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Enhancement of Chemiluminescent Response of Phagocytic Cells from Juvenile Rockfish, *Sebastes schlegeli*, by Oral Administration of Levamisole

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The chemiluminescent (CL) response of phagocytes from juvenile rockfish, *Sebastes schlegeli*, which were administered orally with levamisole was investigated. The fish intubated with doses of levamisole either at 0.5 mg kg^{-} or 1 mg kg^{-} body weight showed significant increase in CL responses at two weeks after the administration. The increased extent of CL in the fish exposed to 0.5 mg kg^{-} body weight was considerably lower than that in the fish exposed to 1 mg kg^{-}. The fish exposed to 5 mg kg^{-} body weight showed a steady and significant increase of CL response after the intubation. The fish intubated with 10 mg of levamisole kg^{-} body weight, however, showed no significant differences in CL response after the administration. In the experiment of feeding experimental diet, a lower dose of levamisole induced immunostimulation of phagocytes, but higher doses of levamisole induced immunosuppression of phagocytes. At one week after marking and blood sampling, plasma glucose level was significantly increased in the control group and the group intubated 0.5 mg levamisole/kg body weight. However, the fish in another groups, which were administered higher levels of levamisole, showed no significant difference in glucose level after marking and blood sampling. The result of the present study suggests that levamisole can be used as a potent immunostimulator in rockfish by oral administration, and the immunomodulating activity of levamisole depends on the dosage used.

Key words: Rockfish, levamisole, immunostimulant, oral administration, chemiluminescent response, plasma glucose

Introduction

Artificial, intensive fish farming system is a highly stressful environment for the fish, and immune-suppression by stress results in an increased susceptibility to diseases (Pickering, 1989; Fevolden et al., 1993). Antibiotics and other chemotherapeutics are widely used by fish farmers to control disease outbreaks. But those therapeutics can induce resistant microbial strains and environmental pollution. Vaccination is a good preventive measure for fish diseases, but vaccines are expensive for fish producers, and are not efficacious at present against many commercially important bacterial and viral diseases (Raa et al., 1992).

Immunostimulants may be effective in fish culture for activating nonspecific defense mechanisms and conferring protection against diseases. Several immunostimulants have been studied in various fish species, including glucans (Yano et al., 1989; Nikl et al., 1993; Duncan and Klesius, 1996; Ogier de Baulny et al., 1996; Santarem et al., 1997), levamisole (Siwicki, 1987; Siwicki et al., 1990; Kajita et al., 1990; Baba et al., 1993), chitin (Anderson and Siwicki, 1994), and saponin (Ninomiya et al., 1995). However, most of these studies were conducted by injection or immersion of the immunostimulants.

Fish, like other vertebrates, respond to infectious pathogens in specific and non-specific ways. However, the non-specific defences are the first a pathogen encounters, and it has been suggested that they are very important in the resistance of fish to infectious agents (Blazer, 1991). Granulocytes and macrophages possess a phagocytic activity which is the initial step in the immune response in fish, and is the major line of defence for all foreign material, including pathogenic agents (Olivier et al., 1986).

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During phagocytosis, fish macrophages increase their oxygen consumption as well as the production of reactive oxygen intermediates (ROIs) (Chung and Secombes, 1988) such as the superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-). These ROIs play an important role in the antimicrobial activity of phagocytic cells (Allen et al., 1972; Babior, 1984). Chemiluminescent response (CL) measures the respiratory burst activity of phagocytic cells in which oxygen is converted into reactive oxygen intermediates. The ROIs can activate probes such as luminol, triggering the emission of photons which can then be measured photometrically.

Levamisole is a drug registered by the U.S. Food and Drug Administration for treating helminth infections in ruminants, and can act alone to elevate the nonspecific defense mechanisms in fish, or can be used as an adjuvant with a vaccine (Anderson, 1992).

The aim of this study was to investigate the effect of levamisole administered orally on the chemiluminescent response of phagocytes in the juvenile rockfish.

Materials and Methods

Fish

Experiment 1

Net-pen reared juvenile rockfish, *Sebastes schlegeli*, were obtained from a local rockfish farm in Tongyoung, Korea. A total of 50 fish were stocked into a 200 ℓ aquarium and were acclimatized for one month prior to the experiment. The seawater had a salinity 33‰ and was maintained at 14 ± 2℃.

Experiment 2

Net-pen reared juvenile rockfish were obtained from a local rockfish farm in Hadong, Korea. A total of 240 fish were group-housed in 12 experimental 50 ℓ aquaria (20 fish/aquarium). Fish were acclimatized for 2 weeks prior to initiating experiment, and the seawater had a salinity 33‰ and was maintained at 20 ± 1℃.

Administration of levamisole(Sigma Chemical Co., U.S.A.)

Experiment 1

Fish in an aquarium were divided into 5 groups by marking on the dorsal fin with 5 kinds of transparent plastic. Each fish in a group could be distinguished by writing Arabian numbers on the plastic. Among fish in five separated groups, four groups were administered 0.5, 1, 5 and 10 mg levamisole/Kg of body weight via oral route with intubation tube. Fish in control group were administered only saline orogastrically.

Experiment 2

The experimental diet was prepared by mixing together basic feed (Table 1) and levamisole. The amount of levamisole added to the feed was 10 g kg^-. Pellets approximately 2 mm in diameter were formed by passing the moistened mixture through a meat grinder. Diets were stored at −20℃ until needed, and prior to use as feed, small quantities were stored at 4℃.

Six different groups with 2 replicates were established corresponding to 6 different treatments. The experimental feed was administered only at the first day of experiment in group 1, at an interval of one week in group 2, for two consecutive days weekly in group 3, at an interval of every other day in group 4, and at every day in group 5. The control group received basal diet. Fish were fed once daily to satiation for 3 weeks.

Blood

In experiment 1, blood samples were taken from all fish in each group for chemiluminescence assay on days 0 (just before intubation of levamisole), 7, and 14 after administration of levamisole. In experiment 2, blood used in the chemiluminescence assay was obtained from 10 fish of each group after 3 weeks of experimental feeding. Fish were anaesthetized with benzocaine and 0.2 mℓ of blood was taken from the caudal vessel using heparinised syringes.

Glucose

To analyse the effect of marking and weekly blood sampling on the stress response of fish in experiment 1, plasma glucose levels were determined using a glucose oxidase/peroxidase enzymatic assay

Table 1. Composition of the basal diet

Ingredients	Percent dry weight
FM white	57.00%
Corn gluten meal	10.00%
Soy bean meal	4.00%
Wheat	19.00%
Vitamin mixture	1.00%
Mineral mixture	1.00%
Vitamin C	0.05%
Squid liver oil	6.75%
CMC	1.00%
Cellulose	0.20%

based upon the method of Werner et al. (1970).

Chemiluminescence (CL) assay

The ROIs produced by stimulated phagocytes was quantified using an automatic photoluminometer (Bio-Orbit 1251, Sweden).

Each test cuvette (4 mℓ) contained 0.4 mℓ luminol (Sigma) made according to the method of Scott and Klesius (1981), 0.5 mℓ HBSS, 0.1 mℓ whole blood, and 0.2 mℓ phorbol-myristate-acetate (PMA, Sigma) as the stimulus. The stock solution of PMA was made by diluting 2 mg PMA in 1 mℓ dimethyl sulphoxide, and before use, diluted further by adding 50 μℓ stock solution to 10 mℓ of PBS. Blank cuvette contained luminol, HBSS and blood, but PMA was replaced with physiological saline. The contents of each cuvette were gently mixed, and cuvettes were immediately placed in the measuring chamber of luminometer. The measurements were made for 100 minutes and the light emission was recorded as mV.

Statistics

Statistical significance was assayed by paired Student's *t*-test in experiment 1, and by unpaired Student's *t*-test in experiment 2. Results of CL responses from the 2 replicates of each group in experiment 2 were analysed for heterogeneity and then grouped together when no statistical difference existed.

Results

Experiment 1

The fish intubated with doses of levamisole at either 0.5 mg kg^{-} or 1 mg kg^{-} body weight showed significant increase in CL responses at two weeks after the administration, when compared with the CL values before the administration of levamisole (Fig. 1). The increased extent of CL in the fish administered 0.5 mg levamisole kg^{-} body weight was considerably lower than that in the fish intubated 1 mg of levamisole kg^{-}. The fish administered 5 mg of levamisole kg^{-} body weight showed a steady and significant increase of CL response after the intubation. The fish intubated with 10 mg of levamisole kg^{-} body weight, however, showed no significant differences in CL values after the administration.

Glucose levels were not significantly different among all experimental groups before administration of levamisole (Fig. 2). At one week after marking and blood sampling, plasma glucose levels

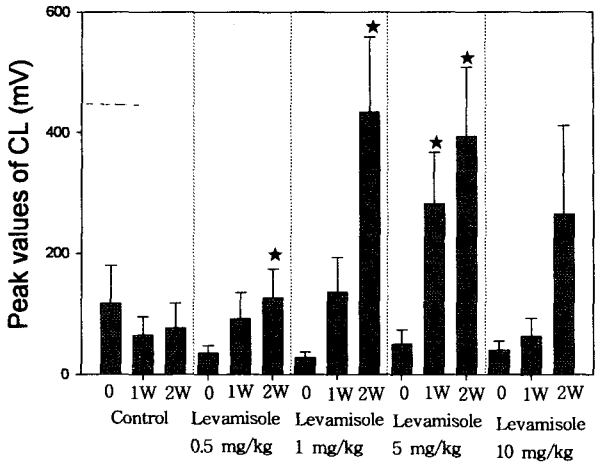

Fig. 1. Effect of the different doses of levamisole on the chemiluminescent response of juvenile rockfish (*Sebastes schlegeli*) phagocytes on days 0, 7 and 14. Results are means+SE. ★ Significant difference [$p<0.05$ using a paired Student's *t*-test to compare between days 0 and days 7 (1W) or days 14 (2W].

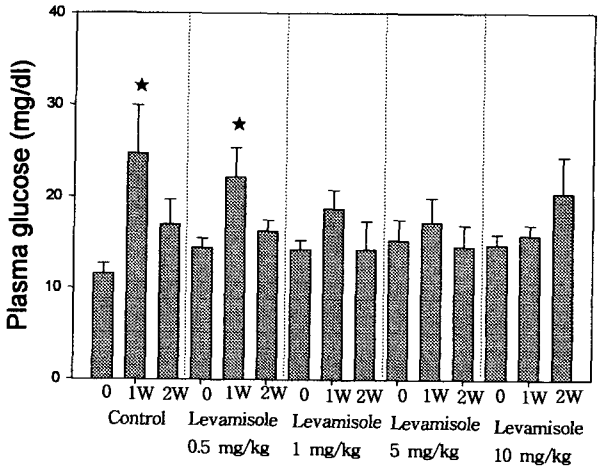

Fig. 2. Effect of the different doses of levamisole on the plasma glucose level of juvenile rockfish (*Sebastes schlegeli*) on days 0, 7 and 14. Results are means+SE. ★ Significant difference [$p<0.05$ using a paired Student's *t*-test to compare between days 0 and days 7 (1W) or days 14 (2W)].

were significantly increased in the control group and the group intubated 0.5 mg levamisole/kg body weight. However, the fish in another groups, which were administered higher levels of levamisole, showed no significant difference in glucose level after marking and blood sampling. The highest level of glucose was found in the control group.

Experiment 2

The mean amount of levamisole taken for 3

weeks in each group was 2.50 mg kg^{-} body weight in group 1, 8.75 mg kg^{-} in group 2, 20.31 mg kg^{-} in group 3, 36.16 mg kg^{-} in group 4, and 37.70 mg kg^{-} in group 5.

The fish of group 1 showed a considerable increase in CL response when compared with the control group ($p<0.1$). In contrast, the CL responses were significantly decreased in the fish of another groups when compared with the control group (Fig. 3).

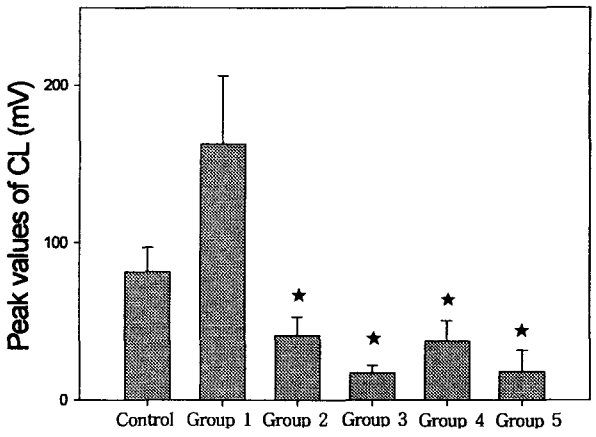

Fig. 3. Chemiluminescent response of juvenile rockfish (*Sebastes schlegeli*) phagocytes at 21 days after feeding diet supplemented with levamisole (Group 1: levamisole 2.5 mg/Kg, Group 2: levamisole 8.75 mg/Kg, Group 3: levamisole 20.31 mg/Kg, Group 4: levamisole 36.16 mg/Kg, Group 5: levamisole 37.7 mg/Kg). Results are means+SE. ★ Significant difference ($p<0.05$ using a Student's *t*-test to compare control group and treated group).

Discussion

In mammals, levamisole is capable of restoring impaired immune responses preferentially of the cell mediated type in compromised hosts, and therapeutic doses of levamisole restore to normal the functions of phagocytes and T lymphocytes (Biniaminov and Ramot, 1975; Symoens and Rosenthal, 1977). According to the study of Kajita et al. (1990), rainbow trout injected with a dose of levamisole at 0.5 mg kg^{-} showed increased phagocytic activity, chemiluminescence response and natural killer cell activity. In addition, fish given levamisole showed an increased protection against an experimental injection challenge with *Vibrio anguillarum*. Carp fed levamisole at 5 mg kg^{-} body weight every 3 days during a 15 day period showed significantly enhanced phagocytosis and nitrobule tetrazolium reduction by blood neutrophils for up to 12 weeks post-treatment (Siwicki, 1989).

The present work revealed that levamisole intubated orally enhanced CL response of juvenile rockfish. Especially, the CL responses of fish intubated with either 1 mg or 5 mg kg^{-} of levamisole were considerably stronger than that of fish intubated with 0.5 mg kg^{-} body weight. On the other hand, fish intubated with 10 mg kg^{-} dose of levamisole did not show significant increase in CL response. This dose dependent effect of levamisole was demonstrated evidently by experiment 2. In fish fed higher doses of levamisole, the CL responses were significantly decreased when compared with the control fish. Therefore, oral administration of levamisole with moderate doses induces immunostimulation of phagocytes, while with high doses induces immunosuppression of phagocytes in juvenile rockfish. Similarly, Anderson et al. (1989) reported that rainbow trout injected with 5 μg kg^{-} of levamisole showed maximal stimulation of nonspecific immune responses, but higher doses of levamisole caused immunosuppression, and lower doses resulted in irregular responses.

Hyperglycemia in response to physical disturbances has been reported in many investigations (Pickering et al., 1982; Vijayan and Moon, 1992; Waring et al., 1992; Biron and Benfey, 1994). Jeney et al. (1997) reported that stress induced by 2 h transportation of rainbow trout caused hyperglycaemia in all experimental groups, but the lowest level of glucose was measured in the group fed the low (0.1%) dose of glucan. They suggested that feeding of glucan in low doses several weeks before transportation can help to prevent negative effects of stress. In the present study, significant increase of plasma glucose was observed in fish administered either only saline or 0.5 mg kg^{-} of levamisole after 1 week of marking and blood sampling, but the fish intubated with higher doses of levamisole showed no significant increase in plasma glucose level after marking and blood sampling. Therefore, it seems that oral administration of levamisole in moderate doses, one or several weeks before handling of rockfish, can reduce harmful effects of stress through immunomodulation.

In conclusion, the result of the present study suggests that levamisole can be used as a potent immunostimulator and an anti-stress agent in juvenile rockfish by oral administration, and the

immunomodulating activity of levamisole depends on the dosage used. Further experiments are required to examine whether fish immunostimulated by oral administration of levamisole can resist against harmful pathogens.

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