ORIGINAL ARTICLE

# Effects of neurotoxin – anatoxin-a on common carp (*Cyprinus carpio* L.) innate immune cells *in vitro*

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## Abstract

**OBJECTIVES:** The aim of this study was to determine if cyanoneurotoxin – anatoxin-a (ANTX-a) alters the essential functions of innate immune cells such as free radicals generation in phagocytic cells and phagocytosis.

**DESIGN:** In the experiments pure ANTX-a was used at concentrations of 0.01, 0.05, 0.1 and 1 μg/ml RPMI–1640 medium. Phagocytes were isolated from carp blood and pronephros. Relative changes in intracellular total free radical presence in fish phagocytes were monitored using a fluorescent probe, dichlorodihydrofluorescin DiOxyQ (DCFH-DiOxyQ) which detects hydrogen peroxide ($H_2O_2$), nitric oxide (NO), peroxyl radical and peroxynitrite anion. Phagocytic activity of fish leukocytes was analyzed with a Vybrant phagocytosis assay kit.

**RESULTS:** The $H_2O_2$ level generated in response to ANTX-a at the highest used concentration was significantly suppressed in pronephros but not in blood phagocytes. Moreover, it was observed that generation of superoxide radicals and nitrite formation was significantly increased in blood and pronephros phagocytes after incubation with lower concentrations of the neurotoxin. The phagocytosis of fish leukocytes was significantly reduced at the two highest used toxin concentrations (0.1 and 1 μg/ml medium).

**CONCLUSION:** This findings suggests that ANTX-a could change innate immunity and reduced adaptive immunity after stress induced by cyanobacterial blooms.

**Abbreviations:**

| | |
|---|---|
| ANTX-a | - anatoxin-a |
| DCFH- DiOxyQ | - dichlorodihydrofluorescin DiOxyQ |
| DCF | - 2′,7′-dichlorofluorescein |
| $LD_{50}$ | - "lethal dose"-the dose that kills half (50%) of the animals tested |
| LPS | - lipopolysaccharide |
| nAChRs | - nicotinic acetylcholine receptors |
| $ONOO^-$ | - peroxynitrite anion |
| $ROO^•$ | - peroxyl radical |
| RNS | - reactive nitrogen species |
| ROS | - reactive oxygen species |

## INTRODUCTION

The occurrence of cyanobacterial blooms, producing a range of toxic metabolites named cyanotoxins, are well-known worldwide problem causing serious health hazards to aquatic animals (Wiegand & Pflugmacher 2005). Even though the adverse effects of cyanotoxins (particularly hepatotoxins) on fish physiological parameters have been reported (Malbrouck & Kestemont 2006), few studies aimed to investigate the natural response of aquatic organisms exposed to water contaminated with cyanoneurotoxins (Oberemm *et al.* 1999; Osswald *et al.* 2007a; 2009; Sierosławska & Rymuszka 2009).

ANTX-a is an alkaloid neurotoxin synthesized by cyanobacteria of *Anabaena, Aphanizomenon, Oscillatoria, Cylindrospermum* and *Microcystis* genera (Osswald *et al.* 2007b). This cyanotoxin is detected in both fresh and brackish waters of Europe, North America, Africa and Asia (James *et al.* 1997; Park *et al.* 1998; Fromme *et al.* 2000; Krienitz *et al.* 2003, Namikoshi *et al.* 2003; Carrasco *et al.* 2007, Sierosławska *et al.* 2010). ANTX-a belongs to the most active compounds ($LD_{50}$ for a mouse is 200 μg/kg via intraperitoneal injection)(Carmichael *et al.* 1979). The toxin acts as a ligand to nicotinic acetylcholine receptors (nAChRs) in the central and peripheral nervous system causing blockage of neuronal signal transmission (summarized by Osswald *et al.* 2009). This cyanoneurotoxin was reported to induce adverse effects in zooplankton, invertebrates, birds, cattle, sheep, horses and dogs (Edwards *et al.* 1992; Hamill 2001; Codd *et al.* 2005; Gugger *et al.* 2005). Only limited laboratory observations performed on carp, goldfish and zebrafish reported the adverse effects of the toxin on behavior, heart rate and on fish reproduction (Oberemm *et al.* 1999; Osswald *et al.* 2007a).

It is well documented that the immune system is sensitive to stress such as environmental contamination and sometimes it reacts more rapidly than other systems, even to concentrations less than those necessary to evoke symptoms of toxicity (Zelikoff *et al.* 2000). The immune response consists of a variety of immune defense mechanisms and protects the host against invasive microorganisms such as bacteria, viruses and also malignant cells. The immune responses are the result of an effective collaboration between innate (relative nonspecific) and acquired (specific) reactions of the immune system. In fish the main immunocompetent organ is pronephros. The cells isolated from this organ (e.g. lymphocytes, macrophages) are important components of the fish immune system (Yoder 2004; Whyte 2007). The innate immune defence of fish is comprised of humoral and cell-mediated mechanisms, in which phagocytosis plays a key role.

The aim of this research was to study the *in vitro* effects of the toxin on the selected mechanisms such as production of reactive oxygen/nitrogen species and phagocytosis by the immune cells isolated from blood and pronephros of common carp (*Cyprinus carpio* L.).

## MATERIAL AND METHODS

*Toxin*

Pure ANTX-a was purchased from TOCRIS Bioscience, USA. ANTX-a was dissolved in phosphate buffered saline (PBS, Biomed, Poland) to give a 1 mg/ml stock solution and kept at –20 °C. Before the immune study the following concentrations of the toxin were prepared: 0.01, 0.05, 0.1 and 1 μg/ml.

*Fish*

In these experiments, 5 fish of 540–640 g were used. Common carp were obtained from a local, commercial farm and acclimated in 100L aerated freshwater tank at 20 °C for 1 week under a natural photoperiod (8 h light : 14 h dark) before experimental manipulation. Fish were fed daily with commercial carp pellets. Carp were anaesthetized by immersion in 0.2% Propiscin (Żabieniec, Poland) diluted in water prior to blood and head kidney (pronephros) collection. All experiments were performed according to the Local Committee of Ethics on animal experimentation (approval number 9/2009).

*Blood leukocyte seperation*

Blood was collected in heparinized tubes from the caudal vessel of anaesthetized fish. Blood samples were diluted 1:1 with PBS without $Ca^{2+}$ and $Mg^{2+}$ and layered carefully on the Gradisol G gradient (AquaMedica, Poland; density 1.117 g/ml). The samples were centrifuged at 400 g for 35 min at 4 °C. Separated leukocytes were gently removed. Cells were then washed twice in PBS without $Ca^{2+}$ and $Mg^{2+}$ and adjusted to $1 \times 10^7$ viable cells/ml.

*Pronephros leukocyte separation*

Head kidney was aseptically collected and leukocyte fraction was separated using a previously described technique (Rymuszka *et al.* 2010a). Briefly, organs were passed through a 100 μm nylon mesh with complete medium containing RPMI-1640 (Biomed, Poland), 10% of fetal calf serum (FCS, Gibco, USA) and 100 U/ml penicillin/streptomycin (Sigma, Aldrich). The cell pellet was gently placed on a Percoll gradient (specific gravity of 1.078 g/ml, Sigma, Aldrich). After centrifugation at 400 × g for 40 min at 10 °C, the cells at the interface were recovered, washed twice and resuspended in PBS without $Ca^{2+}$ and $Mg^{2+}$.

The total leukocyte count and viability were determined using a Nucleo Counter YC-100 (Chemometec, Denmark) according to the manufacturer's procedure. Cell suspensions were adjusted to a concentration of $1 \times 10^7$ cells/ml.

*ROS and RNS generation*

Free radicals including ROS/RNS generation in fish phagocytes were detected using OxiSelect™ *In vitro* ROS/RNS Assay Kit (Cell Biolabs, Inc. San Diego, CA),

which employed the fluorogenic DCFH-DiOxyQ probe reacted with $H_2O_2$, ROO•, NO and $ONOO^-$. Briefly, leukocytes ($1 \times 10^7$ cells/ml) were cultured in the absence or in the presence of ANTX-a for 24 h at concentrations of 0.01, 0.05, 0.1 and 1 μg/ml medium. Samples were measured against a $H_2O_2$ or DCF (measuring free radicals other than $H_2O_2$) standards according to the manufacturer's instruction. Subsequently, the cells or standards were incubated with a catalyst at room temperature for 5 min. The prepared non-fluorescent DCFH probe was added to each well and then rapidly oxidized to highly fluorescent DCF in the presence of ROS and RNS. Fluorescence intensity was measured at the excitation wavelength of 480 nm and emission wavelength of 520 nm using a fluorometer (FLUOstar Optima, BMG Labortechnik, Germany). The free radical content in samples was determined by comparison with the predetermined $H_2O_2$ or DCF standard curve.

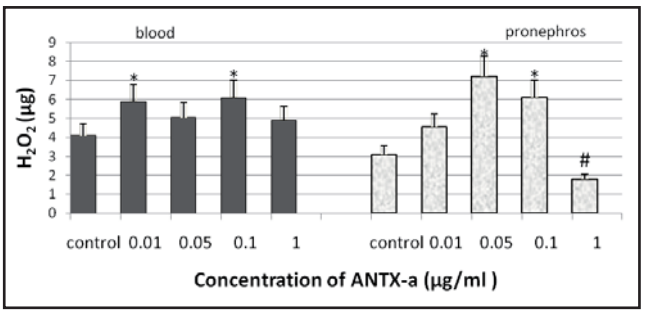


**Fig. 1.** *In vitro* effects of ANTX-a on $H_2O_2$ production in blood and pronephros phagocytes (mean ± SD, n=9, statistically significant up-regulation and down-regulation ($p$<0.05) is denoted by (*) and (#) marks, respectively).

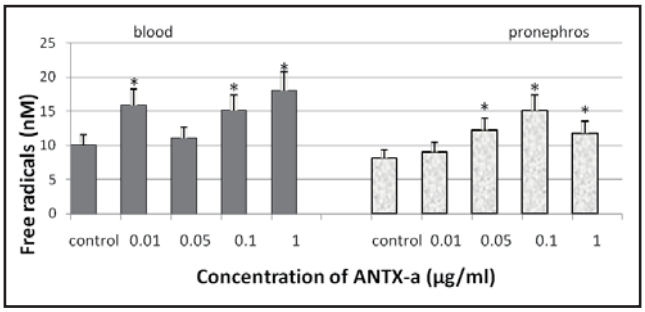


**Fig. 2.** *In vitro* effects of ANTX-a on free radicals (peroxyl radical, nitric oxide, peroxynitrite anion) production in blood and pronephros phagocytes (mean ± SD, n=9, *differences statistically significant when $p$<0.05).

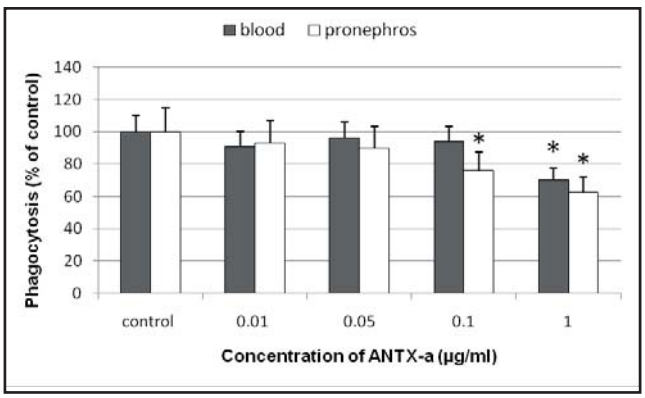


**Fig. 3.** Effect of ANTX-a on phagocytic cells. Phagocytosis was assayed 24 h after exposure to various concentrations of the toxin (0.01, 0.05, 0.1 and 1 μg/ml). Data are shown as mean ± SD, n = 5, *differences statistically significant when $p$<0.05.

*Analysis of phagocytic activity*

Leukocyte phagocytic activity was assessed using a Vybrant Phagocytosis Assay Kit (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's instruction. Briefly, phagocytes were cultured in 96-well black culture plate at $1 \times 10^6$ cells/well. The cells were incubated for 24 h in the presence of different concentrations of ANTX-a (0.01, 0.05, 0.1 and 1 μg/ml). The negative control (only PBS) and positive control (cells stimulated with LPS, 10 μg/ml, Sigma, Aldrich) were prepared. Culture medium was removed and fluorescein-labeled *Escherichia coli* BioParticles were added. After 120 min, supernatant was removed and 100 μl of trypan blue was immediately added to each well for 1 min to quench extracellular fluorescence. Excess of trypan blue dye was removed by aspiration. The plate was read on FLUOstar OPTIMA microplate reader at 480 nm excitation and 520 nm emission. Results are expressed as the percentage of phagocytosis relative to the untreated cells.

*Statistical analysis*

Statistical analysis of the data was carried out using analysis of variance (ANOVA) for repeated measures followed by Duncan's post hoc analysis using Statistica Software (Version 8, StatSoft, Tulsa, OK, USA).

## RESULTS

*Effects of ANTX-a on ROS/RNS generation in fish phagocytes*

As shown in Figure 1, the decrease of $H_2O_2$ generation in pronephros cells was maximal at the highest concentration of the toxin. Further, generation of $H_2O_2$ was found to be enhanced significantly ($p$<0.05) in blood and pronephros cells incubated with lower concentrations (from 0.01 to 0.1 μg/ml) of the ANTX-a as compared to the control cells. Moreover, dose-independent increase of total level of free radicals such as: NO, ROO• and $ONOO^-$ after the exposure of blood and pronephros cells to the toxin at used concentrations was detected (Figure 2).

*Effects of ANTX-a on blood and pronephros cells phagocytosis*

The effect of the toxin on functions of fish phagocytes was tested by measurement of phagocytosis of fluorescent-labeled bacteria after exposure to increasing concentrations of ANTX-a (Figure 3). Uptake of bacteria by blood phagocytes was significantly decreased in the presence of the toxin at 1 μg/ml compared to untreated

controls ($p<0.05$). The phagocytosis in the presence of lower concentrations of the toxin (0.05 and 0.1 μg/ml) was not significantly affected.

Similary, phagocytosis of labeled bacteria was inhibited more than 25% and 38% in the presence of 0.1 and 1 μg/ml ANTX-a, respectively in cultured pronephros macrophages.

## DISCUSSION

The intensive releasing of cyanotoxins from cyanobacterial blooms generates a stressful environment to aquatic animals and in the consequence may effect on the immune system, rendering the fish susceptible to the infectious diseases. Our knowledge on the effects of cyanotoxins on the fish immune system is relatively limited (summarised by Rymuszka & Sierosławska 2009; Rymuszka *et al.* 2010a, b). It has been observed that, although ANTX-a affects mainly the nervous system, it can also interfere with the monkey kidney cells, rat thymocytes and mouse splenocytes by inducing its apoptotic changes (Rao *et al.* 2002; Teneva *et al.* 2005). In our previous study, we found that cyanoneurotoxin ANTX-a is an inducer of apoptosis in fish immune cells. Incubation of lymphocytes and phagocytes with the toxin at concentrations of 0.1 and 1 μg/ml resulted in increase of caspase 3/7 activity. It has also been reported that a short-term exposure of fish to non-lethal concentrations of this neurotoxin can influence the viability of lymphocytes but not phagocytes (Rymuszka & Sierosławska 2010a). It is possible that toxin not only reduces the viability of the cells, but also it may change their functions. The objective of the present work was to investigate whether ANTX-a influences the essential immune parameters of fish phagocytes.

One of important innate immune functions of phagocytes is the respiratory burst which generates ROS and RNS used to kill engulfed microorganisms (Whyte 2007). The changes in intracellular total reactive oxygen species in fish phagocytes were monitored for $H_2O_2$, NO, peroxyl radical and peroxynitrite anion. Results showed that total ROS/RNS activity was modulated when carp phagocytes were exposed to the used concentrations of the neurotoxin. ANTX-a stimulated NO, peroxyl radical and peroxynitrite anion production (Figure 2) but the highest used concentration of the toxin decreased the level of $H_2O_2$ in pronephric cells (Figure 1). In fish, the pronephros is the major hematopoietic organ in which all lines of hematopoietic differentiation and cells in various stages of development are observed. This makes them particularly sensitive to any stressor factors. The exposure of cells at the highest concentration of the toxin leads to a slump in cellular metabolism, and as a result to the decrease of $H_2O_2$ level.

It is known that the ROS produced by activated neutrophils and macrophages are necessary for effective phagocytosis. The process of phagocytosis is a key in the innate defense response to invading pathogens and the ability of leukocytes to kill pathogenic microbes is probably one of the most important protective mechanisms (Yoder 2004). In the present study, it was observed that inducing by the toxin bacterial-killing activity did not correlate with increases in both nitrate and $H_2O_2$ production by phagocytic fish cells. These data indicate that the exposure of fish immune cells to the higher concentrations (0.1 and 1 μg/ml) of the toxin affected the phagocytic ability of the cells (Figure 3) although significantly decreased $H_2O_2$ level was seen only in pronephric phagocytes treated with ANTX-a at 1 μg/ml compared to untreated cells (Figure 1). What is more, the toxin treatment at the other used concentrations (range from 0.01 to 1 μg/ml) increased both $H_2O_2$ (Figure 1) and nitrite (Figure 2) production in cells in a dose-independent manner but it did not increase their phagocytic activity (Figure 3). Such oxidative modifications in phagocytic cells can influence to the loss of its homeostasis, which in turn may be responsible for the changes in the processes of cell signalling and also leads other disorders of cell activities e.g., chemotaxis and adhesiveness affecting the efficiency of phagocytosis. On the other hand, a series of damage may occur when the generation of ROS is too high. Oxidative stress may result in modifications of macromolecules such as proteins, lipids and DNA which lead to cell death (Ziech *et al.* 2010). This is in agreement with our previously study in which ANTX-a induced apoptosis in immune fish cells. Similarly, Rao *et al.* (2002) demonstrated that this toxin caused apoptosis in rat thymocytes and monkey kidney cells possibly by generation of ROS.

In summary, the results of these studies and our previous findings (Rymuszka & Sierosławska 2010a) suggest that the observed changes in the studied cell functions could be attributed to direct action of ANTX-a. Further studies are required to fully explain the mechanisms of modulation of the phagocytic cells caused by cyanoneurotoxin – ANTX-a and its potential effects on fish resistance to disease.

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