Dietary arginine supplementation enhances immune responses to inactivated Pasteurella multocida vaccination in mice

Wenkai Ren1,2†, Lingxiu Zou1†, Nengzhang Li1, Yan Wang1, Gang Liu2, Yuanyi Peng1*, Jiannan Ding3, Lichuang Cai3, Yulong Yin2* and Guoyao Wu2,4

1Chongqing Key Laboratory of Forage & Herbivore, College of Animal Science and Technology, Southwest University, Chongqing 400716, People’s Republic of China
2Key Laboratory for Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, The Chinese Academy of Sciences, 410125 Hunan, People’s Republic of China
3Biological Resources Institute, Jiangxi Academy of Sciences, Nanchang, Jiangxi 330029, People’s Republic of China
4Department of Animal Science, Texas A&M University, College Station, TX 77843, USA

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Abstract
The present study was conducted to determine the adjuvant effect of arginine in mice immunised with inactivated vaccine. Mice immunised with an inactivated Pasteurella multocida vaccine and fed diets supplemented with 0·2 % (vaccine-0·2 %) or 0·5 % (vaccine-0·5 %) arginine exhibited 100 % protection from a challenge with P. multocida serotype A (CQ2) at a dose of 4·4 £ 105 colony-forming units (2LD50; median lethal dose), when compared with mice receiving no arginine supplementation. Meanwhile, antibody titres in the vaccine-0·2 % arginine group were much higher than those in the vaccine-oil adjuvant group before challenge and at 36 h post-infection. Furthermore, immunisation with the inactivated vaccine and dietary supplementation with 0·2 % arginine increased serum levels of glutathione peroxidase, in comparison with immunisation with the inactivated vaccine and an oil adjuvant. Collectively, dietary arginine supplementation confers an immunostimulatory effect in mice immunised with the inactivated P. multocida vaccine. The present results also indicate that optimal supplemental doses of arginine are 0·2–0·5 % in the mouse model.

Key words: Arginine: Pasteurella multocida: Nutrition: Adjuvants

Arginine, a conditionally essential amino acid, exerts an important role in physiological function. Besides serving as an essential building block for tissue protein, arginine is a substrate for several non-protein, N-containing compounds, including agmatine, proline and glutamate. Thus, arginine plays vital roles in the regeneration of adenosine triphosphate, cell proliferation, vasodilatation, neurotransmission and Ca release(1–3). Additionally, arginine metabolism generates several functional substances, such as creatine, polyamines and NO(4,5). Polyamines are essential for proliferation and differentiation of cells, including lymphocytes(6). Creatine is an essential energy source for muscle contraction and neurons. Furthermore, NO plays an important role in physiological events (e.g. ovulation and placentation growth) and in pathological conditions (virus and bacterial infection)(7).

The versatile beneficial roles of arginine are not limited to physiological function; arginine also plays a vital role in the immune system(6). Results of recent studies indicate that arginine stimulates the functional activities of different cell types in the immune system, including natural killer cells, macrophages, lymphokine-activated killer cells, T and B cells(5,8,9). For example, animal experiments have shown that arginine could increase thymic weight, as well as the number and reactivity of thymic lymphocytes(10,11). Likewise, Reynolds et al. (12) also reported that arginine supplementation enhanced thymic weight, splenocyte mitogenesis, natural killer cell activity and

Abbreviations: cfu, colony-forming units; GSH-PX, glutathione peroxidase; V-0·2 %R, mice immunised with an inactivated vaccine and fed diets supplemented with 0·2 % arginine; V-0·5 %R, mice immunised with an inactivated vaccine and fed diets supplemented with 0·5 % arginine; V-1·0 %R, mice immunised with an inactivated vaccine and fed diets supplemented with 1·0 % arginine; V-adjuvant, mice immunised with an inactivated vaccine and an oil adjuvant.

* Corresponding authors: Y. Peng, email pyy2002@sina.com; Y. Yin, email yinyulong@isa.ac.cn
† These authors contributed equally to the present study.
macrophage cytotoxicity. In addition, arginine is also a critical factor affecting the differentiation of pro-B to pre-B cells in bone marrow and the release of these cells from the bone marrow\textsuperscript{13,14}. These immunostimulatory effects of arginine are more pronounced in immunocompromised hosts after trauma, surgical stress or immunosuppression by HIV or other viruses\textsuperscript{15,16}. For example, Kirk \textit{et al.}\textsuperscript{17} reported that arginine therapy resulted in an increased number and mitogenic reactivity of extrathymic T cells in athymic mice. Ren \textit{et al.}\textsuperscript{16} also found that dietary arginine supplementation partially decreased the repressive effect caused by porcine circovirus type 2 infection in the mouse model.

Collectively, arginine has versatile physiological and immunological functions. However, there is no information in the scientific literature regarding an immunomodulatory effect of arginine in vaccine-immunised mammals. Thus, the present study was conducted to evaluate the immunostimulatory effects of dietary L-arginine supplementation in mice immunised with an inactivated \textit{Pasteurella multocida} vaccine. The adjuvant effects of arginine were also compared with those of an oil adjuvant.

### Materials and methods

#### Preparation of the bacterium and inactivated vaccine

The \textit{P. multocida} serotype A (CQ2) strain used in the present study was isolated from clinically infected cattle. The main reason for the use of this strain is that we previously obtained a large amount of relevant data, including the optimal vaccine dose and LD_{50} (median lethal dose), the methods to detect serum antibodies, which greatly facilitated the conduct of the present study. The bacteria isolated were cultured in Martin’s broth agar medium containing 5% equine serum. The pathogenicity of the isolate was tested in healthy mice by intraperitoneal inoculation of a 2d culture, with all mice being succumbed to infection between 36 and 48h post-administration. The bacteria re-isolated from these infected mice were used to prepare the inactivated vaccine. The bacterial inoculum was identified to be \textit{P. multocida} serotype A using the PCR method and biochemical characteristics. Before the animal experiments, the inactivated whole-cell vaccine of \textit{P. multocida} serotype A was prepared. Briefly, bacterial colonies isolated from the liver of mice were cultured in Martin's agar at 37°C overnight, and then an isolated colony from the overnight static culture was transferred to 100 ml of Martin's broth for incubation at 37°C for 12h at 85 rpm. Then, \textit{P. multocida} serotype A strains were inactivated by the addition of 0.4% formalin to the culture mix (10^9 colony-forming units (cfu)/ml), followed by incubation at 37°C for 24h with continuous agitation.

#### Experimental design

A total of ninety-five female Kunming (KM) mice (body weight 18–22 g) were obtained from the Laboratory Animal Center of Central South University. Mice were housed in a pathogen-free mouse colony (temperature, 20–30°C; relative humidity, 45–60%; lighting cycle, 12h/d) and had free access to food (a standard rodent diet) and drinking-water. The animals were randomly divided into six groups (n=19 per group): (1) mice were fed a diet supplemented with 0.2% arginine (0.2% arginine + basal diet; Ajinomoto, Inc.) from day 0 and immunised with the inactivated vaccine at a dose of 10^9 cfu at days 15 and 20 (V-0%R group); (2) mice were fed a diet supplemented with 0.5% arginine from day 0 and immunised with the inactivated vaccine at a dose of 10^9 cfu at days 15 and 20 (V-0.5%R group); (3) mice were fed a diet supplemented with 1.0% arginine from day 0 and immunised with the inactivated vaccine at a dose of 10^9 cfu at days 15 and 20 (V-1.0%R group); (4) mice were immunised with the inactivated vaccine and received an oil adjuvant at a dose of 10^9 cfu at days 15 and 20 (V-adjuvant group); (5) mice immunised with the same volume of PBS at days 15 and 20 (the control group). At day 34, all of the mice were challenged by an intraperitoneal injection of \textit{P. multocida} serotype A (CQ2) at a dose of 4.4×10^9 cfu (2LD_{50}). From each group, ten mice were used to calculate the survival rate, and the other animals were killed to collect serum at 36h post-infection for the determination of cytokine levels and antibody titres. At day 34, serum antibody titres in all groups of mice were also measured before challenge. The present study was performed according to the guidelines of the Laboratory Animal Ethical Commission of the Chinese Academy of Sciences.

#### Analysis of serum cytokines

Serum cytokines, including IL-1β, IL-6, IL-8 and TNF-α, were measured using ELISA kits in accordance with the manufacturers’ instructions (Cusabio Biotech Company Limited). An aliquot (100 μl) of the sample or standard was added to duplicate wells of a microtitre plate, which had been pre-coated with an appropriate antibody. The buffer was used as a negative control. The plate was incubated for 2h at 37°C. A 100 μl sample of biotin antibody was added to each well after the removal of the liquid from each well and incubated for 1h at 37°C. The wells were washed three times with 200 μl of a washing buffer. A 100 μl quantity of horseradish peroxidase–avidin was then added to each well for 1h at 37°C. After the final wash, an aliquot (90 μl) of the 3,3',5,5'-tetratramethylbenzidine substrate was added and incubated for 30 min in the dark at 37°C. The reaction was stopped with 50 μl of the terminating solution and absorbance of the solution measured at 450 nm.

#### Serum glutathione peroxidase

Serum glutathione peroxidase (GSH-PX) was measured using spectrophotometric kits in accordance with the manufacturer’s instructions (Nanjing Jiancheng Biotechnology Institute).

#### Antibody detection by ELISA

ELISA were used for the detection of antibodies. Before detection, the antigens were prepared. A 10 ml bacterial culture was crushed by an ultrasonic wave, and then diluted with a bicarbonate buffer (pH 7.4). A ninety-six-well plate was coated by antigens (100 μl of the prepared antigens) and incubated for
adsorption at 4°C for 16 h. The serum collected from the mice was used as the first antibodies, and the peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) as the secondary antibody. Absorbance was read at 450 nm.

**Statistical analysis**

All statistical analyses were performed using SPSS 17.0 software (SPSS, Inc.). Data are expressed as means with their standard errors. Multiple comparisons were performed using the Student–Newman–Keuls method. Survival rates of mice were evaluated using Kaplan–Meier analysis and Proc Lifetest of SAS 9.2 (SAS Institute Inc.). Differences were considered significant at \( P<0.05 \).

**Results**

**Cytokine profile and glutathione peroxidase**

Concentrations of IL-1β, IL-6, IL-8 and TNF-α in serum were measured at 36 h after infection. As indicated in Table 1, immunisation with the inactivated vaccine, regardless of adjuvant or arginine supplementation, increased \( P<0.05 \) the circulating level of IL-1β, in comparison with the control group, while no difference was found between immunisation with the adjuvant and arginine supplementation groups, or among all the arginine supplementation groups. Unlike IL-1β levels, concentrations of IL-6, IL-8 and TNF-α in the serum of the control group were higher than those in the other groups, but no difference was detected among the arginine groups (Table 1). Furthermore, immunisation along with dietary supplementation with 0.5% arginine \( P<0.01 \) decreased serum GSH-PX levels, when compared with the control group. However, GSH-PX activity in the serum of the V-0.2%R and V-adjvant groups was lower than those in the V-0.2%R and control groups, but no difference was detected between the V-0.2%R and control groups (Fig. 1).

**Serum antibody titres**

Serum antibody titres against *Pasteurella multocida* serotype A were measured before and after being challenged with *P. multocida* serotype A. As shown in Table 2, serum antibody titres in the V-R and V-adjvant groups were much higher \( P<0.001 \) than those in the control group. Meanwhile, immunisation with an inactivated vaccine, along with dietary 0.2% arginine supplementation, increased \( P<0.05 \) serum antibody titres, when compared with immunisation with the inactivated vaccine and an oil adjuvant.

**Survival rates**

The survival rates were calculated every day after infection with *P. multocida* serotype A. All mice were dead in the control group between 36 and 48 h post-administration, and two mice were dead in both the V-1.0%R and V-adjvant groups. Interestingly, no death was observed in the V-0.2%R and V-0.5%R groups. However, Proc Lifetest of SAS 9.2 analysis showed that no difference was observed among the arginine-supplemented and adjuvant groups, while the survival rates in the vaccine group were significantly higher than those in the control group (Fig. 2).

**Discussion**

The present study was conducted to evaluate the immunomodulatory effects of dietary L-arginine supplementation in mice immunised with an inactivated *P. multocida* vaccine. Serum antibody titres were measured to assess the stimulatory effects of arginine on the systemic humoral immune response. A substantial increase was observed in all the mice immunised with the inactivated *P. multocida* vaccine, in comparison with mice immunised with PBS. Meanwhile, the most promising results were that the antibody titres in the V-0.2R group were much higher than those in the V-adjvant group at days 34 and 36 h post-challenge. These compelling results indicate that dietary supplementation with 0.2% arginine increased serum antibody titres when mice were immunised with the inactivated *P. multocida* vaccine. Similar positive results also were observed in the chicken by Tayade’s group, who reported that antibody titres in the intermediate plus l-arginine group (chickens vaccinated with a live intermediate plus strain of the infectious bursal disease virus vaccine and supplemented with 2% l-arginine) were higher than intermediate plus-vaccinated chickens. Additionally, Tan et al. found
that concentrations of total IgG and IgM in serum were higher in piglets supplemented with 0·4–0·8% arginine, compared with unsupplemented piglets. Arginine was involved in the differentiation of pro-B to pre-B cells in the bone marrow and in the release of these cells from the bone marrow\textsuperscript{(13,14)}. Moreover, arginine regulates the secretion of insulin, growth hormone, prolactin and insulin-like growth factor-I, which could mediate an NO-independent effect of arginine on immune mechanisms\textsuperscript{(5)}.

Table 2. Serum antibody titres against Pasteurella multocida serotype A in different groups of control and arginine-supplemented mice (Mean values with their standard errors, n=6)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Before challenge$^a$</th>
<th>After challenge$^a$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
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<tr>
<td>V-0·2 %R, mice immunised with an inactivated vaccine and fed diets supplemented with 0·2% arginine; V-0·5 %R, mice immunised with an inactivated vaccine and fed diets supplemented with 0·5% arginine; V-1·0 %R, mice immunised with an inactivated vaccine and fed diets supplemented with 1·0% arginine; V-adjuvant, mice immunised with an inactivated vaccine and an oil adjuvant; control, mice immunised with PBS.</td>
<td>0·36\textsuperscript{a}</td>
<td>0·01</td>
</tr>
<tr>
<td>V-0·5 %R</td>
<td>0·34\textsuperscript{ab}</td>
<td>0·001</td>
</tr>
<tr>
<td>V-1·0 %R</td>
<td>0·32\textsuperscript{ab}</td>
<td>0·02</td>
</tr>
<tr>
<td>V-adjuvant</td>
<td>0·30\textsuperscript{a}</td>
<td>0·006</td>
</tr>
<tr>
<td>Control</td>
<td>0·10\textsuperscript{a}</td>
<td>0·003</td>
</tr>
</tbody>
</table>

Although there were reports on the effects of arginine on vaccine-immunised chickens, no study has been conducted in this regard with a mammalian species. To our knowledge, the present study is the first to indicate that dietary arginine supplementation can enhance specific antibody titres in the serum of mice. This compelling result supports the view that arginine supplementation confers a useful adjuvant effect in vaccine-immunised mammals.

Cytokines actively participate in the immune response\textsuperscript{(24)}. IL-1β is one of the pivotal early-response pro-inflammatory cytokines that, through up- or down-regulation of other cytokines, enables organisms to respond to infectious non-self challenges and induce a cascade of effects leading to inflammation\textsuperscript{(25)}. IL-6 plays a very complex role in biological events, including immune responses, haematopoiesis, and modulation of endocrine and nervous systems\textsuperscript{(26,27)}. IL-8 is often associated with inflammation by acting preferentially on neutrophils\textsuperscript{(28)}, whereas TNF-α plays a key role in immune regulation, increasing lymphoid development, cell development, cell proliferation, differentiation, activation and death\textsuperscript{(29,30)}. The results of the present study indicate that concentrations of IL-6, IL-8 and TNF-α in the serum of the control group were much higher than those in the other groups. These observations are consistent with the previous finding that pro-inflammatory cytokines were increased in mice after challenge with \textit{P. multocida} serotype A1, while high antibody titres in other groups could protect mice from high levels of pro-inflammatory cytokines\textsuperscript{(24)}. However, serum IL-β levels in the V-R and V-adjuvant groups were much higher than those in the control group. The underlying mechanisms are unknown, but we observed that dietary arginine supplementation increased serum IL-1β levels in mice infected with porcine circovirus (W Ren, Y Yin and G Wu, unpublished results). Notably, serum GSH-PX levels in the control group were higher than those in the V-adjuvant group. This result suggests that immunisation resulted in an unfavourable effect on cellular oxidative defences. Similarly, our previous research also indicated that serum malondialdehyde levels...
were markedly increased, while serum total superoxide dismutase levels were decreased after immunising mice with DNA vaccines (W Ren, Y Yin and G Wu, unpublished results). However, the exact relationship between the immune system and the antioxidant system is not fully understood and warrants further investigation. Notably, we found that dietary supplementation with 0.2% arginine beneficially reversed this unfavourable decrease in serum GSH-PX levels when mice were immunised with the inactivated *P. multocida* vaccine. These results are consistent with the previous notion that arginine regulates cellular redox status and plays a role in oxidative defences either directly or through its metabolic products(1,31–33). Chander & Chopra (34) also reported that arginine administration restored depleted renal antioxidant enzymes in ischaemia–reperfusion-induced rats.

The mortality in each group of mice was calculated after the immunised mice were infected with *P. multocida* serotype A (CQ2) at a dose of 4.4 ¥ 10^5 cfu. No death was observed in both the V-0.2 %R and V-0.5 %R groups, while two mice died in the V-adjuvant group. Also, two dead mice were observed in the V-1.0 %R group, which indicated that an overdose of arginine supplementation maybe has an adverse effect on the host, as reported for cardiovascular and gastrointestinal systems(34). At least, large amounts of NO produced by inducible NO synthase could exert a deleterious effect on mammalian cells and mediate the pathogenesis of many diseases, including the autoimmune destruction of pancreatic B cells in type 1 diabetes mellitus, arthritis, glomerulonephritis, inflammatory bowel disease and neurological disorders(31). Furthermore, several studies have documented that an overdose of NO was responsible for the inhibition of T-cell proliferation and function by blocking the phosphorylation and activation of key signalling molecules, including Janus kinase 1 and 3, signal transducers and activators of transcription 5, extracellular regulated protein kinases and protein kinase B at the level of IL-2 receptor signalling(8,35,36).

Collectively, the results of the present study demonstrate that dietary arginine supplementation confers significant immunostimulatory effects in mice immunised with an inactivated *P. multocida* vaccine. Thus, arginine can be used as an adjuvant for administration of vaccines in animal production. Additionally, based on serum antibody titres and mortality, optimal supplemental doses of arginine are 0.2–0.5% in the mouse model.

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