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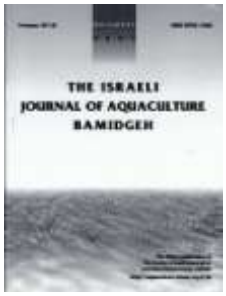

Effects of Methisoprinol on Innate Immunity Parameters in Intensively Reared European Eel (*Anguilla anguilla*)

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Abstract

Stress and chemotherapeutics can negatively affect the immune system of fish in intensive culture. Methods of prevention include immunonutrition. The present study examined the influence of dietary supplementation with the synthetic compound methisoprinol on selected nonspecific immune parameters in intensively cultured juvenile European eel (*Anguilla anguilla*). The fish were fed a commercial pellet containing 0 (control) or 200 mg methisoprinol/kg feed. After four weeks, the following immunological parameters were measured: respiratory burst activity and potential killing activity of the phagocytes, lymphocyte proliferation after stimulation by concanavaline A or lipopolisaccharide, and serum lysozyme activity, ceruloplasmin activity, total protein, and total immunoglobulin. Except for serum ceruloplasmin activity and total protein, all immune parameters were significantly higher ($p<0.05$) in the methisoprinol-treated group than in the control, strongly suggesting that four weeks of feeding methisoprinol to juvenile eels might improve innate immunity.

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Introduction

The European eel (*Anguilla anguilla*) is a catadromous fish with a complicated life cycle. The species migrates over 5000 km to spawning grounds in the Sargasso Sea (Tesch, 1982; van den Thillart and Dufour, 2009). Eel farming is based on wild catches of glass eels that are used for ongrowing. Although EU Council Regulation No. 1100/2007 established a plan for the protection and sustainable use of European eel stock, global production of this species is still large. According to FAO Fishery Statistics, global aquaculture production of *A. anguilla* was nearly 7 tons in 2010. Successful commercial production of eel species has been facilitated by the intensification of larvae and fingerling rearing techniques. However, intensive production systems can expose the animals to stressors, negatively affecting the fish immune system and increasing the susceptibility of fish to infectious disease. Additionally, global transport of aquaculture stocks can induce the spread of pathogens. Infectious diseases occur in eels at every life stage, therefore it is very important to develop effective methods of disease prevention.

Similar to other fish species, the innate immune system in eels comprises a large number of physical, cellular, and humoral factors that act as the first line of defense against invading organisms such as viruses, bacteria, and parasites (Nielsen and Esteve-Gassent, 2006; Uribe et al., 2011). The innate system of fish probably also plays an important role in activating acquired immune mechanisms (Magnadóttir, 2006).

Although much is known about the interactions between nutrition, defense mechanisms, and disease protection, these relationships are far more complex than originally thought. Certain nutrients can be supplemented in the diet and act as immunomodulators by providing building blocks for nonspecific defense mechanisms and enhance specific immune response. Innate immunity is an essential defense system against pathogenic microorganisms in fish and fish depend more strongly on nonspecific defense mechanisms than mammals (Anderson, 1992; Uribe et al., 2011). Thus, immunostimulants might play a significant role in the protection of fish against infectious disease.

Methisoprinol (isoprinosine, inosine pranobex) is a synthetic drug combined from inosine and p-acetamidobenzoate salt of N-N dimethylamino-2-propanol in a 1:3 molar ratio (Campoli-Richards et al., 1986). Studies on antiviral and immunopotentiating properties of methisoprinol began in the 1970s. Currently, methisoprinol is widely used as an immunostimulating drug in human medicine. The present study examined how dietary supplementation of methisoprinol can influence nonspecific immune parameters in intensively reared juvenile European eels.

Materials and Methods

Fish and rearing conditions. Juvenile eels were obtained from a commercial fish farm in Suwałki, Poland. The fish were acclimated in plastic tanks at the Inland Fisheries Institute in Olsztyn, then 400 healthy European eels (mean 25 g) were stocked at 50 kg/m^3 in two tanks. Throughout the experiment, temperature was 27±0.5°C, oxygen content 7.0±1.0 mg/l, and pH 7.0±0.5.

Feed and feeding. The control group of eels was fed commercial pellets (Europa Eel, Skretting, Netherlands) without methisoprinol. The experimental group was fed commercial pellets containing 200 mg methisoprinol/kg feed. The methisoprinol dose was chosen after preliminary studies. Methisoprinol (Polfa Grodzisk, Poland) in a known concentration was dissolved in distilled water and sprayed on the commercial feed. The feed was mixed, allowed to dry for 24 h, then coated with vegetable oil (1% of the feed weight). The fish were fed three times a day (07:00, 15:00, 23:00) and observed daily for unusual behavior, morphological changes, and mortality. The food coefficient was 1.3.

Sample collection. After four weeks of feeding, 20 eels (~40 g) from each group were anesthetized in Propiscin (Kazuń and Siwicki, 2001) and blood samples were taken with heparinized syringes. The spleen and pronephros were aseptically removed. After clotting overnight at 4°C, the blood was centrifuged at 5000 x *g* for 10 min to obtain serum. To obtain individual cells, single cell suspensions were fractionated by Gradisol L (Aqua-Med Poland) or Percoll (Sigma-Aldrich) density centrifugation, according to the manufacturer's

protocol.

Respiratory burst activity. The respiratory burst activity of the pronephros phagocytes, stimulated by phorbol myristate acetate (PMA; Sigma-Aldrich), was measured using the modified Secombes method described in Siwicki et al. (2000). Briefly, aliquots of 100 µl containing 1 x 10^4 cells/ml in RPMI-1640 medium (Sigma-Aldrich) were added to 96-well micro-titer plates (Sarstedt) and incubated for 2 h at room temperature. After incubation, the non-adherent cells were removed by washing with fresh RPMI-1640 medium. The medium was then substituted by 100 µl RPMI and 100 µl nitroblue tetrazolium (NBT) solution (Sigma-Aldrich), both with and without PMA, to final concentrations of 2 mg/ml NBT and 10 µg/ml PMA. The plates were incubated for 30 min at room temperature. The medium with NBT was removed and the wells were washed twice with 70% ethanol. The blue formazan produced in the cells was solubilized in 120 µl 2M KOH and 140 µl dimethyl sulfoxide (DMSO; Sigma-Aldrich). The optical density (OD) of the solution was measured at 620 nm using a microplate reader. Data are expressed as mean values of triplicate determinations.

Potential killing activity. The potential killing activity of the pronephrotic phagocytes was measured by the Rook technique (Rook et al., 1985) with modifications described by Siwicki and Anderson (1993). Briefly, aliquots of 100 µl containing 1 x 10^4 cells/ml in RPMI-1640 medium were added to 96-well micro-titer plates (Sarstedt) and incubated for 2 h at room temperature. After incubation, the non-adherent cells were removed by washing with fresh RPMI-1640 medium. The cells were activated using 100 µl 0.2% NBT solution in phosphate buffered saline containing live *Aeromonas salmonicida* (1 x 10^8/ml) and incubated for 30 min at 22°C. After incubation, the supernatant was removed and the wells were rinsed thrice with 70% ethanol. The microtiter plate was dried by placing it in a warm incubator for 30 min, and then 120 µl 2 M KOH and 140 µl DMSO were added to dissolve the formazan. The OD of the solution was measured at 620 nm using a microplate reader. All samples were performed in duplicate. Data are expressed as mean values of triplicate determinations.

Lymphocyte proliferation. Lymphocyte proliferation was determined by the MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay according to Mosmann (1983) with modifications described by Siwicki et al. (2004). Mitogens concanavaline A (ConA; Sigma) or lipopolisaccharide (LPS; Sigma) were used to stimulate lymphocytes. Isolated lymphocytes were suspended at 5 × 10^6 cells/ml in RPMI-1640 medium containing 2 mM L-glutamine, 0.02 mM 2-mercaptoethanol, 1% Hepes buffer, penicillin/streptomycin (100 U/100 µg/ml), and 10% fetal calf serum. Aliquots of 100 µl cell suspension were distributed in 96-well micro-titer plates (Sarstedt) and 100 µl ConA (64 µg/ml) or LPS (160 µg/ml) was added to each well. The plates were incubated for 72 h at room temperature. After incubation, 50 µl MTT solution was added to each well and the plates were incubated for 4 h at 22°C. After centrifugation of the microplates, the media were removed and 100 µl isopropanol (Sigma-Aldrich) were added to the wells and mixed. Optical density was read on a microreader at 620 nm. To obtain mean values, quadruplicate wells were averaged. Net optical densities were obtained by subtracting the mean OD of negative control cells (without mitogens) from the OD of stimulated cells.

Serum lysozyme activity. Lysozyme activity in the serum was measured using turbidimetric assay as described in Siwicki and Anderson (1993) with further modifications. Briefly, 50 µl serum samples were put into test tubes and diluted with 950 µl 66 mM phosphate buffer pH 6.25. Next, 2 ml solution of *Micrococcus lysodeikticus* (Sigma-Aldrich) in phosphate buffer (66 mM, pH 6.25) was added and mixed. The final concentration of *M. lysodeikticus* was determined by spectrophotometry and made up to 40% T at 450 nm, after 1 h incubation. Absorbance was read at 450 nm immediately after mixing samples with bacteria and after 1 h of incubation. To calculate mean OD values, duplicate determinations were averaged.

Serum ceruloplasmin activity. Ceruloplasmin activity in the serum was determined according to Siwicki and Anderson (1993) with further modifications. Aliquots of 100 µl 0.2% p-phenylenediamine (PPD) solution in acetate buffer (pH 5.2) and 10 µl serum

samples were added to 96-well micro-titer plates (Sarstedt) and mixed. The PPD solution in acetate buffer was freshly prepared and brought to 37°C before mixing with the serum. The plates were incubated for 15 min at 37°C. After incubation, 140 µl 0.02% sodium azide solution was added to each well. Optical density was read immediately at 540 nm. To calculate mean OD values, triplicate determinations were averaged.

Serum total protein. Total protein in serum was measured by standard biuret reaction, using total protein reagent (Sigma-Aldrich) according to the manufacturer's protocol. Absorbance was read by spectrophotometer at 540 nm. To calculate mean OD values, duplicate determinations were averaged.

Serum total immunoglobulin. Total immunoglobulin (Ig) in the serum was measured by the spectrophotometric method (Siwicki and Anderson, 1993) with further modifications. Briefly, 0.1 ml serum samples were put into tubes and 0.1 ml 12% polyethylene glycol 10,000 kDa (Sigma-Aldrich) in distilled water was added. The samples were well mixed and incubated 2 h at room temperature. After incubation, the samples were centrifuged for 10 min at 10,000 RPM. The supernatant was removed and the OD was determined at 540 nm. To calculate mean OD values, duplicate determinations were averaged. To calculate total serum Ig levels, supernatant OD values were subtracted from total protein OD values.

Statistical analysis. Mean values and standard deviations from pooled experiments were used to compare groups. Statistical analysis was performed using Statgraphics 2.1 for Windows and Statistica 5.77 software (analysis of variance, comparison of regression lines, Wilcoxon's twin pair analysis). For all calculations $p<0.05$ was assumed significant.

Results

Most cellular and humoral immune parameters were significantly higher in eels fed the diet supplemented with 200 mg methisoprinol/kg feed than in eels fed the unsupplemented control (Table 1).

Table 1. Nonspecific cellular and humoral immune parameters in European eels (*Anguilla anguilla*) fed a diet containing 200 mg methisoprinol/kg feed for four weeks compared to eels fed an unsupplemented control (means±SD; n = 20).

Cellular immune parameter (OD 620 nm)	*Control*	*Methisoprinol*
Respiratory burst activity of phagocytes	0.40±0.05	0.51±0.03*
Potential killing activity of phagocytes	0.35±0.05	0.42±0.03*
Lymphocyte proliferation, ConA-stimulated	0.39±0.03	0.45±0.04*
Lymphocyte proliferation, LPS-stimulated	0.31±0.03	0.40±0.03*
Humoral immune parameter		
Lysozyme activity in serum (mg/l)	10.5±1.2	15.2±1.0*
Ceruloplasmin activity in serum (IU)	60.3±3.5	63.5±2.8
Total protein in serum (g/l)	58.5±5.0	62.8±4.5
Total immunoglobulin in serum (g/l)	10.5±1.2	17.5±1.5*

* statistically significant difference ($p<0.05$)

Discussion

Macrophages and neutrophils, which are fish phagocytic cells, are essential factors in limiting the growth of fish pathogens. Stimulated phagocytes induce the production of cytokines, which are important signal molecules (Neumann et al., 2001; Magnadóttir, 2010). Respiratory burst activity and potential killing activity of the pronephros phagocytes were significantly higher in eels fed the diet with methisoprinol than in those fed the control. A similar pattern was observed in proliferative response of spleen lymphocytes stimulated by lipopolysaccharide, a B-cell mitogen, and concanavalin A, a T-cell mitogen (Clem et al., 1984; Fujiwara et al., 2001).

T and B lymphocytes are important cells in nonspecific and specific immune responses. Both are responsible for specific pathogen recognition and participate in the regulation of early response mechanisms by producing cytokines and chemokines. After

proliferation, B cells differentiate into memory cells and cells that secrete specific antibodies. Fish B cells might also have *in vitro* and *in vivo* phagocytic activity (Li et al., 2006). The stimulation of lymphocytes by mitogens in the present *in vitro* method may mimic the series of events that occur *in vivo* following stimulation by specific antigens.

In addition to damaging gram-positive bacteria cell walls, fish lysozyme has antibacterial activity against gram-negative bacteria in the absence of a complement as it activates phagocytosis (Saurabh and Sahoo, 2008). Lysozyme in eel is an important part of the innate immune system and exhibits the highest activity during the early stages of eel development (Nielsen and Esteve-Gassent, 2006). Lysozyme activity increases after supplementation with a wide range of immunostimulants in various fish species (Saurabh and Sahoo, 2008). The present study confirms previous experiments and indicates that also methisoprinol increases the level of lysozyme activity in fish.

Total Ig was significantly higher in eels fed the methisoprinol diet, suggesting that methisoprinol activates the production of immunoglobulins. Immunoglobulins are an important part of the acquired immune response, but natural antibodies can also be significant innate system factors.

In the current study, the eels exhibited satisfactory feed intake, which was seemingly uninfluenced by the presence of the immunostimulant. Some innate immunity parameters are lower in intensively cultured eels than in wild eels (Siwicki and Robak, 2011). The present research suggests that methisoprinol can be used as an immunostimulant to enhance cellular and humoral innate immune responses in eels although further studies are needed to determine effective dosing and mode of administration for particular culture conditions. Experiments are also needed to assess potential side affects that could occur during and after immunostimulation.

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