GA et al (2011a) Important roles for ʟ-glutamine in swine nutrition and production. J Anim Sci 89:2017–2030

Wu G, Bazer FW, Burghardt RC et al (2011b) Proline and hydroxyproline metabolism: implications for animal and human nutrition. Amino Acids 40:1053–1063

Xi PB, Jiang ZY, Zheng CT et al (2011) Regulation of protein metabolism by glutamine: implications for nutrition and health. Front Biosci 16:578–597

Yao K, Yin YL, Li XL et al (2011) Alpha-ketoglutarate inhibits glutamine degradation and enhances protein synthesis in intestinal porcine epithelial cells. Amino Acids. doi:10.1007/s00726-011-1060-6

Yin YL, Zhong HY, Huang RL et al (1993) Nutritive value of feedstuffs and diets for pigs. I. Chemical composition, apparent ileal and fecal digestibility. Anim Feed Sci Tech 44:1–27

Yin YL, Li TJ, Huang RL (2008a) Evaluating standardized ileal digestibility of amino acids in growing pigs. Anim Feed Sci Tech 140:385–401

Yin YL, Tang ZR, Sun ZH et al (2008b) Effect of galacto-mannan-oligosaccharides or chitosan supplementation on cytoimmunity and humoral immunity response in early-weaned piglets. Asian-Aust J Anim Sci 21:723–731

Yin FG, Zhang ZZ, Huang J et al (2010a) Digestion rate of dietary starch affects systemic circulation of amino acids in weaned pigs. Br J Nutr 103:1404–1412

Yoo SS, Field CJ, McBurney MI (1997) Glutamine supplementation maintains intramuscular glutamine concentrations and normalizes lymphocyte function in infected early weaned pigs. J Nutr 127:2253–2259

Yoon KJ, Jepsen RJ, Pogranichniy RM et al (2004) A novel approach to intrauterine viral inoculation of swine using PCV type 2 as a model. Theriogenology 61:1025–1037