Journal of Animal and Veterinary Advances 12 (15): 1266-1270, 2013
ISSN: 1680-5593

Effect of Alanine Supplementation on Vaccine Immunized Mice

Pan Zhang and Jianlong Wang
Department of Infectious Disease, The Third Xiangya Hospital,
Central South University, 410013 Changsha, China

Abstract: This study was conducted to test the immunostimulatory effect of graded dose of alanine supplementation on inactivated vaccine immunized mice. Animals were randomly divided into five groups: mice were immunized with inactivated vaccines, mice were treated with dietary 0.5% alanine supplementation and immunized with inactivated vaccines, mice were treated with dietary 1.0% alanine supplementation and immunized with inactivated vaccines, mice were treated with dietary 2.0% alanine supplementation and immunized with inactivated vaccines and all the mice immunized with PBS. Results found that alanine supplementation has little significant effect on serum antibody production, serum interleukin-1 beta and 8 levels and survival rate of mice after challenge. However, higher dose of alanine supplementation increased serum GSH-PX activity compared to the lower dose of alanine supplementation. Collectively, dietary alanine supplementation has little effect on the immune parameters in vaccine immunized mice indicating alanine can be used as isonitrogenous control to study the immune regulatory function of other amino acids.

Key words: Alanine, isonitrogenous control, vaccine, GSH-PX, mice

INTRODUCTION

Classically, amino acids are thought as the building blocks for the synthesis of proteins, as well as the precursors for the biosynthesis of various compounds with numerous physiological functions (Wu *et al*., 2013a, b). For example, compounds like glutathione, creatine and heme of hemoglobin are synthesized from amino acids in different metabolic pathways in the body. However, recent finding have indicated that amino acid also plays various crucial roles like a substrate for various metabolic pathways (Bertolo *et al*., 2003; Van Meijl *et al*., 2010) an energy source for intestinal mucosa (Watford, 2008) a mediator for cell signaling, a regulator for oxidative reasctions (Reeds *et al*., 1997; Wu *et al*., 2004) as well as immune responses (Ren *et al*., 2013a). Interestingly, recent study found that some amino acids, like arginine, glutamine and proline, confers various beneficial function in bacterial or virus infected mice or pigs and these benefits include the alleviation of tissue damage, promotion of immune response and antioxidative capacity and even growth and reproductive performance (Ren *et al*., 2013b-e, 2012a, c).

These exciting findings extend the function of amino acids from traditional nutrition to immune field. These compelling discoveries also promote further study to characterize the immune regulatory function of other amino acids in normal or morbid situation. However, a critic missing question needed to address is whether alanine can be used as isonitrogenous control to study the immune regulatory function of other amino acids. Most of study, alanine was chosen as control for other amino acids research such as arginine, glutamine and proline (Ren *et al*., 2012a, 2013f; Wu *et al*., 2013b).

However, previous study indicated that alanine is not absolutely inert for immune response in body. For example, cell culture medium supplementation with 2 mM-alanine augmented antibody production in B-lymphocyte hybridoma (Duval *et al*., 1991; Franek and Sramkova, 1996). At present, little information is available regarding an effect of dietary supplementation with alanine on the immune response in any animal species. Thus, this study investigate the immune regulatory role of alanine in vaccine immunized mice with graded dose of alanine supplementation.

MATERIALS AND METHODS

Inactivated *Pasteurella multocida* (*P. multocida*) vaccine: The inactivated *Pasteurella multocida* (*P. multocida*) vaccine was kindly provided by Mr. Ren (Chinese Academy of Sciences, China). This vaccine was made from *P. multocida* serotype A strains at dose of 10^9 CFU mL^{-1}. This was isolated from clinically infected cattle.

Experimental design: The experiment was conducted as described previously (Ren *et al*., 2013e, f) with some modifications. In brief, 100 female KM mice (body weight 18~22 g) were obtained from Laboratory Animal Center of

Corresponding Author: Pan Zhang, Department of Infectious Disease, The Third Xiangya Hospital, Central South University, 410013 Changsha, China

Central South University (Hunan, China). The mice were housed in a pathogen-free mouse colony (temperature, 20-30°C; relative humidity, 45-60%; lighting cycle, 12 h day^{-1}) and had free access to food and drinking water. Animals were randomly divided into five groups (n = 20 per group): all mice were immunized with inactivated vaccines at dose of 10^9 CFU at day 15 and 20 (1 group), all mice were treated with dietary 0.5% alanine (Ajinomoto Inc., Tokyo, Japan) supplementation (0.5% alanine+basal diet) from day 0 and immunized with inactivated vaccines at dose of 10^9 CFU at day 15 and 20 (2 group), all mice were treated with dietary 1.0% alanine (Ajinomoto Inc., Tokyo, Japan) supplementation (1.0% alanine+basal diet) from day 0 and immunized with inactivated vaccines at dose of 10^9 CFU at day 15 and 20 (3 group), all mice were treated with dietary 2.0% alanine (Ajinomoto Inc., Tokyo, Japan) supplementation (2.0% alanine+basal diet) from day 0 and immunized with inactivated vaccines at dose of 10^9 CFU at day 15 and 20 (4 group), all the mice immunized with PBS at same volume at day 15 and 20 (5 group). At day 34, all of the mice were challenged by an intraperitioneal injection of *P. multocida* serotype A (CQ2) at dose of 4.4×10^5 CFU (2LD50).

Ten mice in each group were used to calculate survival rate and the others were killed to collect the serum at 36 h post infection for cytokine levels, Glutathione Peroxidase (GSH-PX) levels and antibody titers detection. Meanwhile, the serum antibody titers in all groups also were detected at day 34 before challenge. This study was performed according to the guidelines of the Laboratory Animal Ethical Commission of the Central South University.

Analysis of serum cytokine levels: Interleukin-1 beta (IL-1 β) and interleukin-8 (IL-8) in serum were measured using ELISA kits in accordance with the manufacturer's instructions (CUSABIO BIOTECH Co., Ltd. China) (Ren *et al*., 2013c).

Serum GSH-PX: Serum Glutathione Peroxidase (GSH-PX) was measured using spectrophotometric kits in accordance with the manufacturer's instructions (Nanjing Jiancheng Biotechnology Institute, China) (Ren *et al*., 2012a).

Antibody detection by ELISA: Enzyme-Linked Immunosorbent Assays (ELISA) were used for the detection of antibodies (Ren *et al*., 2013e, f). Prior to detection, the antigens were prepared to coat to a 96 well plate.

Then, primary antisera were diluted 1:40 in PBS containing 0.5% Tween-20 and 1% Bovine Serum Albumin (BSA). Following five washings, horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, USA) diluted 1:10000 in PBS-T containing 1% BSA was added to each well and the plates were incubated at 37°C for 3 h. After the final wash, an aliquot (100 μL) of the TMB substrate was added and incubated for 15 min in the dark at 37°C. The reaction was stopped with 50 μL of the terminating solution and absorbance measured at 450 nm.

Statistical analysis: All statistical analyses were performed using SPSS 17.0 Software. Data are expressed as mean±Standard Error of the Mean (SEM). Multiple comparisons were performed using One-way ANOVA. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Serum antibody level: Like previous study (Ren *et al*., 2013e, f), the inactivated vaccine triggers significant antibody production, compared those with no vaccine immunization (Fig. 1a). However, graded dose of alanine

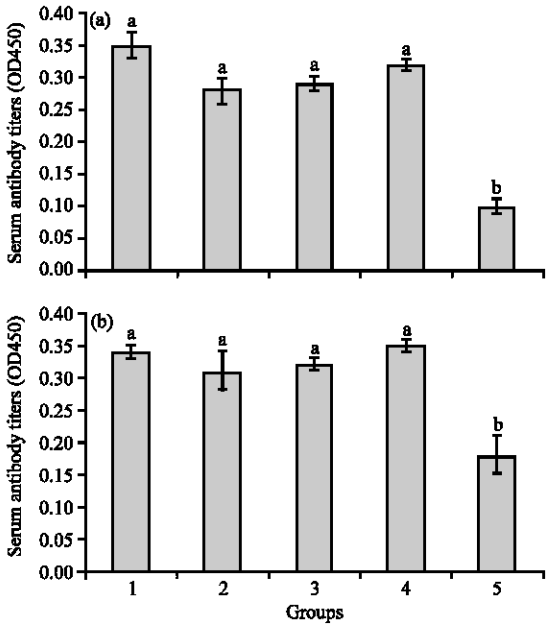

Fig. 1: Serum antibody titers against *P. multocida* serotype A in different groups (OD450). a) before and b) after challenge. Mice were immunized with inactivated vaccines as 1 group; mice were immunized with inactivated vaccines and dietary 0.5% alanine supplementation as 2 group; mice were immunized with inactivated vaccines and dietary 1.0% alanine supplementation as 3 group; mice were immunized with inactivated vaccines and dietary 2.0% alanine supplementation as 4 group; mice immunized with PBS as 5 group. Data are mean±SEM, n = 6. $^{a-b}$Mean values sharing different superscripts within columns differ ($p<0.05$)

supplementation has little effect on the antibody production, compared those vaccine immunized mice with no alanine supplementation (Fig. 1a). Similarly, no obvious difference was found after challenge among different dose of alanine supplementation groups (Fig. 1b). This observation is difference from previous reports that arginine or proline supplementation significantly increases the antibody production before and after *P. multocida* challenge (Ren *et al*., 2013e, f). The reasonable difference may associate with the metabolic difference between alanine and arginine or proline in the body (Wu, 2009). This finding also differed from previous *in vitro* indicated that supplementation with 2 mM-alanine augmented antibody production in B-lymphocyte hybridoma (Duval *et al*., 1991; Franek and Sramkova, 1996). The primarily explanation for this difference may relate to the experimental model in two studies.

Survival rates: Similar to the result of antibody titers, vaccine immunization confers well protection against *P. multocida* challenge, compared to the group without vaccination (Fig. 2). Meanwhile, there is no significant difference was observed among different dose of alanine supplementation about survival rates after challenge (Fig. 2). As little information is available regarding an effect of alanine supplementation on the growth performance and mortality in any animal species thus, the discovery is difficult to compare with other study. However, it still could not rule out the function of alanine supplementation in such situation because the vaccine is enough to provide well protection. Thus, it is interesting to study the impacts of alanine supplementation on other immunosuppressed animal model.

Cytokine profile and GSH-PX: Mouse serum IL-1 β and IL-8 levels were measured at 36 h after infection. Pervious study indicated that serum IL-1 β and IL-8 levels significantly increase in mice after challenged with *P. multocida* serotype A while the high antibody titers protects the mice from high levels of pro-inflammatory cytokines (Praveena *et al*., 2010; Ren *et al*., 2012b). In this study, researchers found that there is no remarkable difference about serum IL-1 β level among different treatments (Fig. 3) while mice immunized with vaccine have lower serum IL-8 level compared those without vaccination (Fig. 4). Current finding is similar to previous study suggested that high level of antibody lows serum IL-8 expression in mice after challenged with *P. multocida* serotype A (Ren *et al*., 2013e, f).

GSH-PX is an important peroxidase enzymes widely spread in the body which can remove harmful peroxides metabolites from the cells and block the lipid peroxidation chain reaction (Yin *et al*., 2013). Results found that higher dose of alanine supplementation increased serum GSH-PX activity compared to the lower dose of alanine supplementation (Fig. 5). It is easy to understand arginine or glutamine supplementation to increase serum GSH-PX activity (Ren *et al*., 2013c, 2012a) however it is difficult to illustrate the underling mechanism about the

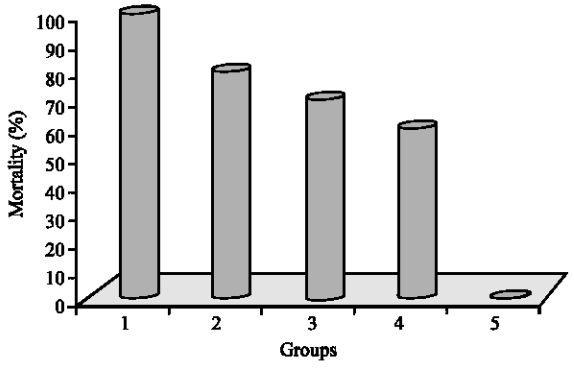

Fig. 2: Survavel rate in different groups after challenge. Mice were immunized with inactivated vaccines as 1 group; mice were immunized with inactivated vaccines and dietary 0.5% alanine supplementation as 2 group; mice were immunized with inactivated vaccines and dietary 1.0% alanine supplementation as 3 group; mice were immunized with inactivated vaccines and dietary 2.0% alanine supplementation as 4 group; mice immunized with PBS as 5 group. Data are mean±SEM, n = 6

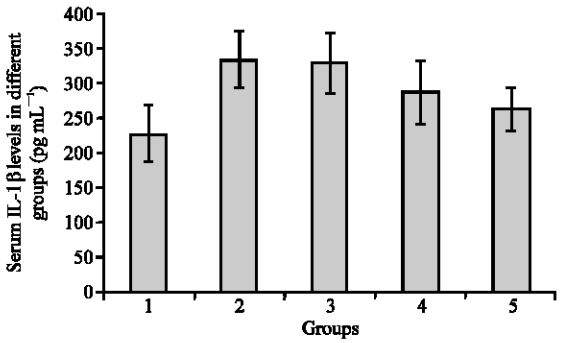

Fig. 3: Serum interleukin-1 beta in different groups. Mice were immunized with inactivated vaccines as 1 group; mice were immunized with inactivated vaccines and dietary 0.5% alanine supplementation as 2 group; mice were immunized with inactivated vaccines and dietary 1.0% alanine supplementation as 3 group; mice were immunized with inactivated vaccines and dietary 2.0% alanine supplementation as 4 group; mice immunized with PBS as 5 group. Data are mean±SEM, n = 6

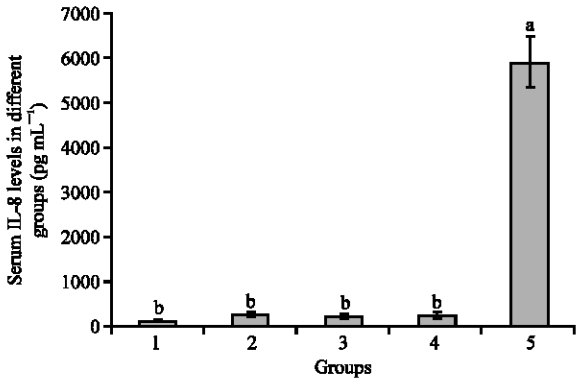

Fig. 4: Serum interleukin-8 in different groups. Mice were immunized with inactivated vaccines as 1 group; mice were immunized with inactivated vaccines and dietary 0.5% alanine supplementation as 2 group; mice were immunized with inactivated vaccines and dietary 1.0% alanine supplementation as 3 group; mice were immunized with inactivated vaccines and dietary 2.0% alanine supplementation as 4 group; mice immunized with PBS as 5 group. Data are mean±SEM, n = 6. $^{a\text{-}b}$Mean values sharing different superscripts within columns differ ($p<0.05$)

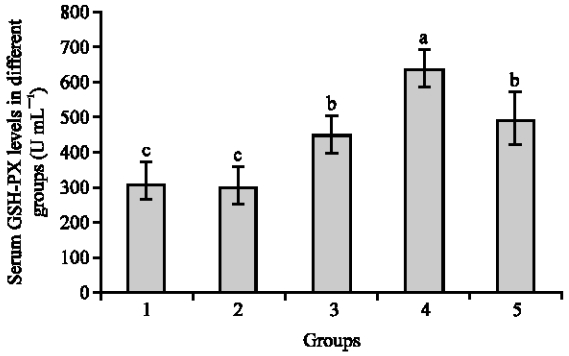

Fig. 5: Serum GSH-PX in different groups. Mice were immunized with inactivated vaccines as 1 group; mice were immunized with inactivated vaccines and dietary 0.5% alanine supplementation as 2 group; mice were immunized with inactivated vaccines and dietary 1.0% alanine supplementation as 3 group; mice were immunized with inactivated vaccines and dietary 2.0% alanine supplementation as 4 group; mice immunized with PBS as 5 group. Data are mean±SEM, n = 6, $^{a\text{-}c}$Mean values sharing different superscripts within columns differ ($p<0.05$)

regulatory role of alanine supplementation on the body because the experiment about the function of alanine supplementation on body is needed.

CONCLUSION

This study found that dietary alanine supplementation has little effect on the immune parameters in vaccine immunized mice indicating alanine can be used as isonitrogenous control to study the immune regulatory function of other amino acids.

REFERENCES

Bertolo, R.F.P., J.A. Brunton, P.B. Pencharz and R.O. Ball, 2003. Arginine, ornithine and proline interconversion is dependent on small intestinal metabolism in neonatal pigs. Am. J. Physiol.-Endocrinol. Metab., 284: E915-E922.

Duval, D., C. Demangel, K. Munierjolain, S. Miossec and I. Geahel, 1991. Factors controlling cell proliferation and antibody production in mouse hybridoma cells. Influence of the amino acid supply. Biotechnol. Bioeng., 38: 561-570.

Franek, F. and K. Sramkova, 1996. Protection of B lymphocyte hybridoma against starvation-induced apoptosis: Survival-signal role of some amino acids. Immunol. Lett., 52: 139-144.

Praveena, P.E., S. Periasamy, A.A. Kumar and N. Singh, 2010. Cytokine profiles, apoptosis and pathology of experimental *Pasteurella multocida* serotype A1 infection in mice. Res. Vet. Sci., 89: 332-339.

Reeds, P.J., D.G. Burrin, B. Stoll, F. Jahoor, L. Wykes, J. Henry and M.E. Frazer, 1997. Enteral glutamate is the preferential source for mucosal glutathione synthesis in fed piglets. Am. J. Physiol.-Endocrinol. Metab., 273: E408-E415.

Ren, W., Y. Yin, G. Liu, X. Yu and Y. Li *et al*., 2012a. Effect of dietary arginine supplementation on reproductive performance of mice with porcine circovirus type 2 infection. Amino Acids, 42: 2089-2094.

Ren, W., L. Zou, N. Li, Y. Wang and G. Liu *et al*., 2012b. Dietary arginine supplementation enhances immune responses to inactivated Pasteurella multocida vaccination in mice. Br. J. Nutr., 19: 1-6.

Ren, W.K., G. Liu, T.J. Li, J. Yin and X.S. Wu *et al*., 2012c. Dietary supplementation with arginine and glutamine confers a positive effect in porcine circovirus-infected pig. J. Food Agric. Environ., 10: 485-490.

Ren, W., Y. Li, X. Yu, W. Luo, G. Liu, H. Shao and Y. Yin, 2013a. Glutamine modifies immune responses of mice infected with porcine circovirus type 2. Br. J. Nutr., 110: 1053-1060.

Ren, W., S. Liu, S. Chen, F. Zhang and N. Li *et al*., 2013b. Dietary L-glutamine supplementation increases *Pasteurella multocida* burden and the expression of its major virulence factors in mice. Amino Acids, 45: 947-955.

Ren, W., M. Wu, W. Luo, R. Huang and Y. Yin *et al*., 2013c. Dietary supplementation with proline confers a positive effect in both porcine circovirus-infected pregnant and non-pregnant mice. Br. J. Nutr., 110: 1492-1499.

Ren, W., L. Zou, N. Li, Y. Wang and G. Liu *et al*., 2013d. Dietary arginine supplementation enhances immune responses to inactivated *Pasteurella multocida* vaccination in mice. Br. J. Nutr., 109: 867-872.

Ren, W., L. Zou, Z. Ruan, N. Li and Y. Wang *et al*., 2013e. Dietary L-proline supplementation confers immunostimulatory effects on inactivated *Pasteurella multocida* vaccine immunized mice. Amino Acids, 45: 555-561.

Ren, W.K., J. Yin, X.P. Zhu, G. Liu, N.Z. Li, Y.Y. Peng and Y.L. Yin, 2013f. Glutamine on intestinal inflammation: A mechanistic perspective. Eur. J. Inflam., 11: 315-326.

Van Meijl, L.E.C., H.E. Popeijus and R.P. Mensink, 2010. Amino acids stimulate Akt phosphorylation and reduce IL-8 production and NF-κB activity in HepG2 liver cells. Mol. Nutr. Food Res., 54: 1568-1573.

Watford, M., 2008. Glutamine metabolism and function in relation to proline synthesis and the safety of glutamine and proline supplementation. J. Nutr., 138: 2003s-2007s.

Wu, G., 2009. Amino acids: Metabolism, functions and nutrition. Amino Acids, 37: 1-17.

Wu, G., Y.Z. Fang, S. Yang, J.R. Lupton and N.D. Turner, 2004. Glutathione metabolism and its implications for health. J. Nutr., 134: 489-492.

Wu, G., Z. Wu, Z. Dai, Y. Yang and W. Wang *et al*., 2013a. Dietary requirements of nutritionally non-essential amino acids by animals and humans. Amino Acids, 44: 1107-1113.

Wu, L., W. Wang, K. Yao, T. Zhou and J. Yin *et al*., 2013b. Effects of dietary arginine and glutamine on alleviating the impairment induced by deoxynivalenol stress and immune relevant cytokines in growing pigs. PLoS ONE, Vol. 8. 10.1371/journal.pone.0069502.

Yin, J., W. Ren, G. Liu, J. Duan and G. Yang *et al*., 2013. Birth oxidative stress and the development of an antioxidant system in newborn piglets. Free Radical Res., 47: 1027-1035.