Effects of Arginine on Carp Immunity in In Vivo and In Vitro Experiments: Postprint

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Abstract

This study investigated the effects of arginine (Arg) on carp immunity using both in vitro and in vivo approaches. In the in vitro experiment, kidney leukocytes were cultured in media supplemented with 0, 0.5, 1.0, 1.5, and 2.0 mmol/L Arg for various durations, after which the proliferation index, respiratory burst activity, phagocytic activity, and bactericidal rate were measured. In the in vivo experiment, carp with an average body weight of 37 g were randomly allocated into five groups (three replicates per group, ten fish per replicate) and injected with Arg at doses of 0, 25, 50, 100, and 200 mg/kg body weight, followed by a 2-week feeding period with commercial feed. Parameters measured included kidney leukocyte proliferation index, respiratory burst activity, and phagocytic activity, as well as serum and hepatopancreatic nitric oxide (NO) content, nitric oxide synthase (NOS) activity, and serum albumin (ALB) content. The in vitro results demonstrated that Arg supplementation enhanced the proliferation index, respiratory burst activity, phagocytic activity, and bactericidal rate of kidney leukocytes. Specifically, after 12 and 24 h of culture, the proliferation index of kidney leukocytes in the 1.0, 1.5, and 2.0 mmol/L Arg groups was significantly higher than that in the 0 mmol/L Arg group ($P<0.05$). Following 6, 12, and 24 h of culture, the respiratory burst activity of kidney leukocytes in the 1.0 mmol/L Arg group was significantly higher than that in the 0 mmol/L Arg group ($P<0.05$). After 12 and 24 h of culture, the phagocytic activity of kidney leukocytes in the 1.0, 1.5, and 2.0 mmol/L Arg groups was significantly higher than that in the 0 mmol/L Arg group ($P<0.05$). At 18 h of culture, the bactericidal rate of kidney leukocytes in the 1.0, 1.5, and 2.0 mmol/L Arg groups was significantly higher than that in the 0 mmol/L Arg group ($P<0.05$). The in vivo results indicated that the respiratory burst activity, phagocytic activity, and proliferation index of kidney leukocytes in the 50, 100, and 200 mg/kg Arg groups were significantly higher than those in the 0 mg/kg Arg group ($P<0.05$). Serum ALB and NO contents in the 100 and 200 mg/kg Arg groups

were significantly higher than those in the 0 mg/kg Arg group (P<0.05), while serum NOS activity in the 50, 100, and 200 mg/kg Arg groups was significantly higher than that in the 0 mg/kg Arg group (P<0.05). Hepatopancreatic NO content in the 50, 100, and 200 mg/kg Arg groups was significantly higher than that in the 0 mg/kg Arg group (P<0.05), and hepatopancreatic NOS activity in the 25 and 50 mg/kg Arg groups was significantly higher than that in the 0 mg/kg Arg group (P<0.05). These findings demonstrate that Arg enhanced the immune function of carp kidney leukocytes, with the in vitro experiment suggesting an optimal Arg concentration of 1.0 mmol/L. Under normal feeding conditions, the optimal injection dosage of Arg for carp was 50–100 mg/kg body weight.

Full Text

Effects of Arginine on Immunity of Carp (Cyprinus carpio) in Vivo and in Vitro

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Abstract

This study investigated the effects of arginine (Arg) on carp immunity using both in vitro and in vivo approaches. In the in vitro experiment, arginine was added to culture media at concentrations of 0, 0.5, 1.0, 1.5, and 2.0 mmol/L. Kidney leukocytes were cultured for various durations before measuring proliferation index, respiratory burst activity, phagocytic activity, and bactericidal rate. In the in vivo experiment, carp with an average body weight of 37 g were randomly divided into five groups (three replicates per group, ten fish per replicate) and intraperitoneally injected with arginine at doses of 0, 25, 50, 100, and 200 mg/kg body weight. Fish were fed commercial diets for two weeks, after which kidney leukocyte proliferation index, respiratory burst activity, and phagocytic activity were assessed, along with serum and hepatopancreatic nitric oxide (NO) content, nitric oxide synthase (NOS) activity, and serum albumin (ALB) content.

The in vitro results demonstrated that arginine supplementation enhanced kidney leukocyte proliferation index, respiratory burst activity, phagocytic activity, and bactericidal rate. After 12 and 24 hours of culture, the proliferation index in the 1.0, 1.5, and 2.0 mmol/L arginine groups was significantly higher than in the 0 mmol/L group (P<0.05). Following 6, 12, and 24 hours of culture, respiratory burst activity in the 1.0 mmol/L arginine group was significantly

elevated compared to the 0 mmol/L group ($P<0.05$). Phagocytic activity after 12 and 24 hours was significantly higher in the 1.0, 1.5, and 2.0 mmol/L groups versus the control ($P<0.05$). After 18 hours of culture, bactericidal rates in the 1.0, 1.5, and 2.0 mmol/L arginine groups were significantly greater than in the 0 mmol/L group ($P<0.05$).

In vivo findings revealed that kidney leukocyte respiratory burst activity, phagocytic activity, and proliferation index were significantly higher in the 50, 100, and 200 mg/kg arginine groups compared to the control ($P<0.05$). Serum ALB and NO contents in the 100 and 200 mg/kg groups were significantly elevated above control levels ($P<0.05$), while serum NOS activity was significantly higher in the 50, 100, and 200 mg/kg groups ($P<0.05$). Hepatopancreatic NO content was significantly increased in the 50, 100, and 200 mg/kg groups ($P<0.05$), and hepatopancreatic NOS activity was significantly higher in the 25 and 50 mg/kg groups compared to control ($P<0.05$). These results indicate that arginine enhances kidney leukocyte immunity in carp, with an optimal concentration of 1.0 mmol/L in vitro. Under normal feeding conditions, the appropriate intraperitoneal injection dose ranges from 50 to 100 mg/kg body weight.

Keywords: arginine; carp; immunity; cell culture

Introduction

Protein constitutes the material basis of life, participating in every cell and all vital components of the organism. Insufficient protein intake compromises antibody formation, and protein deficiency can lead to vitamin or trace element deficiencies, thereby reducing nutritional status and immune capacity and affecting the health of cultured animals. Supplementing with appropriate amino acids can enhance protein synthesis and improve immunity. Among these, functional amino acids—defined as amino acids that serve special functions beyond protein synthesis—are essential not only for normal growth and maintenance but also for the synthesis of many bioactive substances. Arginine represents one such functional amino acid.

While numerous studies have documented arginine' s effects in mammals, research in fish remains limited. In mammals, arginine participates in protein deposition and metabolism through multiple enzymes, exerting multiple nutritional and physiological effects via its metabolites, including nitric oxide (NO) and polyamines. As important bioactive substances, polyamines are involved in protein synthesis, cell proliferation and differentiation, and gene expression regulation. Arginine is an essential amino acid for fish, and both arginine itself and its metabolites (NO, ornithine, and citrulline) can enhance phagocytosis and bactericidal activity while promoting immunoglobulin synthesis. Research has shown that preoperative arginine intake effectively reduces postoperative sepsis and improves infection resistance. In weaned piglets, dietary arginine supplementation reduces intestinal crypt depth and elevates interleukin-2 (IL-

2) gene expression, thereby enhancing intestinal immune function. In aquatic animals, arginine has been shown to increase NO production in channel catfish macrophages and improve resistance against *Edwardsiella* infection. Dietary arginine supplementation also enhances respiratory burst activity, phagocytosis, and lysozyme activity in red drum and hybrid striped bass.

Common carp, a temperate freshwater fish native to Asia, is an important aquaculture species in China due to its rapid growth and palatable flesh. However, research on arginine as a functional amino acid in aquatic animals remains insufficient. This study used carp as a model to investigate arginine' s effects on physiological, biochemical, and immunological parameters. Through in vitro culture media supplementation, we screened sensitive immune indicators to determine optimal arginine concentrations, which were then validated through in vivo injection experiments. These findings provide a reference for green, environmentally friendly, and healthy aquaculture practices.

1.1 Experimental Design

Experimental fish were obtained from the same batch of carp fry at Tianjin Huanxin Aquatic Breeding Farm. After disinfection, fish were acclimated in tanks for one week. Arginine was purchased from MDbio (Taiwan) with 99.4% purity.

For the in vitro experiment, 27 healthy carp of similar size (average weight 34 g) were anesthetized and bled via the caudal vein before dissection to obtain head kidney and trunk kidney tissues. Tissues were placed in 0.85% physiological saline to remove adherent blood cells. RPMI-1640 culture media were supplemented with arginine at concentrations of 0, 0.5, 1.0, 1.5, and 2.0 mmol/L to create five treatment groups for leukocyte culture. Two independent pooled samples (each with three replicates) were used for immunological assays.

In the in vivo experiment, 150 carp of uniform size and weight were randomly allocated into five groups with three replicates per group (ten fish per replicate). Fish were stocked in 110-L tanks and fed commercial diets twice daily. After one week, arginine solutions of varying concentrations were prepared and administered via intraperitoneal injection at doses of 0, 25, 50, 100, and 200 mg/kg body weight in a volume of 0.5 mL. Following injection, fish continued receiving commercial diets for two weeks before sampling.

1.2 Sample Preparation and Analysis

For the in vitro experiment, leukocytes were isolated and cultured according to the method of Cheng et al. Cell suspensions were adjusted to a density of 2$×10^{7}$ cells/mL with viability exceeding 95% as determined by 0.1% trypan blue staining. Proliferation index was measured after 12 and 48 hours of culture, respiratory burst activity after 2, 6, 12, and 24 hours, phagocytic activity after 12 hours, and bactericidal rate after 18 hours, following the protocols established by Cheng et al.

In the in vivo experiment, blood was collected from the caudal vein using syringes rinsed with 7% sodium heparin solution, with each fish' s blood placed in a separate centrifuge tube. Blood samples were centrifuged at 4,000 rpm for 10 minutes, and serum was collected and stored at -80°C for subsequent analysis. Following blood collection, fish were dissected to obtain hepatopancreas tissue, which was homogenized, diluted with 0.85% physiological saline, and centrifuged at 2,500 rpm for 10 minutes. The supernatant was used to determine ALB, NO content, and NOS activity according to the protocols provided by the Nanjing Jiancheng Bioengineering Institute assay kits. Head kidney and trunk kidney tissues were washed in physiological saline to remove surface blood contamination before culture. Proliferation index, respiratory burst activity, and phagocytic activity were determined following the method of Buentello et al.

1.3 Statistical Analysis

Data were analyzed using SPSS 16.0 software via one-way ANOVA. When significant differences were detected ($P<0.05$), Duncan' s multiple range test was applied for intergroup comparisons.

Results

2.1 Effects of Arginine Supplementation in Culture Media on Kidney Leukocyte Proliferation Index

As shown in [Figure 1: see original paper], arginine supplementation in culture media markedly increased the proliferation index of carp kidney leukocytes. After 12 hours of culture, the proliferation index in the 1.0, 1.5, and 2.0 mmol/L arginine groups was significantly higher than in the 0 and 0.5 mmol/L groups ($P<0.05$), with no significant differences between the 0 and 0.5 mmol/L groups or among the 1.0, 1.5, and 2.0 mmol/L groups ($P>0.05$). After 48 hours, the proliferation index increased progressively with arginine concentration, reaching its peak in the 2.0 mmol/L group, which was significantly higher than the 0, 0.5, and 1.0 mmol/L groups ($P<0.05$) but not significantly different from the 1.5 mmol/L group ($P>0.05$). Except for the 1.0 mmol/L group, which showed a lower proliferation index at 48 hours compared to 12 hours, all other groups exhibited increased proliferation over time.

Note: In figures, columns with different lowercase letters indicate significant differences ($P<0.05$), while those with same or no letters indicate no significant difference ($P>0.05$).

2.2 Effects of Arginine Supplementation in Culture Media on Kidney Leukocyte Respiratory Burst Activity

[Figure 2: see original paper] illustrates that arginine supplementation elevated respiratory burst activity in carp kidney leukocytes. After 2 hours of culture, all arginine-treated groups (0.5, 1.0, 1.5, and 2.0 mmol/L) showed increased

activity compared to the control, though differences were not statistically significant ($P>0.05$). Following 6, 12, and 24 hours of culture, the 1.0, 1.5, and 2.0 mmol/L groups exhibited significantly higher respiratory burst activity than the 0 and 0.5 mmol/L groups ($P<0.05$), with the 1.0 mmol/L group showing significantly greater activity than the control ($P<0.05$). No significant differences were observed among the 1.0, 1.5, and 2.0 mmol/L groups ($P>0.05$). Respiratory burst activity in all groups increased initially and then decreased with prolonged culture time, peaking in the 1.0 mmol/L group at 12 hours.

2.3 Effects of Arginine Supplementation in Culture Media on Kidney Leukocyte Phagocytic Activity

As depicted in [Figure 3: see original paper], arginine supplementation enhanced the phagocytic activity of carp kidney leukocytes. After 12 hours of culture, phagocytic activity increased progressively with arginine concentration, with the 2.0 mmol/L group achieving the highest activity, significantly greater than the 0 and 0.5 mmol/L groups ($P<0.05$) but not significantly different from the 1.0 and 1.5 mmol/L groups ($P>0.05$). Similar trends were observed after 24 hours, where the 1.5 and 2.0 mmol/L groups demonstrated significantly higher phagocytic activity than all other groups ($P<0.05$).

2.4 Effects of Arginine Supplementation in Culture Media on Kidney Leukocyte Bactericidal Rate

[Figure 4: see original paper] demonstrates that 18 hours of arginine supplementation significantly improved the bactericidal rate (*Edwardsiella tarda*) of carp kidney leukocytes. While no significant differences were detected among the 1.0, 1.5, and 2.0 mmol/L groups ($P>0.05$), the 1.0 and 1.5 mmol/L groups exhibited significantly higher bactericidal rates than the 0 and 0.5 mmol/L groups ($P<0.05$), with the 1.0 mmol/L group reaching the peak bactericidal activity.

2.5 Effects of Arginine Injection on Kidney Leukocyte Respiratory Burst Activity, Phagocytic Activity, and Proliferation Index

summarizes the effects of arginine injection on carp kidney leukocyte functions. Respiratory burst activity increased initially and then plateaued with rising arginine doses, reaching maximum values in the 100 and 200 mg/kg groups, which were significantly higher than the 0 and 25 mg/kg groups ($P<0.05$) but not significantly different from the 50 mg/kg group ($P>0.05$). Phagocytic activity increased progressively with arginine concentration ($P<0.05$), peaking in the 200 mg/kg group, which was not significantly different from the 100 mg/kg group ($P>0.05$) but significantly exceeded all other groups ($P<0.05$). The proliferation index followed a similar trend, with all groups except the 25 mg/kg group showing significantly higher values than the control ($P<0.05$), and the 200 mg/kg group achieving the highest index.

Note: In tables, values within the same column with different lowercase super-

scripts differ significantly (P<0.05), while those with same or no superscripts show no significant difference (P>0.05).

2.6 Effects of Arginine Injection on ALB, NO Content, and NOS Activity in Hepatopancreas and Serum

presents the effects of arginine injection on biochemical parameters. Serum ALB content increased initially and then decreased with arginine dose, reaching its maximum in the 100 mg/kg group, which was significantly higher than the 0, 25, and 50 mg/kg groups (P<0.05). Hepatopancreatic NO content was highest in the 50 mg/kg group, significantly exceeding all other groups (P<0.05), while the 100 and 200 mg/kg groups were not significantly different from each other (P>0.05) but were significantly higher than the 0 and 25 mg/kg groups (P<0.05). Serum NO content increased progressively with arginine dose, peaking in the 200 mg/kg group, which was significantly higher than the 0, 25, and 50 mg/kg groups (P<0.05) but not significantly different from the 100 mg/kg group (P>0.05). Both serum and hepatopancreatic NOS activities increased initially and then declined with arginine concentration. Hepatopancreatic NOS activity was highest in the 25 mg/kg group, significantly greater than the 0 and 200 mg/kg groups (P<0.05), while serum NOS activity peaked in the 50 mg/kg group, significantly exceeding all other groups (P<0.05).

Discussion

3.1 Effects of Arginine on Kidney Leukocyte Proliferation in Carp

Research indicates that arginine can synthesize polyamines, thereby promoting cell proliferation and serving as a crucial nutritional component for cellular immunity. Intracellular arginine concentration regulates polyamine synthesis. Both our in vitro and in vivo experiments demonstrated that arginine supplementation enhanced the proliferation index of carp kidney leukocytes, suggesting that these cells can utilize arginine as an important nutrient. Moreover, the proliferation index increased with arginine concentration, reaching maximal values at 1 mmol/L in culture medium for 12 hours or at 100 mg/kg via intraperitoneal injection. These findings align with studies in channel catfish and mice, which similarly reported that arginine promotes lymphocyte proliferation.

3.2 Effects of Arginine on Kidney Leukocyte Respiratory Burst Activity in Carp

Respiratory burst represents an oxygen-dependent killing mechanism in which activated neutrophils exhibit dramatically increased oxygen consumption and generate abundant oxygen radicals upon pathogen phagocytosis. When pathogens invade, macrophages produce NO driven by arginine, which stimulates superoxide anion production and triggers respiratory burst. Our in vivo study confirmed this mechanism, as serum and hepatopancreatic NO content increased with arginine dose, while respiratory burst activity rose

correspondingly with both culture time and arginine concentration. These results are consistent with findings in channel catfish and previous studies showing that dietary arginine enhances respiratory burst activity in red drum and hybrid striped bass.

3.3 Effects of Arginine on Kidney Leukocyte Phagocytic and Bactericidal Capacity in Carp

Phagocytic capacity depends on both cell number and activity. In our study, with cell density standardized at 5$×10^{7}$ cells/mL, phagocytic activity increased with arginine concentration. Studies in weaned piglets demonstrated that dietary arginine promotes neutrophil recruitment to inflammatory sites and enhances phagocytic activity. Research also indicates that dietary arginine supports immune organ development in carp, increases leukocyte phagocytic rates, and improves disease resistance, consistent with our findings. Similar results have been reported in channel catfish and Japanese flounder. Reactive oxygen species generated during respiratory burst can kill invading pathogens, with superoxide anion serving as a key reactive oxygen species whose production reflects bactericidal activity. Our experiments showed that arginine supplementation increased leukocyte bactericidal rates, indirectly reflecting elevated NO content and enhanced respiratory burst activity. The increased NO improved resistance against pathogenic bacteria, demonstrating that arginine promotes NO synthesis and enhances disease resistance. In vivo arginine injection also elevated serum and hepatopancreatic NOS activity, enabling phagocytes to produce NO via the NOS pathway, which in turn enhanced the bactericidal activity of reactive oxygen species.

3.4 Effects of Arginine Injection on ALB, NO Content, and NOS Activity in Serum and Hepatopancreas

Our study demonstrated that intraperitoneal injection of appropriate arginine concentrations effectively increased serum ALB content in carp. As arginine serves as a precursor for NO synthesis, increasing arginine doses elevated NO content in both serum and hepatopancreas, with serum NO peaking at 200 mg/kg arginine. Research in gibel carp showed that dietary arginine increased serum NO content in a dose-dependent manner. NOS is the key enzyme for NO synthesis, and its activity reflects tissue capacity for NO production. Studies have shown that dietary arginine enhances inducible NOS (iNOS) activity in Jian carp head kidney and serum NOS activity in cobia. Our in vivo experiments confirmed that arginine injection increased NOS activity in both serum and hepatopancreas, which correspondingly promoted NO production. The arginine-NO pathway is recognized as an effective mechanism for killing intracellular microorganisms and represents a primary mechanism of macrophage cytotoxicity. NO can kill parasites, bacteria, and viruses, inhibit cancer cell proliferation, and enhance immunity and disease resistance. Therefore, supplementation with appropriate arginine concentrations promotes NO production in hepatopancreatic

and serum cells, thereby improving carp immunity and disease resistance.

Conclusion

Arginine enhances kidney leukocyte immunity in carp, with an optimal concentration of 1.0 mmol/L in vitro. Under normal feeding conditions, the appropriate intraperitoneal injection dose for carp ranges from 50 to 100 mg/kg body weight.

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Note: Figure translations are in progress. See original paper for figures.