In vitro effect of Levamisole on Rainbow Trout (Oncorhynchus mykiss) peripheral blood mononuclear cells

Efecto in vitro del levamisol sobre las células mononucleares de la sangre periférica de trucha arcoíris (Oncorhynchus mykiss)

LEONARDO A. GÓMEZ¹, RAUL A. CORTES² & CARLOS T. SMITH¹,*

¹Department of Microbiology, Faculty of Biological Sciences, University of Concepción, Concepción, Chile. P. O. Box 160-C, Concepción, Chile.
²Department of Fish Physiology and Biotechnology, Instituto de Acuicultura de Torre de la Sal (IATS), Consejo Superior de Investigaciones Científicas (CSIC), Castellon, Spain.
*csmith@udec.cl, Telephone: 56 (41) 220 4287, Fax: 56 (41) 224 5975

ABSTRACT

The in vitro effect of levamisole (1, 10 and 100 μg/ml) on cells of the innate immune response of rainbow trout (Oncorhynchus mykiss) was studied, particularly the activity of natural cytotoxic cells and macrophages present in peripheral blood. Levamisole effect on natural cytotoxic cells was evaluated by their capacity to lyse HL-60 tumor cells while activity of macrophages was evaluated measuring production of reactive oxygen species and nitric oxide. Results indicate that natural cytotoxic cells activity was significantly increased by 1 μg/ml levamisole and that higher doses had no effect. On the other hand, reactive oxygen species and nitric oxide production by macrophages was not increased by any of the levamisole doses used and that the higher dose (100 μg/ml) induced a significant decrease of nitric oxide levels, which might be deleterious for the defensive response of these fish.

KEYWORDS: Innate immunity; reactive oxygen species; ROS; nitric oxide.

INTRODUCTION

Immunity against “foreignness” appeared early in evolution. Innate immunity, characterized as non-specific, is the first defense strategy and the most widely distributed and present in plants, invertebrates and vertebrates (Heine 2008). With the appearance of the first vertebrates, particularly gnathostomes, a new defensive strategy, the acquired or adaptive immune response (specific and with memory), acting jointly and coordinately with the innate response, appeared (Flajnik & Du Pasquier 2004; Cooper & Alder 2006). Thus, the adaptive immune response is present in most fish and all the rest of vertebrates and, therefore, fish are a key group for comparative studies to unravel the evolution of adaptive immunity (Plouffe et al. 2005).

Fish immunity is dependent on innate and adaptive (or acquired) responses (Secombes 1996). Innate immunity, being the first defensive line against microorganisms, is fundamental to avoid infectious diseases (Plouffe et al. 2005). Among the cellular components of innate immunity we can include natural cytotoxic cells (NCCs), functionally
The purpose of this study was to evaluate the activity of non-specific cytotoxic cells (NCCs) and peripheral blood macrophages in rainbow trout. Apparently healthy 10 rainbow trouts (Oncorhynchus mykiss Walbaum 1792) of both sexes per group, ranging from 180 to 210 g, were kept at the “Salmones Pangue” pisciculture (Bio Bio Region, Chile) during the summer, under normal conditions of culture. Blood samples were obtained by caudal vein puncture using heparinized syringes after animals were anesthetized with 15 ml of 20% benzocaine (Veterquímica, Santiago, Chile) in 10 l of water. Blood was transported, at 4°C, to the Laboratory of Immunology, Department of Microbiology, Faculty of Biological Sciences, University of Concepción (Concepción, Chile) arriving in not more than 1 h after been drawn.

**MATERIALS AND METHODS**

**FISH AND BLOOD SAMPLES**

Apparantly healthy 10 rainbow trouts (Oncorhynchus mykiss Walbaum 1792) of both sexes per group, ranging from 180 to 210 g, were kept at the “Salmones Pangue” pisciculture (Bio Bio Region, Chile) during the summer, under normal conditions of culture. Blood samples were obtained by caudal vein puncture using heparinized syringes after animals were anesthetized with 15 ml of 20% benzocaine (Veterquímica, Santiago, Chile) in 10 l of water. Blood was transported, at 4°C, to the Laboratory of Immunology, Department of Microbiology, Faculty of Biological Sciences, University of Concepción (Concepción, Chile) arriving in not more than 1 h after been drawn.
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stimulation with 1, 10 or 100 μg/ml levamisole and controls were measured as described by Anderson & Siwicki (1993) with modifications. This method is based on the reduction of nitroblue tetrazoliun (NBT) by the superoxide anions to the colored compound formazan, which can be spectrophotometrically measured. Briefly, 100 μl of the experimental and control cultures containing 1 x 10^6 PBMCs/ml were incubated, in Eppendorf tubes, with 100 μl 0.1% NBT (Sigma Chemical Co., St. Louis., MO., USA) for 60 min. Later on, 1 ml N,N-dimethylformamide (Merck, Darmstadt, Germany) was added and the Eppendorf tubes centrifuged for 10 min at 100 x g. Absorbance of the supernatant was measured at 620 nm. Assays were done in triplicate.

Nitric oxide (NO) production after 24 h of culture in the presence of 1, 10 or 100 μg/ml levamisole and controls was determined using a procedure modified from Walsh et al. (2006), based on detection of nitrite (NO_2^-) derived from the nitric oxide (NO) by means of the Griess reagent (sulphanilamide, naphthylethylenediamine and phosphoric acid) (Sigma-Aldrich Co., St. Louis., MO., USA). Briefly, from the cell cultures (1 x 10^6 PBMC/ml) treated with the different concentrations of levamisole, 200 μl were obtained and centrifuged for 10 min at 100 x g and 100 μl of supernatant was collected. The supernatant was incubated for 10 min with 100 μl de 1% sulphanilamide in 5% phosphoric acid, and 100 μl of 0.1% N-naphthylethylenediamine were then added and incubated for 30 additional min. Nitrite was assessed spectrophotometrically at 540 nm in the supernatant. Assays were done in triplicate.

STATISTICAL ANALYSIS
The effect of the different doses of levamisole on the natural cytotoxic activity and production of ROS and NO was determined using a one-way ANOVA and Tukey’s test. Significant differences were considered those with a value of p < 0.05.

RESULTS
EFFECT OF LEVAMISOLE ON NCCS ACTIVITY
Natural cytotoxic activity by NCCs was determined by lysis of HL-60 tumor cells. Results indicate that a dose of 1 μg/ml significantly increases cytotoxic activity (p= 0.039), but higher doses, 10 or 100 μg/ml, show results not significantly different from controls (Fig. 1).

EFFECT OF LEVAMISOLE ON MACROPHAGES ROS AND NO ACTIVITY
Activity of macrophages was assessed by the production of ROS and NO after 24 h of incubation with levamisole. In this work performed in vitro, ROS production was not affected by any of the levamisole doses used (Fig. 2). On the other hand, in vitro levamisole effect on NO production shows that 1 or 10 μg/ml had no effect on NO production but 100 μg/ml induced a significant reduction in the production of this molecule (p=0.035) (Fig. 3).

DISCUSSION
Levamisole is a synthetic compound with immunostimulatory properties (Sakai 1999). It has been reported that in salmonids, such as Salmo salar and Oncorhynchus mykiss, in vivo, it increases immunity (Kajita et al. 1990; Findlay & Munday 2000; Ispir & Yonar 2007). Nevertheless, some reports indicate that, in vitro, high doses of levamisole induce immunosuppression, especially on the antibody forming cells (AFCs), macrophages and ROS production.

![Figure 1](image1.png)

**Figure 1.** Effect of different doses of levamisole (24 h treatment) on the natural cytotoxic activity by trout peripheral blood mononuclear cells. Bars indicate mean ± standard deviation. * Statistically significant difference (p<0.05).

**Figura 1.** Efecto de diferentes dosis de levamisol (24 h de tratamiento) sobre la actividad citotóxica natural de las células mononucleares de sangre periférica de trucha. Las barras indican el promedio ± desviación estándar. * Diferencias estadísticamente significativas (p<0.05).
FIGURE 2. Effect of the different doses of levamisole (24 h treatment) on the production of reactive oxygen species (ROS) by trout peripheral blood mononuclear cells. Bars indicate mean ± standard deviation.

FIGURA 2. Efecto de diferentes dosis de levamisol (24 h de tratamiento) sobre la producción de especies reactivas del oxígeno (ROS) por las células mononucleares de sangre periférica de trucha. Las barras indican el promedio ± desviación estándar.

FIGURE 3. Effect of the different doses of levamisole (24 h treatment) on the production of nitric oxide (NO) measured as Nitrite (NO$_2^-$) by trout peripheral blood mononuclear cells. Bars indicate mean ± standard deviation. * Statistically significant difference ($p<0.05$).

FIGURA 3. Efecto de diferentes dosis de levamisol (24 h de tratamiento) sobre la producción de óxido nítrico (NO) medido como Nitrito (NO$_2^-$) por las células mononucleares de sangre periférica de trucha. Las barras indican el promedio ± desviación estándar. * Diferencias estadísticamente significativas ($p<0.05$).

Activity of NCCs was evaluated by their cytotoxic activity against HL-60 tumor cells while activity of macrophages was evaluated by the production of ROS and NO. NCCs and macrophages were obtained by isolation of PBMCs. PBMCs include lymphocytes, NCCs and macrophages (Yang et al. 1994). Nevertheless, given the conditions of the assays performed, only NCCs, and not other cellular populations, could exert cytotoxic activity and only macrophages present in the culture were able to produce ROS and NO for defensive purposes (Secombes 1996; Lundén et al. 2002).

The effect of levamisole on NCCs has been reported in various fish species (Findlay & Munday 2000; Cuesta et al. 2002; Choi 2004), including rainbow trout (Kajita et al. 1990; Ispir & Yonar 2007). Our results show a significant increase of trout NCCs activity stimulated in vitro for 24 h with 1 μg/ml levamisole (Fig. 1). These results are in agreement with those reported by Cuesta et al. (2002) for the gilthead seabream (Sparus aurata) and by Choi (2004) for the Japanese flounder (Paralichthys olivaceus) showing that in vitro levamisole doses ranging from 0.001 to 0.1 μg/ml (doses under the lowest used in this work) also significantly increased the activity of NCCs. Hence, the positive effect of low doses of levamisole on NCCs activity is confirmed in different species of teleosts. Furthermore, we can report that high doses of levamisole (up to 100 μg/ml), which might be deleterious for other defensive functions, such as...
the antibody forming cells (AFCs), macrophages and ROS production (Siwicki et al. 1990; Mulero et al. 1998; Li et al. 2004), do not inhibit natural cytotoxic activity (Figure 1). In brief, considering the role played by NCCs in the defense of an organism, we can conclude that low doses of levamisole are favorable in the immune response against tumor cells and intracellular pathogens and that high doses would not negatively affect NCCs.

Besides evaluating the effect of levamisole on trout NCCs, we also studied its effect on macrophages, particularly on ROS and NO production by these cells. When evaluating the in vitro levamisole effect on ROS production by macrophages, contrary to what happened with NCCs activity (Figure 1), there was no ROS increase with any of the three doses used (Figure 2). Our results are similar to those reported by other authors, such as Mulero et al. (1998), reporting that in vitro doses below 1 μg/ml do not increase ROS production in the gilthead seabream (S. aurata). But, they showed a significant decrease in ROS production after 48 h of stimulation with 1 μg/ml levamisole, a time span not evaluated in this work. Also, Li et al. (2004) reported an in vitro decrease in ROS production in the hybrid striped bass (Morone chrysops and Morone saxatilis) after treatment with 1000 μg/ml levamisole for 24 h, a tenfold increase if compared with the highest dose studied in this work. These results suggest that in vitro treatment with levamisole does not produce the same increase in ROS production by macrophages observed in vivo (Kumari & Sahoo 2006; Ispir & Yonar 2007).

In vertebrates in general, activated T lymphocytes produce a series of cytokines able to regulate the activity of NCCs and macrophages (Bird et al. 2006). Thus, we cannot discard that levamisole in vivo activates T lymphocytes which, in turn, exert their regulatory effect on NCCs and macrophages. In vitro work with PBMCs might not provide the same cellular interactions present in vivo, thus explaining the different results obtained when working in vivo or in vitro, that is to say the presence or absence, respectively, of ROS increase after levamisole stimulation.

We have been unable to find other reports evaluating nitric oxide (NO) production by teleost macrophages after levamisole stimulation. Our results indicate that levamisole doses of 1 and 10 μg/ml do not affect nitric oxide production, while 100 μg/ml significantly reduced production of this compound (Fig. 3). This suppression might be related to the already described negative effect of high in vitro levamisole doses on some parameters of the immune response (Siwicki et al. 1990; Mulero et al. 1998; Li et al. 2004).

Since in vivo low doses of levamisole exert a positive effect absent in in vitro experiments, we can support the idea that macrophages may not be the direct target for levamisole effect, but other immunocompetent cells might respond in vivo to levamisole and secrete cytokines which, in turn, will signal macrophages. Some reports suggest that T lymphocytes might be targets for levamisole action (Tizard 2009). For this reason, it becomes necessary to determine if trout T lymphocytes in fact regulate the activity of NCCs and macrophages. If so, studies involving levamisole might help to comprehend the regulatory role of T lymphocytes on the activity of NCCs and macrophages, the interaction of immunocompetent cells in teleost fish and, therefore, to search ways to better stimulate the immune response of these animals against infectious diseases.

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