L-Arginine and L-Glutamine as Immunonutrients and Modulating Agents for *Erysipelothrix rhusiopathiae* Infection

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**Abstract:** L-arginine and L-glutamine were not only building blocks of proteins and polypeptides but also important regulators of key metabolic pathways that were necessary for maintenance, growth, reproduction and immunity in organisms. These compelling findings convinced us that L-arginine and L-glutamine play a vital role in virus and bacteria infection. However, scientific literature about its role on *Erysipelothrix rhusiopathiae* (*E. rhusiopathiae*) infection was unavailable. Thus, this study was conduct to research the effect of dietary L-arginine and L-glutamine supplementation on *E. rhusiopathiae* infection. According to the exciting results, researchers concluded that dietary L-arginine and L-glutamine supplementation ameliorated the cytokines profile and blood parameters and delayed the development process of *E. rhusiopathiae* infection in mouse model.

**Key words:** L-arginine, L-glutamine, *Erysipelothrix rhusiopathiae*, mouse model, growth, reproduction

**INTRODUCTION**

*Erysipelothrix rhusiopathiae* (*E. rhusiopathiae*) species of the genus *Erysipelothrix* was a gram-positive organism which was the causative factor for erysipelas in swine. Three forms of swine disease were described from then they were acute, leading to sudden death and general signs of septicaemia and subacute characterized by cutaneous lesions, urticarial or diamond-skin lesions and chronic form with polyarthritis and endocarditis (Grieco and Sheldon, 1970; Wang *et al.*, 2010). Meanwhile, it also affected a wide variety of vertebrate and invertebrate species such as sheep, cattle, horses, dogs, bears, kangaroos, reindeer, mice, rodents, fresh and salt water fish, turkeys, chickens, ducks and pigeons (Grieco and Sheldon, 1970; Reboli and Farrar, 1989; Wang *et al.*, 2010). More importantly, human also was the victim of this bacterium and this disease traditionally grouped as three forms including a localized cutaneous lesion form, erysipeloïd and a septicaemia form often associated with endocarditis (Gorby and Peacock, 1988; Brooke and Riley, 1999; Wang *et al.*, 2010). Thus, Ingebritson *et al.* (2010) said that *E. rhusiopathiae* infection has an economic and epidemiological impact on animal and human disease research worldwide (Ingebritson *et al.*, 2010). Among numerous control treatments, antibiotics such as penicillin or cephalosporins treatment was effective to control this disease while concerns about development of antimicrobial resistance and transference of antibiotic resistance genes from animal to human microbiota led to the decrease antibiotics usage. This decrease of antibiotic use has focused increasing attention on the development of alternative feed supplements and functional nutrients which performed a vital role in *E. rhusiopathiae* infection.

Functional amino acids not only were building blocks of proteins and polypeptides but also important regulators of key metabolic pathways that were necessary for maintenance, growth, reproduction and immunity in organisms (Wu *et al.*, 2007a, b; Suenaga *et al.*, 2008; Wu, 2009). They were arginine, cysteine, glutamine, leucine, proline and tryptophan. Arginine and glutamine, an immunonutrient and modulator, exerted an important role in physiological and immune function though its metabolites and itself.

The most exciting finding was that dietary L-arginine or L-glutamine supplementation could partially reversed the reproductive failure in mice caused by Porcine Circovirus type 2 (PCV2) infection (Ren *et al.*, 2011). However, its effect on *E. rhusiopathiae* infection was unknown.

Thus, the mainly purpose was study that the effect of dietary L-arginine and L-glutamine supplementation on *E. rhusiopathiae* infection.

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MATERIALS AND METHODS

Animals and feeding: The 92 KunMing female mice with the weight of 18-22 g were obtained from the animal Laboratory Animal Center of Shandong University of Chinese Medicine, Jinan and China. The mice were randomly assigned to three treatment groups after 3 days of adaptive feeding: arginine group (0.6% arginine+basal diet, n = 28), glutamine group (1.0% glutamine+basant diet, n = 28), control group1 (1.22% alanine+basant diet, n = 28) and control group 2 (basalt diet, n = 8). The L-arginine, L-glutamine and alanine were purchased from Beijing Chemclin Biotech, Beijing, China. The amino acids content in the basal diet was measured using Automatic Amino Acid Analyser (AAAA). The mice were housed in a friendly and environmentally controlled pathogen-free colony. 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.

Erysipelotheix rhusiopathiae inoculation strain: The E. rhusiopathiae strain used in this study was isolated from the lung of typical infected pig. The isolate was identified as E. rhusiopathiae by the PCR method and biochemical characteristics.

Experimental design

Experiment 1: To calculate the protection rate, 8 mice from each group were chosen randomly and challenged by E. rhusiopathiae strain with the dose of Least Fatal Dose (LFD, 100 CFU) after 14 days treatment. Their clinical symptoms and their death time were observed and recorded every day after challenged.

Experiment 2: The 20 mice from arginine group, glutamine group and alanine group were also challenged by E. rhusiopathiae strain with the dose of 100 CFU after 14 days treatment. The 6 mice from each group were killed on 3rd and 4th day post infection to collect blood sample for blood routine examination and E. rhusiopathiae enumeration. Meanwhile, serum on third and 4th day post infection also prepared from orbital venous and stored at -80°C for further research.

Serum cytokines detection: Serum Interleukin-1 beta (IL-1 beta), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumor Necrosis Factor alpha (TNF-α) and C-Reactive Protein (CRP) levels in serum were measured using ELISA kit 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 uL 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 uL 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 uL volumes of wash buffer. A 100 uL quantity of HRP-avidin was added to each well for 1 h at 37°C. After a final wash, a 90 uL the supplied TMB substrate was added and incubated for 30 min in the dark at 37°C. The reaction was stopped with 50 uL of supplied stop solution and absorbance measured at 450 nm.

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

Clinical observation: The onset of the symptoms was appeared at 3rd day post infection, became serious at 4th day and existed until death. These symptoms were characterized by dyspnoea, neurologically symptomatic and ataxia. All the mice were dead at 3rd and 4th day post infection in control group 2 and it became most in alanine group. Unlike control group, all the mice were dead until 5th day in arginine group and even existed to 10th day in glutamine group (Table 1). This result was forcefully indicated that dietary function amino acids supplementation could delay the pathogenesis of E. rhusiopathiae infection.

Blood routine examination and E. rhusiopathiae enumeration: General signal of septicemia was associated with E. rhusiopathiae infection. Here, blood sample from

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total mice</th>
<th>4th day</th>
<th>5th day</th>
<th>6th day</th>
<th>7th day</th>
<th>8th day</th>
<th>9th day</th>
<th>10th day</th>
<th>Mortality (%)</th>
</tr>
</thead>
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<tr>
<td>Arginine group</td>
<td>8</td>
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<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Glutamine group</td>
<td>8</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Alanine group</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Control group</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 1: The death time of mice in different group after challenged with E. rhusiopathiae.
the challenged mice were got at 3rd and 4th day after infection for blood routine examination and *E. rhusiopathiae* enumeration. The blood routine examination was finished by the special doctor in Huangxing hospital, Changsha, China. *E. rhusiopathiae* enumeration was conducted using plate count in selective media and PCR.

From Table 2 and 3, researchers found that dietary L-arginine supplementation significant increased the lymphocytes at 4th day (p<0.05) and neutrophilic granulocytes (p<0.05) but significant decreased the mean corpuscular volume (p<0.05) at 4th day, p<0.01 at 3rd day and the mean corpuscular hemoglobin at 4th day post infection (p<0.01). Meanwhile, dietary L-glutamine supplementation significant increased the lymphocytes at 3rd day (p<0.05) and neutrophilic granulocytes (p<0.05) at 4th day, p<0.01 at 3rd day but significant decreased the mean corpuscular volume at 3rd day (p<0.05). Interestingly, no *E. rhusiopathiae* was found in the blood sample for all groups. In plasm, some susceptable colonies existed but all were negative after PCR checking.

**Serum cytokines profile:** Cytokines played a role in both innate and adaptive immune responses. In this study, IL-1β, IL-6, IL-10, TNF-α and CRP levels in serum were measured using ELISA kit. IL-1 beta level was significant higher (p<0.01) in arginine group and glutamine group at 3rd day post infection, compared the alanine group (Fig. 1).

Meanwhile dietary arginine supplementation significant decreased the IL-6 level (p<0.01) at 3rd and 4th day post infection while dietary glutamine supplementation significant (p<0.01) decreased the IL-6 level at 4th day post infection (Fig. 2). Unlike IL-6, dietary arginine supplementation significant increased the TNF-α level (p<0.01) at 3rd and 4th day post infection, meanwhile, dietary glutamine supplementation significant (p<0.01) increased the TNF-α level at 4th day post infection (Fig. 3). Like IL-1β, CRP level was significant...
higher (p<0.01) in arginine group and glutamine group at 3rd day post infection, compared to the alanine group (Fig. 4). In this study, IL-10 was not detected after adding the dilutional serum (1-9).

DISCUSSION

*E. rhusiopathiae* was a gram-positive, non-spore-forming, non-acid-fast bacterium that associated with a variety of diseases in many species of mammals (Wang et al., 2005, 2010). Most importantly, the organism also established as a human pathogen. Thus, this organism has an economic and epidemiological impact on animal production and handling worldwide. Many treatments and preventions were proposed to control *E. rhusiopathiae* infection such as containment and control, sound husbandry, herd management, good sanitation, immunization and antibiotic therapy (Groschup and Timoney, 1990; Fidalgo et al., 2002; Eriksson et al., 2009). However, some disadvantages including difficult to clean, vaccine failures and worry about antibiotic resistance were raised (Imada et al., 2004; Eamens et al., 2006; Wang et al., 2010). Thus, the development of alternative feed supplements and functional nutrients may shed light on control the *E. rhusiopathiae* infection. Glutamine and arginine, displayed various beneficial effect on the host immune. A serial of evidence tested this idea such as Tan found that supplementation with 0.4-0.8% L-arginine enhanced both cellular and humoral immunity in piglets by modulating the production of leukocytes, cytokines and antibodies (Tan et al., 2009). Newsholm (2001) found that glutamine was required in terminally differentiated macrophages for the synthesis of mRNA for producing secretory proteins in immune challenge during pinocytosis or phagocytosis (Newsholm, 2001). Meanwhile, Ren et al. (2011) also found that dietary L-arginine or L-glutamine supplementation play a vital role in FCV2 infection and *E. coli* O157 infection (Ren et al., 2011).

In this study, the effect of dietary L-arginine and L-glutamine supplementation on *E. rhusiopathiae* infection was researched. From the macroscopical result, dietary L-arginine and L-glutamine supplementation significant delay the death time of mouse challenged *E. rhusiopathiae* which indicated that dietary L-arginine and L-glutamine supplementation have a significant immune protection and delayed the development process of *E. rhusiopathiae* infection. Unfortunately, no difference in death rate was observed which was disagreed with Inoue who showed that only 3 of 38 rats in the GLN group died, accounting for a mortality of 8% while in the control group, there were 21 of 38 animal deaths accounting for a mortality of 45% after the rats administered 5×10⁶ colony forming units/200 g body weight of *E. coli* via intraperitoneal injection (Inoue et al., 1993). Although, the animal model and the organism differed, the biggest different was the observation time for only 3 days was used in Inoue’s study. Usually, *E. rhusiopathiae* caused septicemia with leukocytes increase (Grieco and Sheldon, 1970; Wang et al., 2010). No abnormal increase was observed after challenged with *E. rhusiopathiae* using blood routine examination expect in the glutamine group at 3rd day. Meanwhile, dietary L-arginine and L-glutamine supplementation significant increased the lymphocytes and neutrophilic granulocytes which consisted with the macro advantages because lymphocytes and neutrophilic granulocytes played a vital role in clearance of the pathogenic organism.

Cytokines were a large family of proteins and important players in innate and adaptive immune systems. IL-1β was a pre-inflammatory cytokine which secreted by polymorphonuclear leukocyte and monocytes (Oueul et al., 2007). It enabled organisms to respond to infectious non-self challenges and induced a cascade of effects leading to inflammation through up or down
regulation of other cytokines (Dinarello, 1997). IL-6 was a multifunctional cytokine played a very complex role in biological events including immune responses, hematopoiesis and regulation of the endocrine and nervous systems (Biffi et al., 1996; Naugler and Karin, 2008). IL-10’s prime function was to inhibit many functions of NK cells, T cells and macrophage and dendritic cells, reduce production of inflammatory cytokines (Trinchieri, 2007; O’Garra et al., 2008). TNF-α had a key role in immune regulation, increasing lymphoid development, cell proliferation, differentiation, activation and death (Smyth and Johnstone, 2000; Ch’en et al., 2005). CRP played a role in host defense against bacterial pathogens, protection from lethal bacterial infection and endotoxaemia, activation of complement, opsonization and induction of phagocytosis (Szalai et al., 1995, 2000; Vokotakia, 2001; Szalai, 2002). In this study, IL-1β, TNF-α and CRP levels in serum were significant increased with the dietary L-arginine and L-glutamine supplementation, which were beneficial for the clearance of the E. rhusiopathiae and promotion the immune response. This was in agreement with the macro observations of Ren et al. (2011) study.

Interestingly, a significant decrease in IL-6 level with dietary L-arginine and L-glutamine supplementation was observed which was similar with our pervious study in PCV2 infection (submitted). Actually, IL-6, similar with TNF-α and IL-1 was pro-inflammatory cytokines and usually elevated with bacterium infection such as porcine actinobacillosis (Fossum et al., 1998) and A. pleuropneumoniae (Baarsch et al., 1995; Choi et al., 1999). However, IL-6 profile in E. rhusiopathiae infection lacked literature. Judging from this result, dietary L-arginine and L-glutamine supplementation performed its immunomodulatory role though inhibit the Th2 cytokine production but need further study. Meanwhile, IL-10 was not detected after adding the dilutional serum (1-9) which also was in agreement with our pervious study that serum IL-10 could not be detected after dietary L-arginine and L-glutamine supplementation in PCV2 infection.

CONCLUSION

The dietary L-arginine and L-glutamine supplementation played a vital immunomodulatory role in E. rhusiopathiae infection and delayed the development process of E. rhusiopathiae infection in mouse model.

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REFERENCES


