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Original article

Anatoxin-a induces apoptosis of leukocytes and decreases the proliferative ability of lymphocytes of common carp (*Cyprinus carpio* L.) *in vitro*

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Abstract

Cyanobacteria (*Cyanophyta, Cyanoprocaryota, Cyanobacteria*) (blue-green algae) are procaryotic phototrophic microorganisms playing an important ecological role in the freshwater and marine environment as primary producers. However, as a consequence of water eutrophication observed in many reservoirs in different parts of the world, these microorganisms form massive scums, known as water blooms, releasing cyanotoxins hazardous to fish and other aquatic organisms. Cyanotoxins are cyanobacterial secondary metabolites of various chemical structures harmful to humans, terrestial and aquatic animals such as fish. The most abundant cyanotoxins are microcystins and hepatotoxins inducing toxic changes in fish liver, kidney, gills, digestive tract and immune system. Very little is known on the effects of alkaloid neurotoxic anatoxin-a on fish and their immunity. The aim of this study was to assess the *in vitro* influence of anatoxin-a on immune cells isolated from the common carp (*Cyprinus carpio* L.). The leukocyte intracellular level of ATP was reduced only at the highest concentration of anatoxin-a. Apoptotic and necrotic leukocytes were observed at the lower and the highest concentrations of anatoxin-a, respectively. Elevated activity of caspases 3/7 after 2 hours and a concentration-dependent decrease in the proliferative ability of T and B lymphocytes was also observed. The results suggest that anatoxin-a could be a possible immunotoxic agent in the aquatic environment and may increase the susceptibility of fish to infectious and neoplastic diseases. Therefore, constant monitoring of anatoxin-a and its producers in lakes and fish ponds should be performed.

Key words: cyanotoxins, immunotoxicity, fish, anatoxin-a, apoptosis, necrosis

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Introduction

Many species of cyanobacteria (blue-green algae) abundant in eutrophic waters produce various toxic secondary metabolites known as cyanotoxins which, on the basis of their mode of action, can be classified into: hepatotoxins, neurotoxins, cytotoxins, dermatotoxins and irritant toxins. These cyanobacterial products are a serious health hazard to humans, domestic animals, wildlife and also fish (Carmichael and Falconer 1993, Tencalla and Dietrich 1997). Anatoxin-a is an alkaloid neurotoxin (Devlin et al. 1997) produced by cyanobacteria such as *Anabaena* spp. (Camichael et al. 1975), *Oscillatoria* spp. (Edwards et al. 1992), *Aphanizomenon flos-aquae* (Rapala et al. 1993), *Raphidiopsis mediterranea* (Namikoshi et al. 2003), *Planktothrix rubescens* (Viaggiu et al. 2004), *Microcystis aeruginosa* (Park et al. 1993), *Arthrospira fusiformis* (Ballot et al. 2005) and *Phormidium favosum* (Gugger et al. 2005). This toxin is a postsynaptic acetylcholine agonist with depolarizing activity binding irriversibly to the nicotinic receptor in neurons and muscle endplates (Carmichael et al. 1975, Spivak et al. 1980) causing death by respiratory arrest and muscular paralysis (Devlin et al. 1977). Since fish are a group of aquatic vertebrates coexisting with toxic cyanobacterial blooms, these animals can be at high risk of being exposed to cyanotoxins such as anatoxin-a. The purpose of this *in vitro* study was to determine the effects of pure anatoxin-a on the leukocyte viability and the proliferative ability of T and B lymphocytes isolated from common carp (*Cyprinus carpio* L.).

Materials and Methods

Anatoxin-a (fumarate) (Alexis Biochemicals, San Diego, USA) was dissolved in RPMI 1640 medium revealing final concentrations of 0.01, 0.1, 1, 5 and 10 µg/ml. Thirty healthy carp, each weighing about 350 g were purchased from a commercial farm and treated according to the recommendations of the Local Committee of Ethics (approval number 492/2004). Before the study the fish were anaesthetised with 0.2% Propiscin (Żabieniec, Poland) diluted in water.

Pronephros was taken aseptically and pushed through a 60 µm nylon mesh with RPMI 1640 medium with L – glutamin and $NaHCO_3$ (Biomed, Lublin, Poland). The lymphocytes from each individual fish were separated using density gradient centrifugation. The cell suspension was placed on a Gradisol L density gradient (1.077 g/ml, Aqua-Medica, Łódź, Poland), then centrifuged at 400 g for 40 min. The cells were collected from the interface and washed three times with the RPMI 1640 medium at 400g for 5 min. Viability of the cells isolated from the pronephros including lymphocytes (about 88%) and other cells, mostly neutrophils and macrophages (about 12%), was more than 98%. The cells were suspended in RPMI 1640 medium and dispensed into 96 well microplates at a concentration of $1x10^6$ cells/ml. All media were supplemented with 10% fetal calf serum (FCS, GIBCO, UK) and 1% penicillin/streptomycin (Sigma, Aldrich).

Viability Assay

Cytotoxic action of anatoxin-a on lymphocytes was determined by using CellTiter-Glo(R) Luminescent Viability Assay – a homogenous method of determining the number of viable cells in culture based on quantification of intracellular ATP indicating the presence of metabolically active cells. The amount of ATP is directly proportional to the number of viable cells in the culture. 100 µl of the cell suspension were added to a 96-well multiwell plate compatible with the luminometer. The cells were exposed to anatoxin-a at concentrations of 0.1, 1, 5 and 10 µg/ml medium at 22°C for 24 hours. After incubation, 100 µl of CellTiter-Glo reagent were added to each well used. The microplate was mixed for 2 minutes on an orbital shaker to induce cell lysis and left for 10 min incubation at room temperature. The luminescence was measured by using a luminometer.

Determination of cell death type

Characterisation of cell death after 24 hours of incubation with anatoxin-a was obtained using a Cellular DNA Fragmentation ELISA test kit for detection of BrdU (5'-Bromo-2'-deoxy-uridine) – labeled DNA fragments in cell lysates or cell culture supernatants (Roche Applied Science, Switzerland).

Lymphocyte suspension was adjusted to $3x10^5$ cells/ml RPMI. 10 µM of BrdU labeling solution was added to the cell suspension and the mixture was incubated overnight. After incubation the cell culture was centrifuged at 250 g for 10 min and the medium containing BrdU was removed by aspiration. The BrdU-labeled cells were resuspended in RPMI medium at the final concentration of $1x10^5$ cells/ml and 100 µl of cell suspension was transferred to each well of a 96-well round-bottom microplate. 100 µl per well of anatoxin-a at appropriate solution was added to the cell suspension and the microplate was incubated for 24 hours at 22°C. After incubaction the microplate was centrifuged for 10 min at 250 g. 100 µl of the

supernatant was removed for further detection of possible DNA fragments released from necrotic cells in the ELISA procedure. For the detection of possible apoptotic DNA fragments, the supernatants from the remaining wells were removed and 200 µl of incubation solution per well were added to lyse the cells. The microplate was incubated for 30 min at 22°C and centrifuged for 10 min at 250 g.

100 µl of supernatant from the remaining wells were removed to be analysed in the ELISA procedure. A 96-well flat-bottom microplate was coated with anti-DNA antibody solution (100 µl of anti-DNA solution to each well). The plate was then incubated for 1 hour at 22°C and afterwards the coating solution was removed by aspirating the buffer away. After coating the microplate with the anti-DNA antibody, nonspecific binding sites were blocked by adding 200 µl of incubation solution, incubation of the microplate for 30 min at 22°C and removal of the incubation solution. The wells were washed three times with 300 µl of washing solution for 3 min. The ELISA photometric measurment of BrdU fragments was performed at 450 nm (reference wavelenth 690 nm) using a BioRad 550 microreader.

Caspase 3/7 activity

The level of caspase 3/7 was determined using a microplate luminometer method, Caspase-Glo™ 3/7 Assay (Promega, Madison, USA). The study was performed according to the manufacturer's instructions. 50 µl of caspase Glo-reagent was added to each well containing an appopriate concentration of anatoxin-a and 100 µl of leukocyte suspension. The microplate was then gently mixed at 300-500 rpm for 30 seconds, incubated for 30 minutes and the luminescence was measured using a Lumistar Optima luminometer.

Lymphocyte proliferative activity assay

The MTT test was used according to the method described by Mosmann (1983). Lymphocyte suspension at a concentration of $1x10^6$ cells/ml of RPMI 1640 without phenol red was placed in a microtiter plate. 40 µg/ml of concanavalin A – ConA (Sigma, Aldrich) as a T cell mitogen or 20 µg/ml of lipopolysaccharide – LPS (Sigma, Aldrich) as a B cell mitogen were added to the cell suspension (Caspi et al, 1984). Subsequently, anatoxin-a was added at concentrations of 0.1, 1, 5 and 10 µg/ml RPMI – 1640 medium. The mixture was incubated for 72 h at 20°C. After incubation, 20 µl of the solution containing 7 mg/ml of MTT (3 – [4, 5 dimethylthiazoly – 2 yl] – 2,5 – diphenyltetrazolium bromide) (Sigma, Aldrich) in PBS were added and the plate was incubated for 4 hours. The plate was centrifuged (110 g, 5 min) and the cell-free supernatant was removed. 100 µl of DMSO were added to each well and the microplate was mixed. The optical density was measured at 630 nm using a BioRad 550 microreader. Data for 30 individual fish were meaned.

Statistical analysis

Data from the viability assay, type of cell death, caspase 3/7 activity and proliferative ability of lymphocytes were analysed by one-way analysis of variance (ANOVA). When significant differences were detected, Duncan's test was used to compare between the experimental groups and between the experimental groups and the controls. Differences were considered statistically significant when $P < 0.05$.

Results

The study revealed that anatoxin-a at concentrations of 0.1, 1 and 5 µg/ml did not reduce the intracellular ATP level in leukocytes in comparison to the toxin-free control. The ATP level was slightly reduced only at 10 µg/ml (Fig. 1). The cyanotoxin triggered apoptosis in carp leukocytes. A statistically significant number of apoptotic cells after 24-hour exposure to anatoxin-a was found at concentrations of 1, 5 and 10 µg/ml (Fig. 2). Anatoxin-a also induced a slight necrosis of leukocytes but only at a concentration of 10 µg/ml. An early stage of apoptosis in carp leukocytes was also confirmed by increased activity of effector caspases 3/7 (Fig. 3). The highest level of these proteases was observed after 2 hours of incubation with anatoxin-a at concentrations of 0.01, 0.1, 1, 5 and

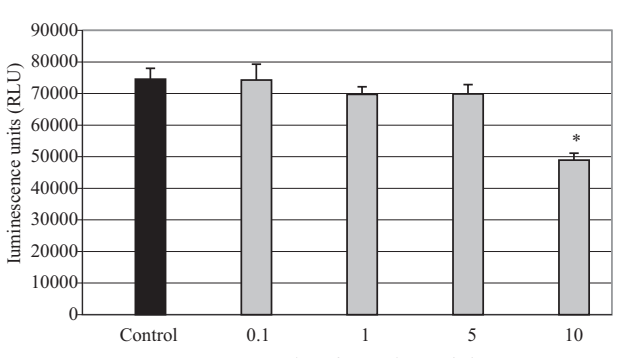

Fig. 1. Intracellular ATP level in leukocytes isolated from pronephros of common carp (*Cyprinus carpio* L.) after 24-hour incubation with anatoxin-a (n=30, mean ± SD, * – statistical significance).

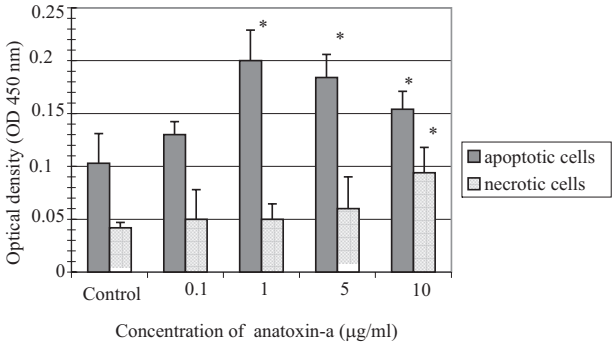

Fig. 2 Type of death of leukocytes isolated from common carp (*Cyprinus carpio* L.) after 24-hour incubation with anatoxin-a (n=30, mean ± SD, * – statistical significance).

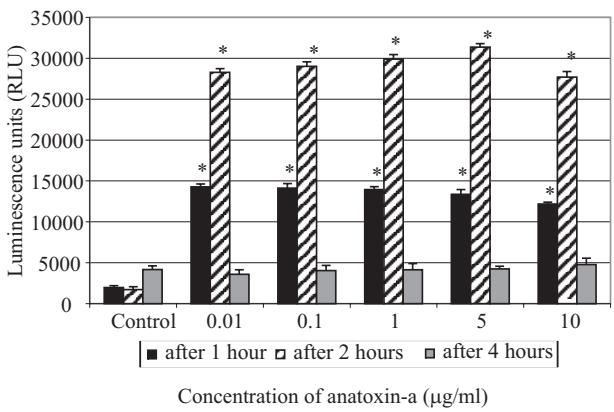

Fig. 3 Caspase 3/7 levels in leukocytes isolated from pronephros of common carp (*Cyprinus carpio* L.) after incubation with anatoxin-a (n=30, mean ± SD, * – statistical significance).

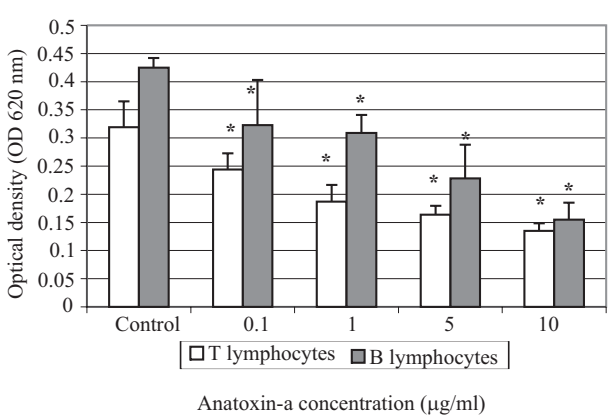

Fig 4. Effect of anatoxin-a on proliferation of lymphocytes isolated from pronephros of common carp (*Cyprinus carpio* L.) (n=30, mean ± SD, * – statistical significance).

10 μg/ml and after 4 hours of exposure to the cyanotoxin the level of the caspases was reduced to the control values.

The study showed the inhibitory influence of cyanobacterial anatoxin-a on the proliferative response of both T and B lymphocytes to mitogens. A significant, concentration-dependent decrease of T lymphocyte proliferation was observed after exposure to anatoxin-a at concentrations of 0.1, 1, 5 and 10 μg/ml (Fig. 4). The cyanotoxin also markedly inhibited the proliferative response of B lymphocytes at anatoxin-a concentrations of 0.1, 1, 5 and 10 μg/ml.

Discussion

Data on the effects of anatoxin-a on vertebrates are very scarce. Subchronic studies on mammals such as rats revealed no disturbances in liver, adrenals, heart, lungs and brain functioning, no loss of body and organ (liver, kidney and spleen) weight and no changes in red and white blood cell count. (Astrachan and Archer 1981). Little information is available on the effects of anatoxin-a on the immune system; only a few studies have been done on its influence on mammalian immune cells. Decreased lymphocyte viability (about 57%) in the mouse after 24 hour incubation with anatoxin-a at a concentration of 0.1 μg/ml was demonstrated by Teneva et al. (2005). In another immunotoxicological study in rats Lakshmana Rao et al. (2002) showed the apoptotic effects of anatoxin-a in thymocytes *in vitro*.

Our study showed a toxic influence of anatoxin-a on the immune system of the common carp; however, the cyanotoxin had little effect on leukocyte viability. The level of intracellular ATP in leukocyte cells after 24 hours of incubation with anatoxin-a was reduced only at the highest concentration (10 μg/ml) of the cyanotoxin. Anatoxin-a at the lower concentrations induced apoptosis in leukocytes. The results from the ELISA fragmentation test show an increased number of apoptotic cells at 1, 5 and 10 μg/ml. Elevated activity of proapoptotic cysteine proteases-caspases 3/7 in leukocytes after 2 hours of incubation with the cyanotoxin also confirmed the early stages of apoptosis. Anatoxin-a at higher concentrations was a necrotic factor for fish immune cells. A rise in necrotic leukocyte number was observed at 10 μg/ml of the cyanotoxin in comparison to the control and this correlated with the reduced level of intracellular ATP. Anatoxin-a should be also considered as a factor suppressing the proliferation of fish lymphocytes-immune cells playing an important role in cellular and humoral immunity. The inhibition of the proliferative response of both T and B cells to mitogens after 24-hours of incubation with this cyanotoxin was observed at all concentrations used in the study. Currently, the mechanisms of anatoxin-a toxic action towards piscine leukocytes are still not known and remain to be investigated.

Anatoxin-a is not very stable in an aquatic environment since it is quickly degradable by sunlight and

high pH, but it tends to accumulate in fish such as juvenile common carp (*Cyprinus carpio* L.) The experimental fish were exposed in laboratory conditions to freeze-dried cells of an anatoxin-a producing strain of *Anabaena* sp. at a density of 10^5 and 10^7 cells/ml. The cyanotoxin level determined in the whole fish was 0.031 and 0.768 µg/g d.w. (dry weight) respectively (Devlin et al. 1977, Osswald at al. 2007). Although the accumulation of anatoxin-a in fish is evident we still need further studies on the determination of its levels in different fish organs such as the pronephros.

The present study has shown that anatoxin-a is an immunotoxic factor, a potent inducer of apoptosis and also a necrotic agent (at high concentrations) in carp immune cells. We need futher *in vivo* studies to confirm the immunotoxic effects of this cyanotoxin in fish. It should be kept in mind that in natural conditions, suppression of fish immunity induced by anatoxin-a could lead to a decreased resistance of fish to bacterial, viral, fungal and neoplastic diseases, causing losses in aquaculture. Therefore anatoxin-a levels in water reservoirs, especially in fish ponds, should be monitored especially during and after cyanobacterial blooms.

Declaration of interest

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