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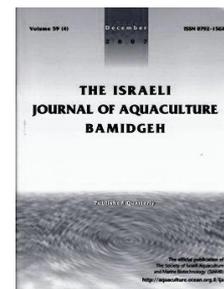
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Astragalus Polysaccharides Enhance Cellular Immune Response and Disease Resistance in Yellow Catfish

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Key words: yellow catfish (*Pelteobagrus fulvidraco*), astragalus polysaccharides, activation of immunological cells, respiratory burst activity, leukocyte proliferation

Abstract

We studied the effects of dietary astragalus polysaccharides on the cellular immune response of yellow catfish (*Pelteobagrus fulvidraco*). 540 Yellow catfish were fed different levels of astragalus polysaccharides (0, 300, 600, 900, 1200, or 1500 mg/kg diet) for eight weeks. Non-specific immune responses were measured at the end of the experimental feeding. We then challenged control and treated fish with an intraperitoneal injection of *Aeromonas hydrophila* and recorded mortality for seven days. Groups treated with 1200 mg/kg displayed a significant increase in oxygen respiratory burst (oxidative burst) activity of head kidney macrophages ($p < 0.05$), along with significant increases in nitrogen respiratory burst activity ($p < 0.01$) and leukocyte proliferation ($p < 0.05$). The 1500 mg/kg group also displayed significant increases in nitrogen respiratory burst activity ($p < 0.01$) and leukocyte proliferation ($p < 0.05$). The lowest mortality was obtained in the 1200 and 1500 mg/kg groups. These data indicate that dietary astragalus polysaccharides may effectively enhance cellular immune responses and disease resistance, and reduce mortality, in yellow catfish against *A. hydrophila* infection.

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Introduction

Yellow catfish (*Pelteobagrus fulvidraco*) is an important small freshwater aquaculture species in China. It is restricted to freshwater habitats, mostly in Asia. It is a favorite food fish because of its excellent meat quality and has a promising market in Japan, South Korea, and East and South Asia (Wang et al., 2006). Exports of yellow catfish to other countries have increased in recent years (Pan et al., 2008). However, expansion of aquaculture has led to increases in aquatic animal diseases that have hampered development and resulted in huge economic losses. Traditional therapeutic agents that are used to control fish diseases, such as antibiotics and chemotherapeutics, can lead to drug-resistant bacteria, environmental pollution, and unwanted residues (Reilly and Käferstein, 1997). Studies show that herbs and herbal extracts can effectively control and prevent fish or shrimp diseases because they are easily accessible and inexpensive, and act against a broad spectrum of pathogens (Edahiro et al., 1990; Bhuvanewari and Balasundaram, 2006; Bai et al., 2009; Guo et al., 2011). Most herbs and herbal extracts can be given orally, which is the most convenient method of supplementation.

Chinese herbs have been used as traditional medicine in China to enhance the human immune system for thousands of years. Polysaccharides are the major active component of Chinese herbs. Astragalus polysaccharide (APS) is one of the major active ingredients obtained from the roots of *Astragalus*. It harbors multiple therapeutic efficacies in humans and animals, such as gastrointestinal protection (Zhang et al., 2010a), and anti-oxidative (Xu, 2008), antiviral (Huang et al., 2008), and immunomodulative activity. Studies have shown an immunostimulant effect of APS (Tan and Vanitha, 2004; Yin et al., 2004; Cao et al., 2008), and its role in specific and non-specific immune responses is an area of further research (Shan et al., 2000). APS can modulate the immune cell function, including T, B, and NK cells and macrophages (Kong et al., 2003). In murine macrophage-like cells, it can enhance the expression of cytokine genes such as IL-1, IL-6, and TNF- α (Song et al., 2000), and the inducible nitric oxide synthase (iNOS) gene (Lee et al., 2005). APS can also significantly enhance the leukocyte phagocytic activity of Nile tilapia (Yin et al., 2006; Ardó et al., 2008) and carp survival after challenge with *Aeromonas hydrophila* (Yin et al., 2009).

In this study, we investigated the effects of different dietary levels of APS on the innate cellular immune response of yellow catfish. We measured the respiratory burst activity of head kidney macrophages, proliferation of peripheral blood leukocytes, and protection against *Aeromonas hydrophila*.

Materials and Methods

Herbal extracts and feed. Astragalus polysaccharides (APS) were extracted by water and alcohol-precipitation as described by Ni et al. (1998). The polysaccharide density was measured by the method of Dubois (1956) and purity was obtained at 90%. Six diets containing different levels of APS were prepared: 0 (control), 300 mg/kg diet, 600 mg/kg diet, 900 mg/kg diet, 1200 mg/kg diet, and 1500 mg/kg diet (Table 1). For all feeds, moisture, crude ash, crude protein, and crude lipid compositions were 12.36%, 12.37%, 38.14%, and 3.08%, respectively.

Fish and rearing conditions. Five hundred and forty healthy juvenile yellow catfish (61.6 ± 5.4 g, 19.5 ± 3.2 cm) were obtained from a pond at Tianjin Agricultural University. The juveniles were randomly distributed into 18 cages ($1.5 \times 1.5 \times 1$ m) with triplicates of each treatment. Water was maintained at $26 \pm 1^\circ\text{C}$ and pH at 5.5–6.0. Dissolved oxygen was monitored daily and maintained above 3.0 mg/l. Water was exchanged at a daily rate of 30%. Fish were fed twice daily (9:00, 17:00) for eight weeks at 2% of their body weight.

At the end of the feeding trial, 12 fish in each treatment (four from each of the triplicate cages) were randomly sampled to assay for respiratory burst of head kidney macrophages and peripheral blood leukocyte proliferation. An additional 36 fish in each treatment were challenged with *Aeromonas hydrophila* isolated from yellow catfish and obtained from the Fishery Institute of Tianjin, China.

Table 1. Composition of the basal diet (air dry basis, %).

| Ingredient | Diet (mg APS/kg feed) | | | | | |
|--|-----------------------|------|------|------|------|------|
| | Control | 300 | 600 | 900 | 1200 | 1500 |
| Fishmeal | 25 | 25 | 25 | 25 | 25 | 25 |
| Soybean meal | 40 | 40 | 40 | 40 | 40 | 40 |
| Shrimp meal | 5 | 5 | 5 | 5 | 5 | 5 |
| Yeast meal | 1 | 1 | 1 | 1 | 1 | 1 |
| Wheat flour | 23.5 | 23.5 | 23.5 | 23.5 | 23.5 | 23.5 |
| Fish oil | 1 | 1 | 1 | 1 | 1 | 1 |
| Soybean oil | 1 | 1 | 1 | 1 | 1 | 1 |
| APS | 0 | 0.03 | 0.06 | 0.09 | 0.12 | 0.15 |
| Cellulose | 0.5 | 0.47 | 0.44 | 0.41 | 0.38 | 0.35 |
| Ca(H ₂ PO ₄) ₂ | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| NaH ₂ PO ₄ | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Premix ¹ | 1 | 1 | 1 | 1 | 1 | 1 |

¹ per kg: Fe 150 mg, Zn 30 mg, Mn 13 mg, Cu 3 mg, Co 0.1 mg, I 0.6 mg, Se 0.15 mg, vitamin C 100 mg, vitamin B₁ 3 mg, vitamin B₂ 10 mg, vitamin B₆ 12 mg, calcium pantothenate 30 mg, nicotinic acid 30 mg, biotin 0.1 mg, folic acid 2 mg, vitamin B₁₂ 0.01 mg, inositol 400 mg, choline 1000 mg, vitamin A 2000 IU, vitamin D₃ 1000 IU, vitamin E 60 mg, vitamin K 6 mg. Ingredients were diluted with wheat flour.

Isolation of macrophages and leukocytes. Immune parameters were measured after eight weeks. Macrophages were isolated from head kidney samples (12 fish/treatment) based on the method of Bayne (1986). Head kidneys were excised using a mortar after adding Leibovitz's L-15 medium (Sigma, USA), and ground on ice. Cell suspensions were obtained by pressing samples through a 150-µm stainless steel mesh with a syringe rod, layered carefully onto a histopaque 1.077 g/cm³ (Sigma), and centrifuged at 400 g for 30 min at 4°C. Isolated macrophages were removed gently, then washed twice with Hanks solution, i.e., 1% penicillin and streptomycin, 0.2% heparin, and 10% fetal calf serum (FCS). Purified macrophages were counted using a hemocytometer and

cell viability was determined by the trypan blue exclusion method. The macrophages were resuspended in L-15 growth medium and adjusted to 1×10⁷ cells/ml for further analysis.

Blood samples (12 fish/treatment) were collected after eight weeks from the caudal vein. Heparin was used as an anticoagulant. Blood and L-15 medium were mixed at a ratio of 1:1. Peripheral blood leukocytes were separated by Percol (1.077 g/cm³/1.119 g/cm³) continuous density gradient centrifugation from the head kidney macrophages.

Respiratory burst activity. The oxygen respiratory burst activity of head kidney macrophages was evaluated using nitroblue tetrazolium (NBT, Sigma) reduction that measures intracellular oxidative free radicals using a method described by Secombes (1990) with slight modifications. One hundred µl macrophages (1×10⁷) were plated into the wells of U-bottom microtiter plates and incubated at 27°C for 3 h to allow adhesion of cells. After incubation, the 96-well plates were centrifuged three times at 400 g for 10 min. The supernatant was then removed and the wells were washed three times with Hanks' balanced salt solution (HBSS). After washing, 100 µl 0.1% NBT (Sigma, USA) and 1 µg/ml phorbol 12-myristate 13-acetate (PMA, Sigma, USA) were added for 1 h; the positive control group contained 100 µg/ml lipopolysaccharide (LPS). Absolute methanol was added to terminate the staining. Each tube was washed three times with 70% methanol and air-dried. One hundred twenty µl 2 M KOH and 140 µl dimethyl sulfoxide (DMSO, Sigma, USA) were then added and the color was measured at 620 nm. To detect cell activity during culture, 500 µg/ml LPS was added to the cell solution and used as a positive control. Optical density was recorded in a microplate reader (Therme) at 620 nm.

Nitrogen respiratory burst activity was evaluated using Griess reagent coloration (Green et al., 1982). Incubation and washing of macrophages was as described for oxygen respiratory burst, above. After washing, 100 µl L-15 medium (1% penicillin and streptomycin, 0.2% heparin, 10% FCS) was added and incubated for an additional 24 h at 27°C (positive control group contained 100 µg/ml LPS). Cells were washed twice with HBSS and 100 µl Griess (Beyotime Institute of Biotechnology) was added and incubated for 10 min. Optical density was recorded at 540 nm.

Proliferation of peripheral blood leukocytes. Proliferation of peripheral blood leukocytes was measured by methylthiazolyl-diphenyl-tetrazolium bromide (MTT; Sigma, USA; Mosmann, 1983). The leukocytes were incubated and washed as described for oxygen respiratory burst of head kidney macrophages, above. After washing, 100 µl L-15 media (1% penicillin and streptomycin, 0.2% heparin, and 10% FCS) was added and

incubated for an additional 24 h at 27°C (positive control group contained 100 µg/ml LPS). One hundred µl of L-15 media and 20 µl MTT were added to each well and incubated for another 4 h at 27°C. After incubation, plates were centrifuged to remove the L-15 medium, washed twice with HBSS, and 150 µl DMSO was added to each well. Optical density was measured at 570 nm.

Infection challenge. *Aeromonas hydrophila* were inoculated into 10 ml liquid tryptic soy broth (TSB, Sigma) and incubated overnight at 28°C. Cultures were centrifuged at 850 g for 15 min. The supernatant was removed and the pelleted bacteria were washed twice in sterile phosphate buffered saline (PBS) solution. The bacterial concentration was adjusted to 1×10^6 by optical density of suspension and 0.1 ml of the suspended bacteria was injected into the peritoneal cavity of 24 fish per group. Mortality was recorded for seven days following infection.

Statistical analysis. Experimental results are presented as average \pm standard error for 12 fish and were compared at each time point using one-way analysis of variance (ANOVA) in the SPSS (Statistical Products Service and Solutions) version 17.0 for Microsoft Windows. Significant differences between the control and experimental groups were expressed as $p < 0.05$ or $p < 0.01$.

Results

LPS in the positive control group significantly increased the oxygen respiratory burst activity of head kidney macrophages, indicating that isolation of macrophages from the head kidney was successful (Fig. 1). Nitrogen respiratory burst activity and proliferation of peripheral blood leukocytes increased with the level of APS ($p < 0.05$).

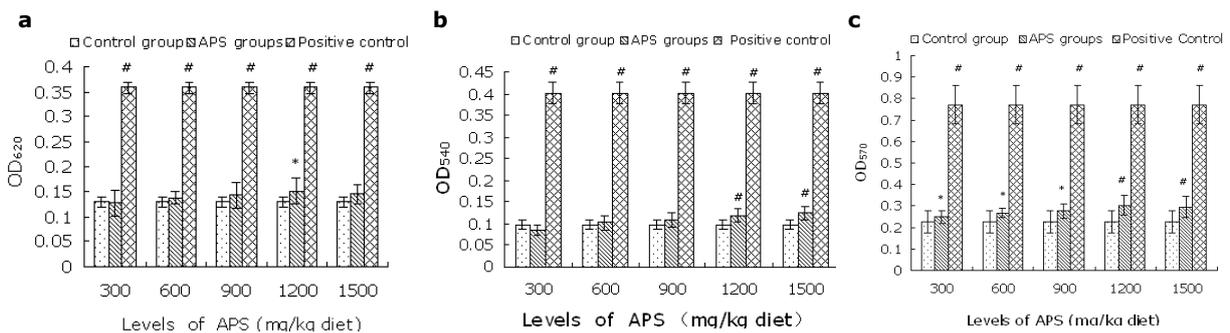


Fig. 1. Effects of *Astragalus* polysaccharides on (a) oxygen and (b) nitrogen respiratory burst activity of head kidney macrophages and (c) proliferation of peripheral blood leukocytes in yellow catfish; significant difference from the control: * $p < 0.05$, # $p < 0.01$

Fish challenged with *A. hydrophila* started to die 36 h post-infection (Fig. 2). Fifty percent of the control fish survived 48 h after infection and the cumulative mortality over the 6-day treatment period was 88%. In the treated groups, mortality occurred 48 h after infection and cumulative mortality was significantly lower than the control: 69%, 65%, and 63% in the 300, 600, and 900 mg/kg groups, respectively ($p < 0.05$), and 55% and 58% in the 1200 and 1500 mg/kg treatments ($p < 0.01$).

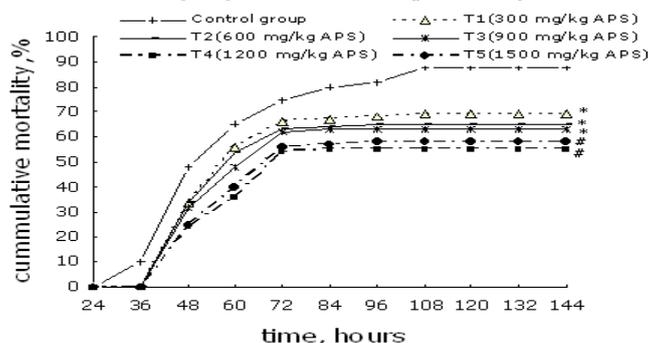


Fig. 2. Mortality in yellow catfish after challenge with *Aeromonas hydrophila*; significant difference from the control: * $p < 0.05$, # $p < 0.01$

Discussion

In the fish immune system, macrophages, lymphocytes, and granulocytes play an important role in pathogen resistance and killing. Respiratory burst, phagocytosis, and leukocyte proliferation are important indicators of immunostimulants. The results of our study indicate that a suitable level of APS can significantly enhance oxygen and nitrogen respiratory burst of head kidney macrophages in yellow catfish. Dietary APS can also significantly enhance disease resistance against *A. hydrophila* in yellow catfish, particularly at 1200 and 1500 mg/kg. Oxygen and nitrogen respiratory burst activity of head kidney macrophages was significantly enhanced in carp fed 0.5% APS and 0.5% lycium bararum polysaccharides for five weeks (Yin et al., 2004, 2009). Lycium bararum polysaccharides induced respiratory burst activity of head kidney macrophages in carp *in vitro* (Cao et al., 2008). Feeding 0.1% *Astragalus membranaceus* significantly enhanced phagocytic and respiratory burst activity of blood phagocytic cells in the fourth week of treatment in tilapia (Ardó, 2008).

In this study, feeding yellow catfish a suitable level of APS effectively enhanced proliferation of peripheral blood leukocytes. Similarly, APS significantly enhanced proliferation of peripheral blood leukocytes in carp (Cao et al., 2008). Five groups of tilapia were fed a basal diet supplemented with 0, 500, 1000, 1500, or 2000 mg/kg astragalus polysaccharide for 40 days and the length of the intestinal villus, the depth of the crypt, the thickness of the muscle, and the level of intestinal mucous cells and intraepithelial lymphocytes increased; the optimal dietary concentration of APS was recommended at 1000 and 1500 mg/kg (Huang et al., 2010). Suitable dosages of APS significantly stimulated proliferation in peripheral blood and splenic lymphocyte in broilers at a concentration of 100 µg/ml (Zhang et al., 2010b). Astragalan, ganoderan, and lichenan promoted IgG secretion and proliferation of spleen lymphocytes by LPS while lichenan and astragalan LPS significantly enhanced peritoneal macrophage and splenic NK activation (Wang et al., 2008). APS (300 µg/ml) significantly stimulated splenic lymphocyte proliferation of chicken *in vitro* (Zhang et al., 2009). Together, these studies indicate APS can serve as an immunopotentiator during humoral immunity.

In our study, mortality was reduced in all treated groups challenged with *A. hydrophila* compared to the control; the lowest mortality was in the 1200 mg/kg treatment. The amount of bacteria used to challenge the fish was high since mortality was 88% in the control fish. Intramuscular injection of *A. hydrophila* at a concentration of 5×10^6 cells/ml caused infection in tilapia, and mortality began within 12 h (Sarder et al., 2001). The first mortality in carp, injected intraperitoneally with an *A. hydrophila* suspension (5.3×10^6 cells/ml), was observed within 12 h, with 25% dying between 12 and 24 h post-infection (Sharifpour, 1997). Carp infected with *A. hydrophila* started to die after 36 h and cumulative mortalities in fish fed *Astragalus radix* and *Ganoderma lucidum* were significantly lower than in control groups.(Yin et al., 2009).

Overall, APS effectively enhanced activation of various immune cells suggesting that APS can be used as an immunostimulant to enhance the cellular immune response of cultured yellow catfish and enhance disease resistance. The optimal APS dietary concentration to add is 1200-1500 mg/kg.

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