Immunomodulating Effect of Methisoprinol on the Pronephros Macrophage and Lymphocyte Activity after Suppression Induced by Infectious Haematopoietic Necrosis Virus (IHNV) in Rainbow Trout *(Oncorhynchus mykiss)*

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Abstract

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The purpose of this *in vitro* study was to determine the influence of methisoprinol on the activity of pronephros macrophages and lymphocytes after suppression induced by infectious haematopoietic necrosis virus (IHNV). For this study IHNV-free rainbow trout were used. Pronephros from 20 fish were removed and single leukocyte suspensions were separated. The IHNV significantly ($P < 0.05$) decreased the respiratory burst activity and potential killing activity of pronephros macrophages and proliferative response of lymphocytes stimulated by mitogens ConA and LPS. The results of our *in vitro* study showed that methisoprinol at a concentration of 50 mg/ml modulated (restored) the metabolic and potential killing activity of macrophages and proliferative response of lymphocytes suppressed by IHNV.

In vitro study, phagocyte activity, lymphocyte proliferation

Rhabdoviruses constitute one of the largest groups of viruses isolated from fish, and are mostly associated with epizootics and heavy losses in intensive fish farming. Infectious haematopoietic necrosis (IHN) is the most important viral disease and produces high losses in rainbow trout and other salmon species farmed in North America and in several European countries. Infectious haematopoietic necrosis virus (IHNV) is a member of *Rhabdoviridae* and the type species of the genus Novirhabdovirus. Like other mononegavirales, it has a single molecule of linear, negative-sense ssRNA genome (Kurath et al. 1997). The multiplication of virus takes place in endothelial cells of blood capillaries leading to haemorrhages in haematopoietic tissues and nephron cells (Amend and Smith 1974). The transmission of IHNV takes place horizontally, vertically, and by biological vectors such as fish parasites. Acutely infected fish release the virus by external mucus, faeces and urine. Carriers shed the agent via sexual products (Kim et al. 1999).

The protection against viral diseases by specific vaccines against IHN is being developed for the last few years (Corbeil et al. 2000; Lorenzen et al. 2002; Prost 2003) and some successful vaccines have been developed (Purcell et al. 2006; Miller et al. 2007). Rodriguez Saint-Jean and Perez-Prieto (2007) examined the ability of several fish viruses to induce protection against homologous or heterologous viruses in single or double infections, and assessed whether such protection is correlated with innate immunity or expression of the *Mx* gene. The results of this study indicate that activation of the immune response could explain the interference and loss of IHNV in the IPNV-IHNV co-infections. In fact, the DNA vaccines against IHN virus only have a scientific aspect and the application of these vaccines in rainbow trout culture is

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limited. However, for an ideal preventive approach, specific drugs should be developed to inhibit selectively virus replication or to stimulate the antiviral protection. The use of natural and synthetic immunomodulators in fish offers a wide range of attractive methods for inducing and building up protection against viral diseases and is a promising new development in aquaculture (Anderson 1992; Siwicki et al. 1998). Several promising drugs and biological response modifiers such as carbohydrates and other synthetics have been tested on fish immunocompetence cells *in vitro*. A dose-dependent immunomodulatory effect of levamisole, nitrogranulogen, lysozyme dimer and HMB were observed on macrophage activity, proliferative response of lymphocytes and antibody secreting cells in rainbow trout and other fish species (Siwicki et al. 2003; Terech-Majewska et al. 2004; Siwicki et al. 2006).

Methisoprinol is a synthetic compound formed from the p-acetamidobenzoate salt of N-N-dimethylamino-2-propanol and inosine in a 3 : 1 molar ratio. Methisoprinol presents low toxicity and has been shown to act *in vitro* by inhibiting the replication of salmonid rhabdoviruses (Siwicki et al. 2002). It exerts antiviral and antitumour activities *in vitro* and *in vivo*, which are secondary to the immunomodulating influence on non-specific cellular and humoral defence mechanisms and protection against viral diseases (Delogu et al. 1982; Fudenberg and Whitten 1984; Siwicki et al. 2003).

The aim of the present study was to determine the *in vitro* influence of methisoprinol on the pronephros macrophage and lymphocyte activity after suppression induced by infectious haematopoietic necrosis virus (IHNV) in rainbow trout (*Oncorhynchus mykiss*).

Materials and Methods

In this *in vitro* study 20 healthy (IHNV-free) rainbow trout (*Oncorhynchus mykiss*) with a mean weight of 50 g were used. The fish were held in a 500 l tank in 14 ºC spring water and fed twice daily with commercial pellets. The pronephros were removed from fish aseptically and single leukocyte suspensions were separated on Histopaque-1077 (Sigma, USA) gradient or Gradisol (Polfa, Poland), according to the method presented by Siwicki et al. (1996). Viable cells from the pronephros were counted with 0.1% of trypan blue staining after washing three times in RPMI 1640 medium, and 90–94% of cell vitality were ascertained. The Laboratoire Departemental d'Analyses France (LDA 39) IHNV isolate was used in this *in vitro* study and quantified by plaque assay using epithelioma papulosum cyprini (EPC) cells incubated at 14 °C for 72 h.

Methisoprinol (Polfa Grodzisk, Poland) was used at a concentration of 50 mg/ml of RMPI-1640 medium (Sigma).

The spectrophotometric assay presented by Siwicki et al. (1996) was used to study the respiratory burst activity of pronephros macrophages stimulated by phorbol myristate acetate (PMA, Sigma). One hundred ml of cell suspension (1×10^6 in RPMI-1640) were added to 96-well culture plates (Nunclon, Denmark) and incubated for 2 h at 22 ºC with 50 ml of methisoprinol or 50 ml of 1×10^7 plaque-forming units/ml RPMI-1640 of IHN virus or with 50 ml of IHN virus + 50 ml of methisoprinol. The control group comprised cells only stimulated by PMA.

The potential killing activity of pronephros macrophages was determined with a microcolorimetric method presented by Anderson and Siwicki (1994). The 0.2% nitroblue tetrazolium (NBT, Sigma, USA) in PBS solution containing live 1×10^7 *Aeromonas salmonicida* cells was used for stimulation of pronephros macrophages. The cells (100 ml) were added to 96-well culture plates (Nunclon, Denmark) and incubated for 30 min at 22 ºC with 50 ml of methisoprinol or 1×10^7 plaque-forming units/ml RPMI-1640 of IHN virus or with 50 ml of IHN virus + 50 ml of methisoprinol. The control group comprised cells only stimulated by 0.2% NBT solution containing live bacteria *A. salmonicida*.

The proliferative response of pronephros lymphocytes was determined by the MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay described by Mosmann (1983), as modified for use with fish lymphocytes by Siwicki et al. (1996). Briefly, lymphocytes were isolated from pronephros and distributed (100 ml) into 96-well culture plates (Nunclon, Denmark) at 5×10^6 cell/ml of RPMI-1640. The mitogen concanavalin A (ConA, Sigma) at a concentration of 64 mg/ml or lipopolysaccharide (LPS, Sigma) at a concentration of 160 mg/ml was added (20 ml) into each well. The cells were incubated for 72 h with 50 ml of methisoprinol or 50 ml of IHN virus (1×10^7 pfu/ml) or with 50 ml of IHN virus + 50 ml of methisoprinol. The control group comprised cells only stimulated by mitogens: Con A or LPS.

The results were verified statistically by one-way ANOVA analysis of variance (GraphPad Prism software

package), and the significance of differences between the groups verified with Bonferroni test. Differences between the means were considered significant if $P < 0.05$.

Results and Discussion

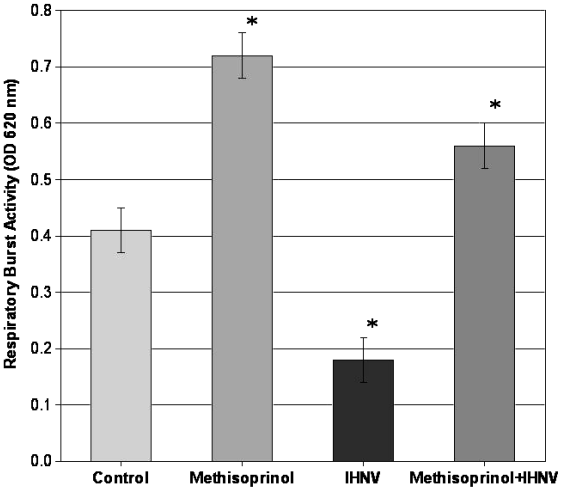

Fig. 1. *In vitro* immunomodulatory effect of methisoprinol on the pronephros macrophage respiratory burst activity (RBA) after suppression induced by IHN virus (IHNV) (n = 20, mean ± SD, * significant differences $P < 0.05$)

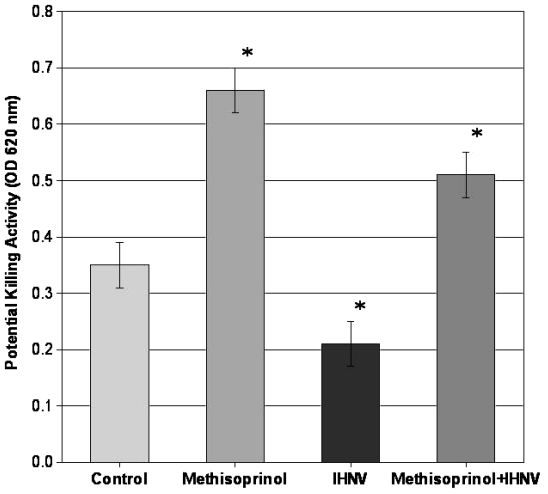

Fig. 2. *In vitro* immunomodulatory effect of methisoprinol on the pronephros macrophage potential killing activity (PKA) after suppression induced by IHN virus (IHNV) (n = 20, mean ± SD, * significant differences $P < 0.05$)

The current study used an *in vitro* model to examine the influence of IHNV and methisoprinol on the pronephros macrophage and lymphocyte activity stimulated by mitogens. The aim of the first part of our study was to determine the *in vitro* effects of IHNV on the activity of pronephros macrophages and lymphocytes. As shown in Fig. 1, the IHNV significantly decreased the metabolic activity of pronephros macrophages and potential killing activity of pronephros macrophages (Fig. 2). Similar pattern was observed with pronephric lymphocytes. The IHNV significantly decreased the proliferative response of lymphocytes stimulated by mitogens ConA and LPS (Fig. 3).

The mechanisms involved in the establishment of rhabdovirus infections in fish are not well understood. Very few data have been obtained on the influence of IHNV on the cell-mediated immunity in fish (Amend and Smith 1974; La Patra et al. 1993). Chilmonczyk and Winton (1994) suggested that leukocytes may serve as target cells for the initial phase of IHNV infection. In our study we clearly demonstrated a suppressive effect of IHNV on the pronephros macrophage activity. This may have been due to an impairment of the phagocyte function. The disease may affect the phagocyte function, and particularly the respiratory burst activity and potential killing activity. The data obtained for IHNV corroborated those previously obtained with viral haemorrhagic septicaemia virus (VHSV) and confirmed that leukocytes represent one of the cell types involved in both rhabdovirus infections. It has been previously shown that rhabdoviruses replicate within the macrophages (Estepa et al. 1992).

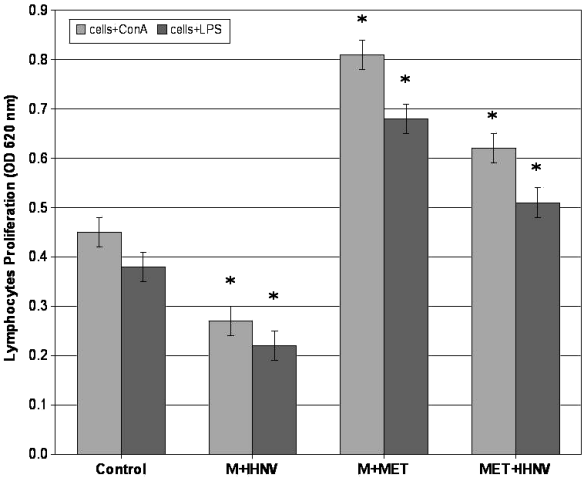

Fig. 3. *In vitro* immunomodulatory influence of methisoprinol (MET) on the proliferative response of pronephros lymphocytes stimulated by mitogens (M): ConA or LPS, after suppression induced by IHN virus (IHNV) (n = 20, mean ± SD, * significant differences $P < 0.05$)

In the second part of our study, we analysed the *in vitro* effects of methisoprinol on the metabolic activity of pronephros macrophages and proliferative response of lymphocytes stimulated by mitogens after suppression induced by IHNV. The influence of methisoprinol on the respiratory burst activity of pronephros macrophages after suppression induced by IHNV are presented in Fig. 1, and on the potential killing activity of pronephros macrophages after suppression induced by IHNV are presented in Fig. 2. Similar pattern was observed with lymphocyte activity. The effect of methisoprinol on the proliferative response of pronephros lymphocytes stimulated by ConA and LPS after suppression induced by IHNV are presented in Fig. 3. In this *in vitro* study we observed that methisoprinol modulated (restored) the macrophage and lymphocyte activity strongly suppressed by IHNV. The *in vitro* infection of cells by IHNV and incubation with methisoprinol significantly increased the metabolic activity and potential killing activity of pronephros macrophages, compared to the control cells and cells only infected by IHNV. Similar results were observed with proliferative response of lymphocytes infected by IHNV and incubated with methisoprinol (Fig. 3).

In this study we clearly demonstrated that methisoprinol stimulated the respiratory burst activity and potential killing activity of pronephros macrophages and proliferative response of lymphocytes stimulated by mitogens ConA and LPS. Also an immunomodulating influence of methisoprinol on the pronephros macrophages and lymphocytes after suppression induced by IHNV was observed. Similar results were observed with *in vitro* proliferative response of lymphocytes and macrophage activity infected by VHSV in rainbow trout and incubated with methisoprinol (Siwicki et al. 2003). The results of our *in vitro* study suggest that methisoprinol restores the function of phagocytes and lymphocytes suppressed by IHNV. These results and observations are important, especially in view of the possibility that this product could by used to eliminate the depressive influence of viral infection on the cell-mediated immunity responses. A future study will include determination of optimal doses and protocol of application of methisoprinol to maximise the *in vivo* immunomodulatory effects and protection against IHNV in rainbow trout.

Imunomodulační účinky methisoprinolu na aktivitu makrofágů a lymfocytů pronefros po supresi způsobené virem infekční nekrózy hematopoetické tkáně (IHNV) u pstruha duhového (*Oncorhynchus mykiss)*

Cílem této *in vitro* studie bylo určit vliv methisoprinolu na aktivitu makrofágů a lymfocytů pronefros po supresi způsobené virem infekční nekrózy hematopoetické tkáně

(IHNV) s využitím pstruha duhového prostého IHNV (IHNV-free). Tkáň pronefros byla odebrána z 20 ryb pro získání suspenzí jednotlivých leukocytárních linií. Virus infekční nekrózy krvetvorné tkáně významně ($P < 0.05$) snižuje aktivitu oxidačního vzplanutí a zneškodnění cizorodého agens makrofágů pocházejících z pronefros a proliferativní odpověď lymfocytů po stimulaci mitogeny ConA a LPS. Výsledky naší *in vitro* studie ukázaly, že methisoprinol v koncentraci 50 µg/ml reguluje (obnovuje) metabolickou a potenciální obrannou aktivitu makrofágů pro zneškodnění cizorodé agens a proliferativní odpověď lymfocytů suprimovaných virem infekční nekrózy hematopoetické tkáně u pstruha duhového.

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