Dietary L-Arginine Supplementation Improves the Immune Responses in Mouse Model Infected Porcine Circovirus Types 2

1Gang Liu, 1Wenkai Ren, 1Dingding Su, 1Miaomiao Wu, 1Yinghui Li
2Wei Luo, 2Xinglong Yu, 2Tiejun Li and 1Jun Fang
3Key Laboratory for Agro-Ecological Processes in Subtropical Region, The Chinese Academy of Sciences, Research Center for Healthy Breeding of Livestock and Poultry, Hunan Engineering and Research Center of Animal and Poultry Science, Institute of Subtropical Agriculture, 410125 Hunan, China
3Hunan Provincial Key Laboratory for Germplasm Innovation and Utilization of Crop, Hunan Agricultural University, Hunan, 410128 Changsha, China
3College of Veterinarian, Hunan Agricultural University, Changsha, 410128 Hunan, China
3Biological Technology College, Hunan Agricultural University, Changsha, 410128 Hunan, China

Abstract: This study was conducted to test the hypotheses that dietary L-arginine supplementation may enhance the immune responses and result in a clearance against PCV2 in experimentally infected mice. The measured variables include: the PCV2 virus load in liver, spleen, heart, lung, kidney, ovary and serum on 3rd, 5th, 7th, 9th and 11th day post infection (dpi); serum Interleukin-2 (IL-2), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interferon alpha (IFN-α), Interferon gamma (IFN-γ) and C-Reactive Protein (CRP) levels on 3rd, 5th, 7th, 9th and 11th dpi; serum Total Superoxide Dismutase (T-SOD) activity on 3rd, 5th, 7th, 9th and 11th dpi. Results showed that arginine supplementation could significantly increase the serum IL-2 levels on the 9th and 11th dpi; significantly increase the serum IFN-α and CRP levels on the 11th dpi; significantly increase the serum IFN-γ levels on the 7th dpi and significantly decrease the serum IL-6 levels on the 9th dpi. Meanwhile, the PCV2 virus genome was detected sporadically. Collectively, dietary L-arginine supplementation had beneficial effects on the cytokines profile in the PCV2 infected mouse and maybe could delay the PCV2 replication and/or clear the PCV2 in mouse model.

Key words: L-arginine, porcine circovirus types 2, C-reactive protein, mouse model, dietary

INTRODUCTION

Arginine usually regarded as non-essential dietary amino acid for it could be synthesized from L-ornithine or L-citrulline involving the intestinal-renal axis in humans and most other mammals (Yin et al., 1993; Wu et al., 2009). Two kinds of enzymes (Nitric Oxide Synthase (NOS) and arginase) were existed for arginine metabolism. NOS catalyzed the arginine to produce Nitric Oxide (NO) after stimulated by Th1 cytokines including Interferon-gamma (IFN-γ) and Tumor Necrosis Factor-alpha (TNF-α) and several Th1-favored chemokines including Macrophage Inflammatory Protein-1α (MIP-1α/CCL3), Macrophage Chemotactic Protein-1 (MCP-1/CCL2) and gamma Interferon inducible Protein-10 (IP-10/CXCL10) (Zhang et al., 2012; Green et al., 1990; Bhattacharyya et al., 2002; Kim et al., 2007; Li et al., 2007; Wu et al., 2009). Promising findings indicated that NO played a role in the host defense against bacteria, fungi, parasites and virus (Tan et al., 2009, 2010; Nathan and Hibbs, 1991; Liu, et al., 2008). Meanwhile arginine can be catabolized by arginase for polyamine synthesis after stimulated by Th2 cytokines such as Interleukin (IL)-4, IL-10, IL-13 and Transforming Growth Factor-β (TGF-β) (Frieiota et al., 2002, Barksdale et al., 2004; Wu et al., 2009). Polyamine was deeply involved in the regulation of cellular functions, cell growth and death (Minarini et al., 2010; Thomas and Thomas, 2001). Moreover, other metabolites such as ornithine, proline, creatine, agmatine and glutamate also generated. Thus, arginine exerted an important role in physiological and immune function and regarded as an immunonutrient and modulator. Porcine Circovirus types 2 (PCV2) was presumed as the essential pathogen for the Post-wearing

Corresponding Author: Wenkai Ren, Key Laboratory for Agro-Ecological Processes in Subtropical Region, The Chinese Academy of Sciences, Research Center for Healthy Breeding of Livestock and Poultry, Hunan Engineering and Research Center of Animal and Poultry Science, Institute of Subtropical Agriculture, 410125 Hunan, China
Multi-systemic Wasting Syndrome (PMWS) which existed worldwide and caused big loss in pig-industry (Allan et al., 1998, Ellis et al., 1998). PMWS, immunosuppressive disease was characterized by progressive weight loss, generalized enlargement of lymph nodes, the symptoms of pallor, jaundice and diarrhea (O’Connor et al., 2001; Stevenson et al., 2001). Moreover, PCV2 also associated with a serial of other syndromes which all were now termed as porcine circovirus-associated diseases or porcine circovirus diseases (Segales et al., 2005; Opriessnig et al., 2007). These syndromes included Porcine Dermatitis and Nephropathy Syndrome (PDNS), reproductive disorders, enteritis (Kim et al., 2004), Proliferative and Necrotising Pneumonia (PNP) and Porcine Respiratory Disease Complex (PRDC).

Despite the compelling findings that arginine as an immunonutrient and modulator, played a role in immune function but the scientific literature on the immune enhancing effects and the virus clear ability of L-arginine supplement in infected models was unavailable. This study was conducted to evaluate the immune enhancing effects of dietary L-arginine supplement in PCV2 infected mice and to demonstrate the clearance effects of arginine against PCV2 in experimentally infected mice.

**MATERIALS AND METHODS**

**Animals and feeding:** About 60 KunMing female mice (body weight 18-22 g) were obtained from the Laboratory Animal Center of Central South University, Hunan, China. The mice were randomly assigned to two treatment groups after 3 days of adaptive feeding: Arginine group (0.6% arginine+basal diet, n = 30) and control group (1.22% alanine+basal diet, n = 30). The arginine and alanine were purchased from Beijing Chemlin Biotech, Beijing, China. The amino acids content in the basal diet was measured using Automatic Amino Acid Analyzer (AAA). The mice were housed in an environmentally controlled pathogen-free condition. All of the animals had free access to diets and drinking water. This study was carried out in full compliance with the Chinese guidelines for animal welfare and was approved by the Animal Care and Use Committee of the Chinese Academy of Sciences.

**Preparation of PCV2 stock:** A PCV2 infectious clone constructed by self-ligation of the PCV2 genome via a Sac I enzyme site was used to generate the virus stock pools required for experimental infection (Ren et al., 2011). Briefly, the continuous porcine kidney cell line PK-15, free of PCV1 and PCV2 was cultured in RPMI medium 1640 supplemented with 6% (vol/vol.) Fetal Calf Serum (FCS).

The cell monolayer was dispersed by using trypsin-EDTA and suspended in RPMI medium 1640 supplemented with 6% (vol/vol.) FCS. Cells were simultaneously infected with the PCV2 infectious clone. After 72 h of incubation, the infected cells were frozen and thawed three times and the cell mixture was tested by PCR before being stored at -20°C. PCV2 stocks were titrated on PK-15 cells.

**Experiment design:** All the mice infected with PCV2 at the dose of 100 TCID50 (50% tissue culture infection dose, TCID50) with intraperitoneal injection after 2 weeks feeding L-arginine. Six mice on 3rd, 5th, 7th, 9th and 11th dpi from each group were killed to collect liver, spleen, heart, lung, kidney and ovary, meanwhile serum on 3rd, 5th, 7th, 9th and 11th dpi also prepared from orbital venous. All the samples were stored at -80°C for further research.

**Serum cytokines detection:** Serum Interleukin-2 (IL-2), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interferon alpha (IFN-α), Interferon gamma (IFN-γ) and C-Reactive Protein (CRP) levels in serum were measured using ELISA kits in accordance with the manufacturer’s instructions (Cusabio Biotech Co., Ltd. China). Supplied diluent buffer was used to dilute standards and serum samples. Next, 100 μL volumes of sample or standard were added to duplicate wells of the microtiter plate which had been pre-coated with antibody. Diluent buffer was used as a negative control. The plate was incubated for 2 h at 37°C. A 100 μL of biotin-antibody was added to each well after removing the liquid of each well and incubated for 1 h at 37°C. The wells were washed three times with 200 μL volumes of wash buffer. A 100 μL quantity of HRP-avidin was added to each well for 1 h at 37°C. After a final wash, a 90 μL the supplied chromogen was added and incubated for 30 min in the dark at 37°C. The reaction was stopped with 50 μL of supplied stop solution and absorbance measured at 450 nm.

**DNA extraction and PCV2 quantitative PCR (qPCR):**
DNA from samples (liver (10 mg), spleen (5 mg), heart (10 mg), lung (10 mg), kidney (10 mg) and ovary (10 mg)) was extracted using Tissue Genomic DNA Extraction kits (Betines 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 for further use. DNA extracts were used for quantification of PCV2 genomes using the real-time PCR. Prior to quantification of the PCV2 genomes in the collected samples, a PCV2 real-time PCR standard was established. Briefly, a PCV2 genome was cloned in the pMD18-T
Vector (TaKaRa) after PCR amplification with the following primers: forward, 5'-CGCGGGCTGCTGCTGAACATTTTGAAG-3' and reverse, 5'- CGCGGAAATTTCGGACACATC-3' (GenBank accession number: EU058020) and transformed in TOP10 competent cells (Invitrogen). The plasmid was prepared using a PureLinkTM HiPure Plasmid Midiprep kit (Invitrogen). The PCV2 plasmid was mixed with the mouse DNA extracted from a PCV2 PCR-negative blood sample. The 10 fold dilutions of this mixture (from 10^4 to 10^6 PCV2 copy numbers/μL) were used as a standard for PCV2 quantitation. PCR was performed using a SYBR Green detection kit (Takara, China), containing MgCl₂, dNTP and Hotstar Taq polymerase. About 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). Researchers followed the protocol: pre-denaturation (30 sec at 95°C); amplification and quantification, repeated 40 cycles (5 sec at 95°C, 34 sec at 60°C) and melting (60-99°C at a heating rate of 0.1°C sec⁻¹ and fluorescence measurement).

**Total Superoxide Dismutase (T-SOD) activity detection:**
Total Superoxide Dismutase (T-SOD) activity in the serum was determined by using spectrophotometric kits at 550 nm (Nanjing Jiancheng Biotechnology Institute, China) according to the manufacturer’s instructions. The results were expressed as units per milliliter of serum (U mL⁻¹).

**Statistical analysis:** All statistical analyses were performed using SPSS 16.0 Software. Group comparisons were performed using student’s t-test. Differences were considered significant at p<0.05. Data are expressed as mean±Standard Error of the Mean (SEM).

**RESULTS AND DISCUSSION**

In this study, a serial of cytokines related with the pathogenesis of PMWS were measured to evaluate immune enhancing effects of arginine supplementation. Meanwhile, the Total Superoxide Dismutase (T-SOD) activity in the serum also checked to profile the redox status in the body. Result showed that arginine supplementation significantly increased (p<0.05) the serum IL-2 levels on the 9th and 11th dpi (Table 1). Meanwhile, serum IFN-α (Table 1) and IFN-γ (Table 1) levels in arginine group were much higher (p<0.05) than those in alanine group on the 11th dpi. Furthermore, dietary arginine supplementation significantly increased (p<0.05) the serum CRP levels on the 11th dpi (Table 1).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>3 dpi</th>
<th>5 dpi</th>
<th>7 dpi</th>
<th>9 dpi</th>
<th>11 dpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/C</td>
<td>52.31</td>
<td>55.63</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>1/2% Ala (μg mL⁻¹)</td>
<td>19.06</td>
<td>45.54</td>
<td>20.61</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Mice in Arg group were fed with 0.6% arginine basal diet while mice in Ala group were fed with 1.2% alanine basal diet. IL-2: Interleukin-2, IL-6: Interleukin-6, IFN-α: Interferon alpha, IFN-γ: Interferon gamma, CRP: C-Reactive Protein, SOD: Superoxide Dismutase, dpi: days post infection. Data are means±SEM, n = 6, *p<0.05 versus Ala group

While serum IL-6 levels in alanine group were much higher than those in the arginine group on the 9th dpi (Table 1). Meanwhile, research found that arginine supplementation have little effect on T-SOD activity (Table 1). Moreover, serum IL-10 levels were detected sporadically from 3rd day to 11th dpi, resulting in statistical difference was unavailable (Table 2). In this study, to explore the clearance of arginine against PCV2 in mouse model, PCR and qPCR were used to detect the PCV2 virus load in liver, spleen, heart, lung, kidney and ovary tissue and serum on the 3rd, 5th, 7th, 9th and 11th dpi. For this goal, PCV2 real-time PCR standard and the DNA template from each sample were prepared firstly.

PCR was used first to screen the template but all most samples were negative. Further, researchers used quantitative real-time PCR to detected it, the result was that the PCV2 virus genome was detected sporadically in liver, spleen, heart, lung, kidney and ovary tissue and serum on the 3rd, 5th, 7th, 9th and 11th dpi. Arginine participated in the regeneration of adenosine triphosphate, cell proliferation, vasodilatation, neurotransmission, calcium release and ultimately immunity (Tan et al., 2009). Actually, arginine played an important role in both innate and acquired immunity. It regulated the production of antibodies, T-cell receptor expression and B-cell development (Wu et al., 2009). NO, its metabolites, performed promising antimicrobial effect via inactivate (4Fe-4S)-cluster-containing enzymes, damage DNA, oxidize cellular membranes, inhibition of the viral DNA synthesis, late protein synthesis, virus particle formation and viral protease.

Usually, arginine be synthesized from proline, glutamine and glutamate and was enough to meet body’s need. However, in some pathological situations in which L-arginine biosynthesis cannot compensate for depletion or inadequate supply, it became a conditionally essential amino acid and thus dietary intake remains the primary determinant of plasma L-arginine levels. For example, Wu found that arginine supplied in current diets was not enough for maximal growth of milk-fed piglets or maximal reproductive performance of swine. Enough arginine content was required to perform its immune enhancing
Table 2: IL-10 levels in arginine group and alanine group

<table>
<thead>
<tr>
<th></th>
<th>3 dpi</th>
<th>5 dpi</th>
<th>7 dpi</th>
<th>9 dpi</th>
<th>11 dpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 (μg mL⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg group</td>
<td>904.3±41.2</td>
<td>1017.3±63.9</td>
<td>971.5±53.7</td>
<td>1013.5±84.0*</td>
<td>1012.3±49.1*</td>
</tr>
<tr>
<td>Ala group</td>
<td>943.8±60.1</td>
<td>981.3±57.6</td>
<td>954.8±30.2</td>
<td>901.2±26.2</td>
<td>863.8±17.8</td>
</tr>
<tr>
<td>IL-6 (μg mL⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg group</td>
<td>255.3±1.9</td>
<td>279.5±11.9</td>
<td>226.2±6.5</td>
<td>224.6±11.8*</td>
<td>260.5±9.1</td>
</tr>
<tr>
<td>Ala group</td>
<td>248.8±4.5</td>
<td>230.7±28.6</td>
<td>268.3±9.0</td>
<td>281.3±14.5</td>
<td>270.1±5.6</td>
</tr>
<tr>
<td>IFN-α (μg mL⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg group</td>
<td>945.8±79.5</td>
<td>819.2±39.3</td>
<td>889.4±134.5</td>
<td>1149.1±70.7</td>
<td>1150.7±46.1*</td>
</tr>
<tr>
<td>Ala group</td>
<td>1096.7±64.2</td>
<td>879.6±34.1</td>
<td>1061.6±49.4</td>
<td>1157.2±55.5</td>
<td>963.8±47.5</td>
</tr>
<tr>
<td>IFN-γ (μg mL⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg group</td>
<td>4.8±0.3</td>
<td>4.1±0.2</td>
<td>4.5±0.5*</td>
<td>5.8±0.1</td>
<td>5.5±0.3</td>
</tr>
<tr>
<td>Ala group</td>
<td>4.9±0.3</td>
<td>4.4±0.5</td>
<td>3.6±0.3</td>
<td>6.0±0.4</td>
<td>4.7±0.4</td>
</tr>
<tr>
<td>CRP (μg mL⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg group</td>
<td>25.9±2.9</td>
<td>26.3±3.1</td>
<td>24.9±5.3</td>
<td>45.2±4.8</td>
<td>46.3±2.3*</td>
</tr>
<tr>
<td>Ala group</td>
<td>29.4±2.8</td>
<td>22.1±1.8</td>
<td>36.2±2.3</td>
<td>42.2±4.5</td>
<td>33.3±3.4</td>
</tr>
<tr>
<td>SOD (U mL⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg group</td>
<td>264±6.0</td>
<td>57.4±5.0</td>
<td>79.2±2.7</td>
<td>71.8±12.0</td>
<td>67.2±3.7</td>
</tr>
<tr>
<td>Ala group</td>
<td>46±5.4</td>
<td>58.2±10.8</td>
<td>82.3±6.0</td>
<td>85.4±9.9</td>
<td>86.7±8.9</td>
</tr>
</tbody>
</table>

*Mice in Arg group were fed with 0.8% arginine+basal diet while mice in Ala group were fed with 1.22% alanine+basal diet. IL-10: Interleukin-10, NT: the IL-10 detected mouse number/total number. dpi: days post infection. The dates were shown as mean, n = 6

function such as lymphocyte proliferation and development. Thus, researchers hypothesized that the arginine concentration in diet was not enough for swine infected with virus (porcine circovirus) and/or bacteria resulting in inhibition the immune responses and the virus and bacteria clearance.

In fact, compelling studies indicated that dietary arginine supplementation enhanced immune responses in various models of immunological challenges, increased thymic weight, the number of thymic lymphocytes, T-lymphocyte proliferation, the cytotoxicity of specific cells (T-lymphocyte, macrophages and NK cells), IL-2 production, IL-2 receptor expression on T-lymphocytes and the delayed type hypersensitivity response and reduced morbidity and mortality in response to infectious pathogens. So, researchers hypothesized that dietary L-arginine supplement enhanced the immune effects in Porcine Circovirus Types 2 (PCV2) infected mice and resulting in the clearance against PCV2 in experimentally infected mice.

Cytokines were a large family of proteins and important players in innate and adaptive immune systems. Thus, many papers involved the cytokines profile in PCV2 infection. These exciting findings indicated that PCV2 infection could down-regulate the IL-2 mRNA expression such as Darwin which found IL-2 expression level decreased in spleen and inguinal lymph nodes of the pigs infected with PCV2. Sipos found that IL-2 mRNA level trend to be down-regulated in the pigs suffering from the natural PMWS, PDNS and PMWS pigs. IL-6 was up-regulated in most PCV2 infection, for example Shi found that IL-6 mRNA level had significant increased in PCV2 infected piglets at 7, 14, 21, 28 and 42 days post infection (dpi). Large number of evidences indicated IL-10 expression increased in PCV2 infection. This idea was tested by researchers who showed that elevated IL-10 level was consistently detected in the PMWS-affected piglets from and IL-10 was notably increased in the nature PMWS affected animals. Other compelling finding was that a transient correlation was existed between IL-10 level and the viral load of PCV2 in pig.

IFN-α and IFN-γ were members of the interferon family which were active as an antiviral and immune-modulatory cytokine. PCV2 infection could significant inhibit IFN-α and IFN-γ production. For example, Zhang showed that IFN-α was decreased in PCV2 infection and PCV2/Mycoplasma hyopneumoniae co-infection pigs; Kekarainen showed that PCV2 and to a lesser extent PCV1, Inhibited IFN-γ secretion upon a recall stimulation of PBMCs.

From the results, researchers concluded that dietary L-arginine supplementation had beneficial effects on the cytokines profile in the PCV2 infected mouse. Moreover, dietary L-arginine supplementation significant increased (p<0.05) the serum CRP level on the 11th dpi while CRP played a role in host defense against bacterial pathogens, protection from lethal bacterial infection and endotoxemia, activation of complement, opsonization and induction of phagocytosis. Thus, these compelling results indicated that L-arginine supplementation was beneficial to clear the PCV2 in mouse model. So, researchers measured the PCV2 virus load in liver, spleen, heart, lung, kidney and ovari tissue and serum on the 3rd, 5th, 7th, 9th and 11th dpi using PCR and qPCR. Researchers hope that significant difference existed between the arginine group and control group. But beyond the precondition, the PCV2 virus genome was detected sporadically, resulting in no law was found in tissue and time level and statistical
difference was unavailable. The first line of reasons should responsible for this phenomenon was that the virus stock, the dose was used to challenge and the time researchers chose to check the virus load. Actually, these selections were made according to the lab’s result in normal mouse meanwhile, similar phenomenon also happened in the other experiments in the similar mouse model. The second reason should focus on the animal model. Although, these years witnessed a promising progress in PCV2 research, little information was known about PCV2 viral performance in species other than swine and the opinions about using mouse as model was mixed. Quintana indicated that porcine circoviruses did not cause any disease or microscopic lesions in inoculated mice during the experimental period. However, Li found that PCV2 viral replication, seroconversion and microscopic lesions were found in inoculated KM mice and the KM mouse could be infected by PCV2 virus and used as a PCV2 infected experimental mode. Judging from the lab’s result, normal KM mouse could be a model for PCV2 replication. The third reason as explained in the literature (Wu et al., 2007; Yao et al., 2008; Han et al., 2009; He et al., 2009) was amino acids supplementation could delay the virus replication and/or clear the virus but this presumption needs further evidences.

CONCLUSION

It is concluded that dietary L-arginine supplementation had beneficial effect on the cytokines profile in the PCV2 infected mouse and maybe could delay the PCV2 replication and/or clear the PCV2 in mouse model.

ACKNOWLEDGEMENTS

The present research was supported by the National Key Technology R&D Program in the 11th Five year Plan of China (No.: 2008BADA7B00); the National Basic Research Program of China (973 Program) (No.: 2009CB118800), Ministry of Science and Technology of the People’s Republic of China (2010GB2D200322) and Hunan Provincial Natural Science Foundation of China (10JJ2028, 2011NK2011).

REFERENCES


