World Journal of Fish and Marine Sciences 3 (6): 509-513, 2011
ISSN 2078-4589

Effect of Chitin Supplemented Diet on Innate Immune Response of Rainbow Trout

[1]Ghasem Vahedi and [2]Soroush Ghodratizadeh

[1]Faculty of Veterinary Medicine, Islamic Azad University, Tabriz Branch, Tabriz, Iran
[2]Faculty of Veterinary Medicine, Islamic Azad University, Urmia Branch, Urmia, Iran

Abstract: Chitin is a natural polymer found abundantly in the shells of crustaceans, insects and in fungi. Chitin and its deacetylated product chitosan are commercially manufactured from shells of shrimp and crab. Four experimental diets containing 0 (control), 10, 25 and 50 mg/kg chitin were prepared in the laboratory from a commercial pellet diet. A total of 50 rainbow trout were divided randomly into four groups, each receiving one of the above mentioned diets. Six fish from each group were randomly sampled. Blood samples were collected from the caudal vein on days 0, 7, 14, 21, 28, 35 for haemolytic complement activity and NBT assay. When fish were fed with chitin supplemented diets for 5 weeks, this activity increased to a statistically significant degree. Studies on neutrophil activity showed the enhancing effect of dietary supplements on neutrophils respiratory burst activity as evidenced from the increased NBT reduction. Based on the results of the present study, it can be concluded that the administration of a chitin diet (10, 25 or 50 mg/kg) enhances rainbow trout immune activity through the non-specific modulation of haemolytic complement activity and leukocyte respiratory burst activity.

Key words: Chitin % Complement Activity % Superoxide Anion % Rainbow Tarout

INTRODUCTION

The use of antimicrobials for disease control and growth promotion in animals increases the selective pressure exerted on the microbial world and encourages the natural emergence of bacterial resistance. Vaccination may be the most effective method of controlling fish diseases, even though disease caused by bacteria like A. hydrophila has not been controlled by vaccination due to their heterogeneity. However, when applied to hatchery conditions, some immunization techniques are not as effective as they should be. Immunostimulants may represent an alternative and a supplemental treatment to vaccination in the prevention of diseases in aquatic animals. Immunostimulants and immunomodulators comprise a group of biological and synthetic compounds that enhance the nonspecific cellular and humoral defence mechanisms in mammals. These substances, such as levamisole, $-glucan, peptidoglycan, chitin, chitosan yeast and vitamin combinations, as well as various products derived from plants and animals are effective in preventing diseases [1-6] Most of the research on immunostimulants has been focused on the treatment of human and animals [7,8]. The basis for this approach in therapy is the fact that natural or synthetic immunostimulants activate macrophages, neutrophils, natural killer cells and T cell mediated immunity. Immunostimulants also have the ability to increase resistance to viral, bacterial and fungal infections [9,10].

Chitin is a natural polymer found abundantly in the shells of crustaceans, insects and in fungi. Chitin and its deacetylated product chitosan are commercially manufactured from shells of shrimp and crab. Chitosan has many applications in medicine, agriculture and aquaculture. In aquaculture, it is used as an immunostimulant to protect salmonids against bacterial diseases [10,11], enhancing the respiratory burst and phagocytic activities in gilthead sea bream [1,12], immersion and dietary supplements [13]. The present study was undertaken to evaluate the efficacy of chitin, on enhancing nonspecific immunity of rainbow trout under field conditions.

Corresponding Author: Ghasem Vahedi, Faculty of veterinary medicine, Islamic Azad University, Tabriz branch, Tabriz, Iran. E-mail: g.vahedi@ymail.com.

MATERIALS AND METHODS

Fish: A total of 40 rainbow trout averaging 110 g was obtained from a commercial fish farm. Fish were maintained in outdoor tanks with running fresh water at 18°C for 2 weeks and fed commercial diets twice daily. The health status was examined throughout the acclimatization period. Water pH was measured by using electric digital pH meter and water temperature was recorded daily using a glass thermometer.

Feed: Four experimental diets containing 0 (control), 10, 25 and 50 mg/kg chitin (poly [1-4]-$-N-acetyl-D-glucosamine) powder purified from crab shells (Sigma) were prepared in the laboratory from a commercial pellet diet. The fish were divided randomly into four groups (10 fish per each), each receiving one of the above mentioned diets. Fish were fed at a rate of 10 g dry commercial diet/kg biomass (approximately 1%) each day.

Sample Collection: Six fish from each group were randomly sampled. Blood samples were collected from the caudal vein on days 0, 7, 14, 21, 28, 35.

Haemolytic Complement Activity: The activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [14]. Aliquots (500 µl) of test serum as complement source, serially diluted in Hank's buffer (HBSS), were added to 500 µl of SRBC (final concentrations 10-0.078%). After incubation for 1 h at 22°C, the samples were centrifuged (800 x g for 5 min at 4°C) to remove non-lysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 540 nm in a spectrophotometer. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 500 µl of distilled water or HBSS to 500 µl samples of SRBC, respectively. The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting Y/(1-Y) against the volume of serum added (ml) on a log-log scaled graph. The volume of serum producing 50% haemolysis (ACH50) was determined and the number of ACH50 units/ml was obtained for each experimental group.

NBT Assay: The superoxide anion from phagocytic cells was determined by the reduction of the redox dye nitroblue tetrazolium (NBT) as described by [15]. The kidney cells suspended in RPMI 1640 containing 10% Fetal Bovine Derum (Biowhittaker, USA) and 1% S/P were collected as described above. One hundred microliters of this suspension was added to each well of a 96 well microtiter plates. After 2 h at 20 °C of incubation the cells were washed by RPMI 1640 medium to remove non-adherent cells. The total adhered cell number per well was about 105 cells. One hundred microliters of NBT solution (1 mg/ml in RPMI 1640 medium) and phorbol 13-myristate 12-acetate (50 ng/ml) (PMA, Sigma) were added to each well and incubated for 60 min at 20 °C. The reduction was stopped by the addition of methanol, after removal of the medium from the cells. The formazan in each well was dissolved in 120 µl of 2 M KOH and 140 µl DMSO and the optical density was measured by a multiscan spectrophotometer (Pharmacia, Sweden) at 620 nm.

Statistical Analysis: Results are expressed as mean ± SEM Multiple comparisons were performed by ANOVA and followed by the Tukey honestly significant difference (HSD) test. In all analyses, the level of significance was set to ($P<0.05$ or 0.01).

RESULTS

Haemolytic Complement Activity: Serum complement activity, measured by the mean number of ACH50 units/ml serum, was increased by the chitin supplement (Table 1). When fish were fed with chitin supplemented diets for 5 weeks, this activity increased to a statistically significant

Table 1: Effect of chitin supplemented diet on haemolytic complement activity of rainbow trout. Data are expressed as mean ± SEM.

	Day					
Group	0	7	14	21	28	35
1	68 ± 6.4	71 ± 7.4	74 ± 6.8	71 ± 7.3	69 ± 8.5	74 ± 6.5
2	72 ±8.3	79 ± 8.5	165 ± 11.3*	187 ± 18.5*	203 ± 18.4**	205 ± 18.9**
3	71 ±7.6	82 ± 5.8	184 ± 12.7*	192 ± 19.4*	205 ± 24.3**	203 ± 26.3**
4	67 ± 5.5	85 ± 8.8	177 ± 14.3*	195 ± 22.2*	210 ± 21.1**	211 ± 27.6**

* mean statistically significant differences (ANOVA, P % 0.5) among groups according to the Tukey's comparison of means test.

Table 2: Effect of chitin supplemented diet on NBT assay of rainbow trout. Data are expressed as mean ± SEM.

	Day					
Group	0	7	14	21	28	35
1	0.4 ± 0.02	0.5 ± 0.05	0.4 ± 0.04	0.5 ± 0.06	0.5 ± 0.05	0.4 ± 0.08
2	0.5 ± 0.04	0.6 ± 0.06	0.8 ± 11.3*	1.1 ± 0.06*	1.4 ± 0.10*	1.5 ± 0.20*
3	0.4 ± 0.03	0.6 ± 0.05	0.9 ± 12.7*	1.00 ± 0.08*	1.5 ± 0.20*	1.5 ± 0.30*
4	0.4 ± 0.02	0.6 ± 0.03	1.0 ± 14.3*	1.3 ± 0.10*	1.7 ± 0.40**	1.7 ± 0.20**

* mean statistically significant differences (ANOVA, P % 0.5) among groups according to the Tukey's comparison of means test.

degree (P>0•05), although not in a dose-dependent way, for all the chitin concentrations assayed. However, no significant increase was found in this activity after 4 or 5 weeks of treatment.

NBT Assay: Studies on neutrophil activity showed the enhancing effect of dietary supplements on neutrophils respiratory burst activity as evidenced from the increased NBT reduction (Table 2). The neutrophil activity was enhanced in all the treatments. The highest significant NBT reduction was observed on the 28 and 35th days in 50 mg/kg chitin fed fish (P>0.01). In all the treatments, a gradual significant increase of NBT activity (P>0.01) was observed which reached a maximum on the 35th day.

DISCUSSION

Immunological approaches to prevent fish diseases have normally involved treatment with antibiotics, chemicals or vaccination against specific pathogens [16], while the use of immunostimulants is a relatively new and developing area [17,18]. It is known that many external (environmental) and internal factors may influence the effects of a particular immunostimulant on the fish immune system [19]. One such factor is the way in which the immunostimulant is administered. Different administration protocols (e.g. immersion, injection, oral) have produced different results, even when the same substance is being studied. However, perhaps the most appropriate method for aquaculture practice is oral administration, which is non-stressful and which permits a larger number of fish to be treated with the minimum cost and effort. With this in mind, the present work focuses on the administration of chitin to rainbow trout by incorporating it in the diet. Few papers have reported on its immunostimulant properties in fish. Also the effects of chitin on innate immune response of human are documented [20]. In rainbow trout, chitin stimulated macrophage activities [21] and in seabream it stimulated the main innate immune responses, including respiratory burst, phagocytic and also cytotoxic activities [1]. Furthermore, there is some evidence that chitin confers protection against infections [22, 23]. Rainbow trout and yellow tail injected with chitin showed increased resistance to V. anguillarum [21] and Pasteurella piscida [13], respectively. One of the advantages of using chitin, besides its low cost, is the fact that it is not a usual constituent of fish feed and that it is very stable. This makes it easier to work with a known chitin concentration, compared to using other soluble substances such as vitamins, which exist as micronutrients in feed and which are very sensitive to different factors. Haemolytic complement activity seems to vary widely in fish as a consequence of the administration of immunostimulants. Usually, this activity increases when immunostimulants are administered orally [24-27] and, in agreement with these results, the haemolytic complement activity of fish fed chitin-supplemented diets was the immune response to increase most in the present study. Some of the values found in fish fed diets containing 10, 25 or 50 g chitin/kg of diet were higher than those found in control fish after 2 weeks of treatment. The respiratory burst activity of leucocytes increased and peaked after 4 weeks post fed the chitin-supplemented diet, although the increases observed were not dose-dependent.

Based on the results of the present study, it can be concluded that the administration of a chitin diet (10, 20 or 50 g/kg) enhances rainbow trout immune activity through the non-specific modulation of haemolytic complement activity, leukocyte respiratory burst activity. The fact that chitin is not a normal constituent of fish feed and is very stable in its particular form makes it easier to work than other immunostimulants which are soluble and more sensitive to different physico-chemical agents.

REFERENCES

1. Esteban, M.A., V. Mulero. A. Cuesta. J. Ortuno and J. Meseguer, 2000. Effects of injecting chitin particles on the innate immune responses of gilthead seabream (*Sparus aurata* L.). Fish and Shellfish Immunol., 10: 543-554.

2. Ortuno, J., A. Cuesta. M.A. Esteban and J. Meseguer, 2001. Effect of oral administration of high vitamin C and E dosages on the gilthead seabream (*Sparus aurata* L.) innate immune system. Veterinary Immunology and Immunopathol., 79: 167-180.
3. Miles, D.J.C., J. Polchana, J.H. Lilly, S. Kanchanakhan, K.D. Thaompson and A. Adams, 2001. Immunostimulation of stiped snakehead *Channa striata* against epizootic atcerative syndrome. Aquaculture, 195: 1-15.
4. Esteban, M.A., A. Cuesta, J. Ortuno and J. Meseguer, 2001. Immunomodulatory effects of dietary intake of chitin in gilhead seabream (*Sparus aurata* L.) innate immune response. Fish and Shellfish Immunol., 11: 303-315.
5. Villamil, L., A. Figueras and B. Novoa, 2003. Immunomodulatory effects of nisin in turbot (*Scopthalmus maximus* L.). Fish and Shellfish Immunol., 14: 157-169.
6. Dautremepuits, C., S. Paris-Palacios. S. Betoulle and G. Vernet, 2004. Modulation in hepatic and head kidney parameters of carp (*Cyprinus carpio* L) induced by copper and chitosan. Comparative Biochemistry and Physiology. Part C, Pharmacology Toxicology and Endocrinol., 137: 325-333.
7. Nishimura, K., S. Nishimura, N. Nishi, I. Sakai, S. Tokura and I. Azuma, 1984. Immunological activity of chitin and its derivatives. Vaccine, 2: 93-98.
8. Azuma, I. and G. Jolles, 1987. Development of immunostimulants in Japan. In: Immunostimulants Now and Tomorrow. Jpn. Sci. Soc. Press. Tokoyo, Springer Verlag, Berlin, pp: 41-56.
9. Siwicki, A.K. and M. Dunier, 1994. In vitro restoration of antibodysecreting cells and lymphocyte proliferation activity by nitrogranulogen after *in vivo* immunosuppression due to lindane in rainbow trout *Oncorhynchus mykiss*. Ecotoxicology and Environmental Safety. 27: 316-323.
10. Siwicki, A.K., D.P. Anderson and G.L. Rumsey, 1994. Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. Veterinary Immunology and Immunopathol., 41: 125-139.
11. Anderson, D.P. and A.K. Siwicki, 1994. Duration of protection against *Aeromonas salmonicida* in brook trout immunostimulated with glucan or chitosan by injection or immersion. Progressive Fish- Culturist. 56: 258-261.
12. Cuesta, A.M.A. Esteban and J. Meseguer, 2003. *In vitro* effect of chitin particles on the innate cellular immune system of gilhead seabream (*Sparus aurata* L.). Fish and Shellfish Immunol., 15: 1-11.
13. Kawakami, H., N. Shinohara and M. Sakai, 1998. The non-specific immunostimulation and adjuvant effects of *Vibrio anguillarum* bacterin, M-glucan, chitin and Freund's complete adjuvant against Pasteurella piscicida infection in yellowtail. Fish Pathol., 33: 287-292.
14. Ortuno, J., M.A. Esteban. V. Mulero and J. Meseguer, 1998. Methods for studying the haemolytic chemoattractant and opsonic activities of seabream (*Sparus aurata* L.) serum. In Methodology in Fish Diseases Research (A. C. Barnes, G. A. Davidson, M.P. Hiney and D. McIntosh, eds) pp: 97-100. Aberdeen: Albion Press.
15. Chung, S. and C.J. Secombes, 1988. Analysis of events occurring within teleost macrophages during the respiratory burst. Comparative Biochemistry and Physiol., 89(b): 539-544.
16. Ellis, A.E., 1999. Fish immune system. In Encyclopedia of Immunology (P. J. Delves and I. M. Roitt, eds) pp: 920-926. London: Academic Press.
17. Robertsen, B., 1999. Modulation of the non-specific defence of fish by structurally conserved microbial polymers. Fish and Shellfish Immunol., 9: 269-290.
18. Sakai, M., 1999. Current research status of fish immunostimulants. Aquaculture, 172: 63-92.
19. Anderson, D.P., 1996. Environmental factors in fish health: Immunological aspects. In The fish immune system. Organism, pathogen and environment (G. Iwama and T. Nakanishi, eds) pp: 289-310. London: Academic Press.
20. Koller, B., A.S. Müller-Wiefel . R. Rupec. H.C. Korting and T. Ruzicka, 2011. Chitin modulates innate immune responses of keratinocytes. PLoS One. 6: e16594.
21. Sakai, M., H. Kamiya, S. Ishii, S. Atsuta and M. Kobayashi, 1992. The immunostimulating effects of chitin in rainbow trout, *Oncorhynchus mykiss*. Diseases in Asian Aquaculture, 1: 413-417.
22. Maqsood, S., P. Singh, M.H. Samoon and A.K. Balange, 2010. Effect of dietary chitosan on non-specific immune response andgrowth of *Cyprinus carpio* challenged with Aeromonas hydrophila. Int. Aquat. Res., 2: 77-85.

23. Powella, A. and A.F. Rowley, 2007. The effect of dietary chitin supplementation on the survival and immune reactivity of the shore crab, *Carcinus maenas*. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiol., 147: 122-128.
24. Verlhac, V., J. Gabaudan. A. Obach. W. Schüep and R. Hole, 1996. Influence of dietary glucan and vitamin C on non-specific and specific immune responses of rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 143: 123-133.
25. Mulero, V., M.A. Esteban. J. Munoz and J. Meseguer, 1998. Dietary intake of levamisole enhances the immune response and disease resistance of the marine teleost gilthead seabream (*Sparus aurata* L.). Fish and Shellfish Immunol., 8: 49-62.
26. Ortuno, J., M.A. Esteban and J. Meseguer, 1999. Effect of high dietary intake of vitamin C on non-specific immune response of gilthead seabream (*Sparus aurata* L.). Fish and Shellfish Immunol., 9: 429-443.
27. Ortuno, J., M.A. Esteban and J. Meseguer, 2000. High dietary intake of alphatocopherol acetate enhances the non-specific immune response of gilthead seabream (*Sparus aurata* L.). Fish and Shellfish Immunol., 10: 293-307.