

Impact of iridovirus and methisoprinol on immunocompetent cells isolated from African catfish, *Clarias gariepinus* (Burchell), under *in vitro* conditions

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Abstract. Experiments to assess the effectiveness of the immunostimulator methisoprinol (Polfa Grodzisk Pharmaceuticals, Poland) focused on its impact on the innate immune response. The impact of different doses of methisoprinol on organ leukocytes isolated from the kidneys and spleens of African catfish that were subjected to or not subjected to the suppressive impact of iridovirus. The results indicate that the addition of methisoprinol causes increased respiratory burst activity (RBA), potential killing activity (PKA), and proliferative activity of lymphocytes T and B in response to mitogens. Methisoprinol stimulates the mechanisms of cellular immunity that are linked to the activation of T lymphocytes, which can impact the antiviral activity of this preparation after its application *in vivo*.

Keywords: methisoprinol, immunostimulation, African catfish

Introduction

Many immunostimulatory preparations of natural origin are used in medicine; these can be isolated from fungi, bacteria, and crustaceans and include polysaccharides, lipopolysaccharides (LPS), peptides,

lysozyme dimer (Anderson et al. 1995, Morand et al. 1999), and synthetic substances including levamisole, isoprinosine, FK-565, etc. (Kowalski 1989, Jeney and Anderson 1993, Siwicki et al. 2002, 2003).

Studies to date on the effectiveness of using immunostimulators in fish have focused on attempts to increase the innate immune response to bacterial disease (Kitao and Yoshida 1986, Siwicki 1989, Yano et al. 1989, Kajita et al. 1990, Robertsen et al. 1990). They are also used as adjuvants aimed at increasing antigen activity in vaccines (Kitao et al. 1987, Chen and Ainsworth 1992, Jeney and Anderson 1993, Rørstad et al. 1993).

In recent years, increased fish susceptibility to viral diseases has been noted (Żelazny 2010). Currently, there is a lack of effective anti-viral medication that could be used in fisheries. This means that it is crucial to find effective methods for preventing viral infections. Reports in the literature indicate that isoprinosine, a substance that inhibits viral infection, could be applied in aquaculture (Siwicki et al. 2002, 2003).

There has been a significant increase in African catfish, *Clarias gariepinus* (Burchell), production in Poland in recent years (Adamek 2001). Fish farmers are expressing growing interest in this species because of its characteristics and usefulness. The build of the African catfish gill is such that this fish can breathe atmospheric air, inhabit waters with low

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oxygen contents, and tolerate high concentrations of ammonia and organic matter. It can be reared at high densities, which translates into high production results. Market demand for this species is increasing as more and more consumers develop an appreciation for the taste of this fish and its dietary advantages of low fat and high protein contents (Adamek 2001).

Cellular defense mechanisms play key roles in fighting viral infections (Siwicki et al. 1994), and organ leukocyte activity is a good indicator of the condition of the immune system. This is why the current study of the effectiveness of the synthetic immunostimulator methisoprinol, with its active ingredient of isoprinosine, focused its impact on non-specific cellular response. The aim of the studies conducted *in vitro* on leukocytes isolated from the spleen and head kidneys of African catfish was to assess together and separately the impact of methisoprinol and iridovirus on selected parameters of cellular defense mechanisms.

Materials and methods

The study was conducted at the Department of Fish Pathology and Immunology in Żabieniec of the Inland Fisheries Institute in Olsztyn and at the Department of Microbiology and Clinical Immunology of the Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn. The study was performed on 180 clinically healthy African catfish with body weights of about 100 g. The fish were obtained from the Department of Ichthyology and Fisheries Management of the Polish Academy of Sciences in Gołysz. The catfish used in the study were in good condition and clinically healthy. The fish were fed manually three times per day; the daily ration (2% of the stock biomass) of Aller Møele feed was divided so the last daily portion of feed was the largest. Basic water quality parameters were monitored in the tanks stocked with fish: water temperature was $27 \pm 1^\circ\text{C}$; dissolved oxygen was 4 mg dm^{-3} ; water pH ranged from 6.5 to 7.0 pH. The concentration of ammonia nitrogen (NH_4), nitrite nitrogen (NO_2), and

nitrate nitrogen (NO_3) oscillated around 8 mg dm^{-3} , 0.25 mg dm^{-3} , and 250 mg dm^{-3} , respectively.

The fish used in the study were anesthetized with Propiscin (IFI Żabieniec) at a dose of 0.5 ml dm^{-3} water, and the head kidney and spleen were excised aseptically. Methisoprinol (Polfa Grodzisk Pharmaceuticals, Poland), the active ingredient of which is isoprinosine, was used in the study. Methisoprinol is a synthetic complex of inosine and 1-(dimethylamino/2-propanol/4-acetamidobenzene) at a ratio of 1:3. The lymphocytes were infected experimentally with iridovirus 59.90 at a dose of $10^4 \text{ TCID}_{50} \text{ ml}^{-1}$ obtained from the Laboratoire Vétérinaire Départemental (LVD 39) in Lons-Le-Sounier, France; this virus is from the genus *Ranavirus* and belongs to a group of epizootic hematopoietic necrosis viruses (Whittington et al. 2010).

The impact of different doses of methisoprinol on the activity of organ leukocytes isolated from the kidneys and spleens of African catfish that were subjected or not to the suppressive impact of iridovirus was assessed based on three tests conducted under *in vitro* conditions. The MTT (3-(4,5-dimethylthiazol-2-yl)/2,5-diphenyltetrazolium bromide) assay was used to assess the impact of methisoprinol on the proliferative response of lymphocytes isolated from head kidneys to the sum of mitogens concanavalin A (ConA, Sigma-Aldrich) and lipopolysaccharide (LPS, Sigma-Aldrich). The proliferative response of the lymphocytes isolated from the head kidneys to the mitogens was evaluated with spectrophotometric measurements of the reduction degree of MTT tetrazolium salts by the mitochondrial dehydrogenase of living leukocytes (Mossmann 1983, Siwicki et al. 1996). The metabolic activity of macrophages and neutrophils isolated from the spleen was assessed with the Respiratory Burst Activity (RBA) test (Secombes 1990, Siwicki et al. 1996). The level of metabolic activity of phagocytic cells was assessed by their ability to create reactive oxygen compounds. In the RBA test, the phagocytes were stimulated with phorbol myristate acetate (PMA, Sigma-Aldrich) in a 0.1% solution of NBT (Nitrotetrazolium blue chloride, Sigma-Aldrich). The

phagocytic ability of macrophages and neutrophils isolated from the spleen was assessed with the Potential Killing Activity (PKA) test (Rook et al. 1985, Siwicki and Anderson 1993). The method assesses the ability of the neutrophils and macrophages of the spleen to kill intracellular bacteria. To this end, a suspension of suitably prepared cells of *A. hydrophila* bacteria were added in a 0.1% solution of NBT.

The lymphocytes obtained from the fish were divided into control group M, in which the lymphocytes were not exposed to iridovirus, and group M+W, in which the lymphocytes were exposed to iridovirus. Lymphocyte group M was divided into six groups as follows: control group M0 and experimental groups M100, M200, M300, M400, and M500, in which the lymphocytes were subjected to methisoprinol at respective doses of 100, 200, 300, 400, and 500 $\mu\text{g ml}^{-1}$ medium. The lymphocytes from group M+W were also divided into six groups. Group M0+W comprised lymphocytes exposed to iridovirus at a dose of 10^4 TCID₅₀ ml^{-1} , while the experimental groups of M100+W, M200+W, M300+W, M400+W, and M500+W comprised lymphocytes exposed to iridovirus at the same respective doses as those of methisoprinol at 100, 200, 300, 400, and 500 $\mu\text{g ml}^{-1}$ medium. Simultaneously, the impact of various doses of methisoprinol on the respiratory burst activity (RBA) and potential killing ability (PKA) of African catfish phagocytes after suppression caused by iridovirus was assessed. The cells used to test RBA and PKA were isolated from the spleen in a gradient with Gradisol G (Aqua-Medica). The phagocytes were divided into groups analogous to those of the lymphocytes.

The results were analyzed statistically using analysis of variance to compare multiple means (LSD test) at $P < 0.05$ and $P < 0.01$ and to determine standard deviations.

Results and discussion

The study assessed the impact under *in vitro* conditions of various concentrations of methisoprinol on

the proliferative response of lymphocytes obtained from the head kidney of African catfish. The lymphocytes were stimulated with concanavalin and lipopolysaccharide mitogens. Increases were observed in the proliferative activity of T lymphocytes in response to concanavalin in experimental groups M200, M300, M400, and M500 in comparison to control group M0 (without methisoprinol) (Fig. 1a). The stimulation of cellular response mechanisms by isoprinosine is confirmed by data in the literature (Ohnishi et al. 1983, Cabrera and Sergio 1986, Kowalski 1989). Increased activity of the B lymphocytes stimulated with lipopolysaccharides was only noted in groups M200 and M300 (Fig. 1a). This suggests that methisoprinol applied in a wider spectrum would stimulate more effectively the cellular immune mechanisms associated with T lymphocyte activity, which could indicate the antiviral activity of this compound when used *in vivo*.

The subsequent stage of the experiment assessed the impact of different concentrations of methisoprinol on the proliferative response of lymphocytes in the presence of iridovirus. The studies indicated that iridovirus decreases the proliferative capabilities of lymphocytes that were stimulated with either ConA or LPS (Fig. 1b), which is confirmation of reports to this effect in the literature (Siwicki et al. 1999, 2000, 2001). Weakened lymphocyte capabilities to antigen stimulation impacts the functioning of the entire fish immune system and its defense capabilities. For this reason, attempts were made to evaluate the impact of methisoprinol on the proliferation capabilities of lymphocytes impaired by the virus. The results of the current studies indicated that the addition of isoprinosine in doses of 200 and 300 $\mu\text{g ml}^{-1}$ significantly statistically increased the proliferative response of African catfish lymphocytes stimulated with both ConA and LPS mitogens in comparison to the control group (without isoprinosine) (Fig. 1b). Thus, it is justified to administer methisoprinol to cultured African catfish even when a viral infection has been confirmed. Similar observations were reported for turkeys infected with hemorrhagic enteritis virus (HEV) (Rumińska-Groda 2002).

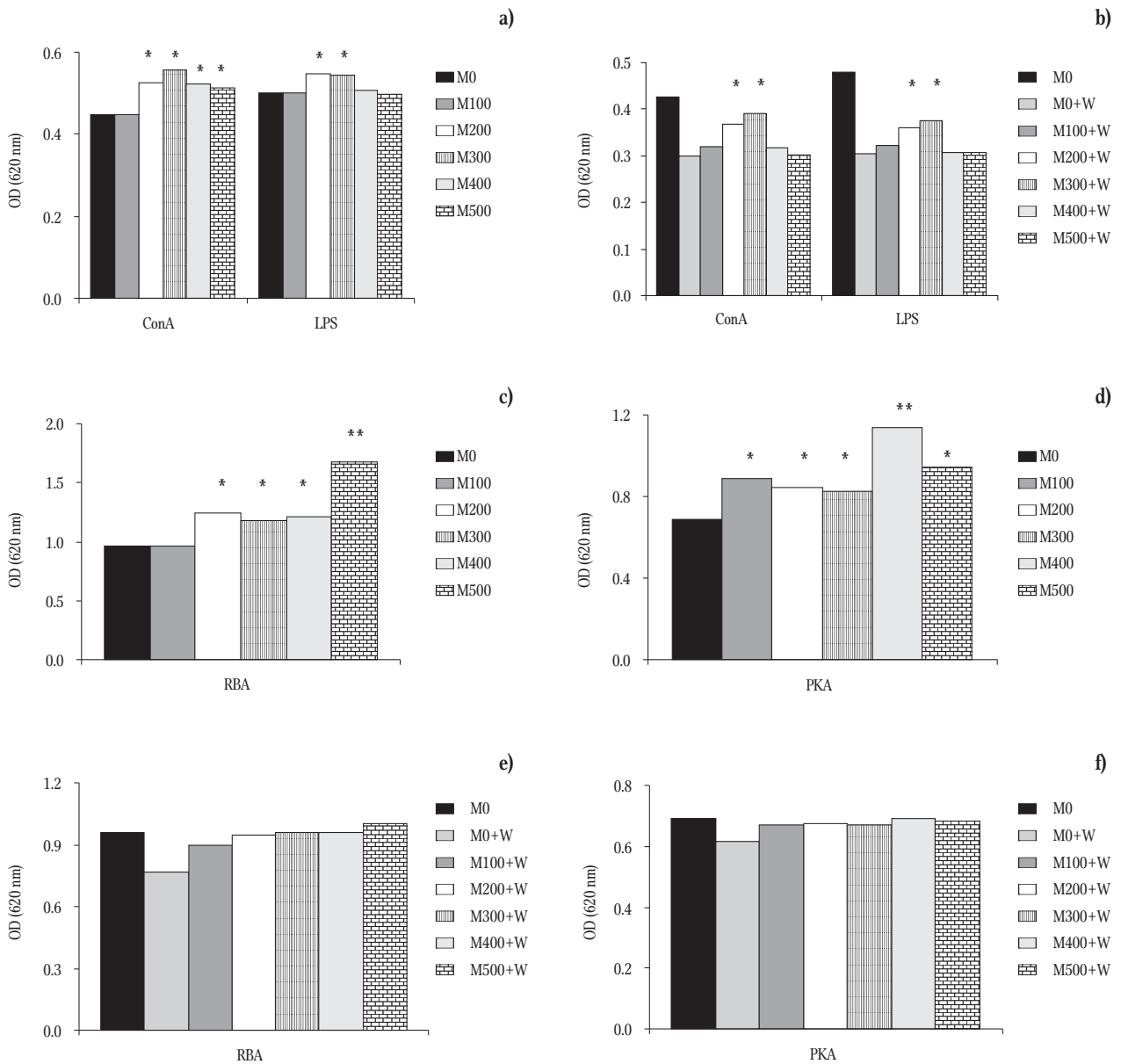


Figure 1. *In vitro* impact of iridovirus and methisoprinol on immunocompetent cells isolated from African catfish. a-f. Impact of methisoprinol on the lymphocyte proliferative response of African catfish stimulated with mitogens ConA and LPS: (a) impact of methisoprinol on the lymphocyte proliferative response stimulated with mitogens ConA and LPS and subjected to iridovirus; (b) impact of methisoprinol on phagocyte metabolism; (c) impact of methisoprinol on the phagocytic activity of macrophages and neutrophils; (d) impact of methisoprinol on phagocyte activity subjected to iridovirus; (e) impact of methisoprinol on phagocyte activity (f). M0 – lymphocytes/phagocytes not subjected to iridovirus or methisoprinol, M100 – lymphocytes/phagocytes subjected to methisoprinol at a dose of 100 $\mu\text{g ml}^{-1}$ medium, M200 – lymphocytes/phagocytes subjected to methisoprinol at a dose of 200 $\mu\text{g ml}^{-1}$ medium, M300 – lymphocytes/phagocytes subjected to methisoprinol at a dose of 300 $\mu\text{g ml}^{-1}$ medium, M400 – lymphocytes/phagocytes subjected to methisoprinol at a dose of 400 $\mu\text{g ml}^{-1}$ medium, M500 – lymphocytes/phagocytes subjected to methisoprinol at a dose of 500 $\mu\text{g ml}^{-1}$ medium, M0+W – lymphocytes/phagocytes with iridovirus at a dose of 10^4 TCID₅₀ not subjected to methisoprinol, M100+W – lymphocytes/phagocytes with iridovirus at a dose of 10^4 TCID₅₀ subjected to methisoprinol at a dose of 100 $\mu\text{g ml}^{-1}$ medium, M200+W – lymphocytes/phagocytes with iridovirus at a dose of 10^4 TCID₅₀ subjected to methisoprinol at a dose of 200 $\mu\text{g ml}^{-1}$ medium, M300+W – lymphocytes/phagocytes with iridovirus at a dose of 10^4 TCID₅₀ subjected to methisoprinol at a dose of 300 $\mu\text{g ml}^{-1}$ medium, M400+W – lymphocytes/phagocytes with iridovirus at a dose of 10^4 TCID₅₀ subjected to methisoprinol at a dose of 400 $\mu\text{g ml}^{-1}$ medium, M500+W – lymphocytes/phagocytes with iridovirus at a dose of 10^4 TCID₅₀ subjected to methisoprinol at a dose of 500 $\mu\text{g ml}^{-1}$ medium, * – $P < 0.05$, ** – $P < 0.01$.

The first line of defense in viral infections are the phagocytes, which include monocytes, macrophages, and neutrophils (Secombes et al. 2001). Considering the crucial importance of innate immunity in fish and in comparison to analogous parameters in mammals, phagocytes play a very important role in the smooth functioning of the entire immunological system. In order to illustrate the activity of the cells isolated from African catfish, PKA and RBA tests were conducted in the subsequent stage of the study. These tests conducted on phagocytes indicated there was increased metabolic activity (RBA) in the experimental groups with the addition of methisoprinol at doses of 200, 300, 400, and 500 $\mu\text{g ml}^{-1}$. No significant differences were noted in the activity of the cells in group M100 in comparison to control group M0 (Fig. 1c). After stimulating the phagocytes with phorbol ester (PKA), a statistically significant increase in killing activity was noted in all experimental groups (Fig. 1d), which is confirmed by the results of other authors published in the literature (Ohnishi et al. 1983, Kowalski 1989). The authors' previous studies have also confirmed the immunosuppressive effects of iridoviruses manifested by decreased activity of phagocytes (Fig. 1e and 1f); these are described in previously cited results of research conducted under laboratory conditions (Siwicki et al. 1999, 2000).

The results of the current study indicate that the addition of methisoprinol results in increased respiratory burst activity (RBA) and potential killing activity (PKA) as well as the increased proliferative activity of lymphocytes T and B in the response to mitogens. Methisoprinol stimulates the innate immune mechanism affiliated with the activation of T lymphocytes, which might influence the antiviral activity of this preparation after its application *in vivo*.

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Streszczenie

Wpływ iridowirusa oraz methisoprinolu na komórki immunokompetentne izolowane od suma afrykańskiego *Clarias gariepinus* (Burchell) w warunkach *in vitro*

Przeprowadzono doświadczenia nad skutecznością działania preparatu immunostymulującego methisoprinol (Grodziskie Zakłady Farmaceutyczne Polfa), którego substancją czynną jest izoprinozyna, będąca kompleksem inozyny i 1-(dimetyloamino)/2-propanolo/4-acetamidobenzoesanu) w stosunku 1:3. W badaniach koncentrowano się na działaniu methisoprinolu na nieswoistą odpowiedź komórkową. Określano wpływ różnych jego dawek na aktywność leukocytów narządowych izolowanych z nerki i śledziony suma afrykań-

skiego, poddanych lub niepoddanych supresyjnemu działaniu iridowirusa. Uzyskane wyniki wskazują, iż dodatek methisoprinolu powoduje wzrost zarówno aktywności metabolicznej (RBA), jak i bójczej (PKA) oraz aktywności proliferacyjnej limfocytów T i B w odpowiedzi na mitogeny: konkanawalinę oraz lipopolisacharyd. Methisoprinol pobudza szczególnie mechanizmy odporności komórkowej związanej z aktywacją limfocytów T, co przekładać się może na działanie przeciwwirusowe tego preparatu po jego zastosowaniu *in vivo*.