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ORIGINAL ARTICLE

In vitro cytotoxicity of cyanobacteria from water ecosystems of Serbia

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Summary

Purpose: *The purpose of this study was to investigate whether water samples from water ecosystems of Serbia, unknown so far with regard to cyanotoxin levels, are the source of toxic compounds originating from the biological activity of cyanobacteria.*

Materials and methods: *The growth inhibition activity was evaluated using in vitro toxicity assay in Neuro-2a (mouse neuroblastoma) and MRC-5 (human fetal lung) cell lines, after 48 h of exposure time. Cell growth was evaluated by the colorimetric sulforhodamine B (SRB) assay.*

Results: *Our experiments revealed that some of the investigated water samples are toxigenic and alter cell growth of Neuro-2 and MRC-5 cell lines in vitro.*

Conclusion: *Neuro-2a and MRC-5 cell lines responded to the presence of secondary metabolites of cyanobacteria. Significant cytotoxic effects were detected in the samples from lakes (Ludos and Palic), reservoirs (Zobnatica) and rivers (Krivaja).*

Key words: biological assay, cell line, colorimetry, cyanobacteria, toxicity tests, water

Introduction

Blue-green algae (cyanobacteria) have been observed in aquatic environments around the world. Several strains of cyanobacteria are known to produce a wide variety of toxins and/or biomedically interesting, bioactive compounds. The chemical structure of these compounds and their mechanism of action in biological systems *in vitro/in vivo* have been intensively investigated during the recent years. Most investigations in this field examined species belonging to the genera *Microcystis, Cylindrospermopsis, Anabaena, Oscillatoria (Planktotix) and Aphanisomenon* [1].

Cyanotoxins cause direct intoxication of animals and humans through contact with bloom water or indirect poisoning due to consumption of contaminated food [2,3]. They differ in primary target organs and mechanisms of toxicity in mammals. Toxins produced by cyanobacteria may be categorized according to their toxicological properties as neurotoxins (anatoxin-a, anatoxin-a(s), saxitoxin and neosaxitoxin), tumor promoters (microcystins and lipopolysaccharides), dermatotoxins/irritant toxins (lyngbyatoxin A, apysiatoxins and lipopolysaccharides) and hepatotoxins (microcystins, nodularins and cylindrospermopsin). Based on their chemical structure, cyanotoxins fall into 3 main groups: cyclic peptides (microcystins and nodularins), alkaloids (neurotoxins and cylindrospermopsin) and lipopolysaccharides [4].

Some scientists have been more concerned about the cyclic peptides hepatotoxins than neurotoxic alkaloids or lipopolysaccharides, because the latter are not considered to be widespread in water supplies [5]. However, during the past years, the frequency and global distribution of toxic algal incidents appear to have increased, and human intoxications from novel algal sources have occurred. This has led to the revelation that numerous cyanobacterial species, not commonly investigated may be the source of potent toxins [6].

Microcystin-LR is a potent inhibitor of eukary-

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otic protein serine/threonine phosphatases PP1 and PP2A, both *in vitro* and *in vivo*. Substances that inhibit these enzymes are considered to be nonphorbol ester (TPA)-type tumor promoters. PPs serve an important regulatory role to maintain homeostasis in the cell. PP inhibition results in a shift in the balance towards higher phosphorylation of target proteins such as tumor suppressor proteins. This is a major posttranslational modification, which may result in an excessive signaling and may lead toward cell proliferation, cell transformation and tumor promotion [7].

Currently, there is no single method which will provide adequate monitoring for all cyanotoxins in the increasing range of sample types which have to be evaluated. For many years, the mouse bioassay alone has been used to determine bloom toxicity. Although it provides a measure of the total toxicity (response) within a few hours, it is generally not very sensitive or specific [8].

Established cell lines represent useful test systems for toxicological studies [9]. Bioassays using mammalian cells have received attention as suitable replacements for mouse toxicity tests. The well documented fact that microcystins have caused acute liver damage has prompted studies using hepatocytes. Freshly isolated rat hepatocytes were first investigated by Aune and Berg [10] who reported good correlation between toxicity measured by leakage of the enzyme lactate dehydrogenase (LDH) from hepatocytes and results from mouse bioassay. A neuroblastoma cell line technique for sodium channel blocking activity has been developed as a replacement for the mouse in the detection of saxitoxins [11,12].

The purpose of this study was to investigate whether 18 water samples from water ecosystems of Serbia, unknown so far with regard to cyanotoxin levels are a source of toxic compounds originating from intracellular and/or extracellular toxins of cyanobacteria. Samples were collected in spring and autumn of 2007 to devise seasonal differences in their toxic profiles. These experiments revealed that some of the investigated water samples are toxigenic indeed and alter cell growth of Neuro-2 (mouse neuroblastoma) and MRC-5 (human fetal lung) cell lines *in vitro*.

Materials and methods

Water samples

Water samples were collected in spring and autumn 2007 from 18 localities, 17 from the Vojvodina region and one locality (Gruza, the only reservoir for water supply) from central Serbia. The investigation covered different types of surface water ecosystems such as lakes (Palic, Ludos), reservoirs (Gruza, Zobnatica, Provala), channels (Jegricka, Koviljski rit, DTD-Becej, Vrbas-DTD, Sr. Miletic-DTD, B. Gradiste-DTD, Ruski Krstur-DTD, Sombor-DTD) and rivers (Krivaja, Tisa-N. Knezevac, Begej-Sr. Itebej, Tamis-Botos, Mrtva Tisa-B. Gradiste).

Preparation of samples

Samples from water ecosystems of Serbia were freezed / thawed twice, sonicated 3×15 sec on ice and filtered through 0.22 μm Millipore filters. In that way total cyanobacterial toxins (both intracellular and extracellular) were tested.

Cell lines

Cell lines Neuro-2a (ATCC CCL-131, mouse neuroblastoma) and MRC-5 (ATCC CCL-171, human fetal lung) were cultured in RPMI 1640 medium (Gibco, UK) and Dulbecco's Modified Eagle's Medium (DMEM, Gibco, UK), respectively, supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS, PAA Laboratories GmbH, Austria), 100 IU/mL penicillin and 100 μg/mL streptomycin (Galenika, Serbia). Cells were cultured in 25 cm^2 flasks (Corning, New York, USA) at 37° C with 5% CO_2 in air and high humidity, and were sub-cultured twice a week. A single cell suspension was obtained using 0.1% trypsin in 0.04% EDTA (Sigma, USA). Trypsin treatment and subculturing were done according to the Invittox protocols [13,14]. Cell viability was measured with the trypan blue exclusion test [15] prior to seeding. Exponentially growing cells were used throughout the assay.

Exposure conditions

Cells were harvested and plated into 96-well microtiter plates (Corning, New York, USA) at seeding density of 3×10^3 cells per well, in a volume of 180 μL, and preincubated in complete medium supplemented with 5% FCS, at 37° C for 24 h.

Exposure to water samples

Mammalian cells were exposed to one concentration of water samples at a final concentration of 10% that was obtained by adding 20 μL of the sample to 180 μL RPMI 1640 or DMEM culture medium. Control wells were prepared by adding 20 μL distilled water. The cells were exposed to the water samples for 48 h (37° C) prior to analysis by the SRB assay.

Cell growth assay

Cell growth was evaluated by the colorimetric Sulforhodamine B (SRB) assay according to Skehan et al. [16]. Cells were fixed (50% trichloroacetic acid (TCA), 50 µL/well, 1h, +4° C), washed 4 times with distilled water (Wellwash 4, Labsystem, Helsinki, Finland) and stained (0.4% SRB, $C_{27}H_{29}N_2O_7S_2Na$, 100 µL/well, 30 min, room temperature). The plates were then washed 4 times with 1% acetic acid to remove unbound dye. Protein-bound dye was extracted with 10 mM TRIS base (200 µL/well). Absorbance (A) was measured on a microplate reader (Multiscan Ascent, Labsystems, Helsinki, Finland) at 540/620 nm. The effect on cell growth was expressed as a percent of the control, and calculated as:

$(At/Ac) \times 100$ [%]

where: At= absorbance of the test sample

Ac= absorbance of the control

Statistical analysis

Data were expressed as mean ±SD of 2 experiments carried out in quadruplicate. Significant differences compared to control were determined using two-tailed Student's t-test. The significance levels were 95% ($p < 0.05$) or 99% ($p < 0.01$).

Results

Both cytotoxic (antiproliferative) and stimulatory (proliferative) effects were observed in Neuro-2a and MRC-5 cell line after 48 h of exposure to water samples (Figures 1,2).

In Neuro-2a cell line significant antiproliferative response was induced only in September samples from Ludos and Palic, generating 81.72% and 94.31% of cell growth, respectively (Figure 1). Of all May samples only a sample from Provala induced some antiproliferative effect but without significance. Significant stimulatory (proliferative) responses were observed in both examined time periods, the most pronounced being a September sample from Srpski Miletic inducing 109.98% of cell growth. A sample from Mrtva Tisa did not generate neither stimulatory (proliferative) nor cytotoxic (antiproliferative) response in both examined time periods.

Samples collected in May 2007 from Backo Gradiste and Krivaja induced some antiproliferative response but without significance. May samples from Ruski Krstur, Sombor, Srpski Miletic, Provala, Zobnatica, Palic, Ludos, Novi Knezevac and Srpski Itebej induced significant proliferation of MRC-5 cell line (Figure 2), the most pronounced (Zobnatica) reaching 113.17% of cell growth. On the other hand, samples collected in September 2007 did not show any proliferative effects on the examined cell line. The most pronounced antiproliferative effects were induced by samples from Ludos (68.94% of cell growth), Palic (82.45%), Krivaja (87.64%) and Zobnatica (88.74%). Samples from Mrtva Tisa and Becej did not generate neither proliferative nor antiproliferative response in both examined time periods.

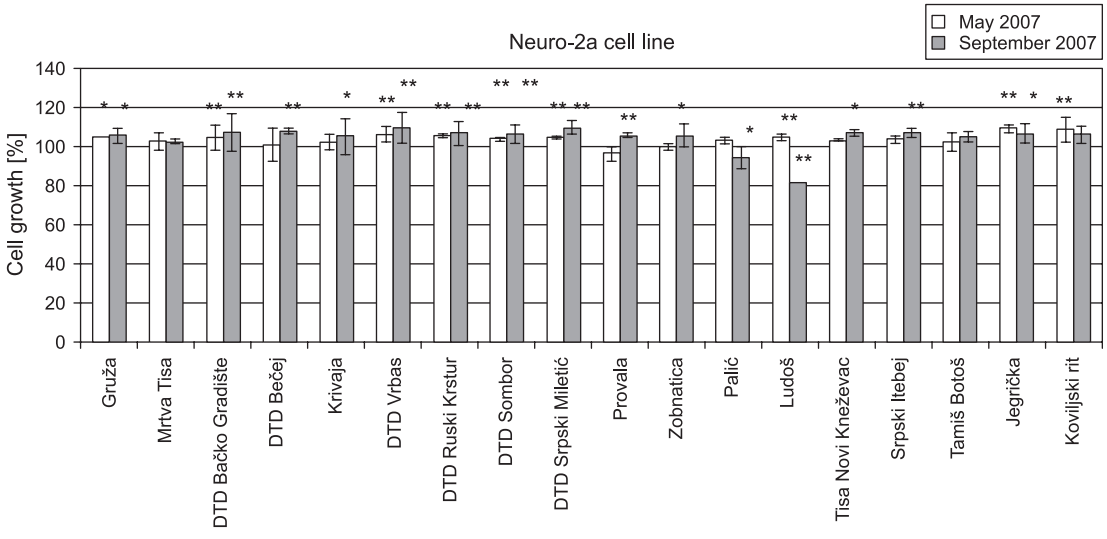

Figure 1. Cell growth activity of the tested water samples in Neuro-2a cell line. Data are the mean ± SD of 2 experiments, performed in quadruplicate (*$p < 0.05$, **$p < 0.01$; Student's t-test, significantly different from the control).

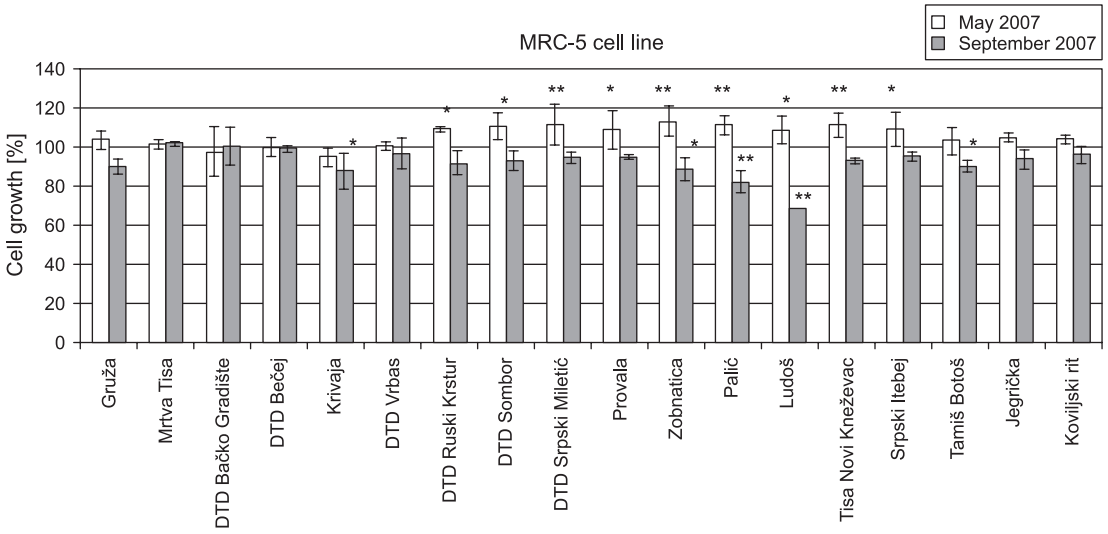

Figure 2. Cell growth activity of the tested water samples in MRC-5 cell line. Data are the mean ± SD of 2 experiments, performed in quadruplicate (*p < 0.05, **p < 0.01; Student's t-test, significantly different from the control).

Discussion

Considerable research efforts have been made to find suitable alternative methods to the mouse bioassay as a routine monitoring assay for cyanotoxins and many novel and sensitive methods have become available in recent years. Different methods provide often complementary information, sensitivity and selectivity being important criteria for their selection. However, no single method is currently available to replace the mouse bioassay for the detection of all cyanotoxins using a single assay, and further validation and comparison of methods is needed [8]. It is also crucial to test different bioassays with regard to their suitability for locally-prevalent cyanotoxins.

The growth inhibition activity of water samples collected from 18 localities of Serbia in spring and autumn 2007 was evaluated using *in vitro* toxicity assay in Neuro-2a and MRC-5 cell line, after 48 h of exposure time. The results presented herein were obtained by assessing cell growth with the SRB colorimetric assay. SRB dye binds to protein basic amino acid residues providing a sensitive index of cellular protein content [16,17].

The results of our work showed that Neuro-2a and MRC-5 cells are sensitive for the detection of cytotoxins even to the diluted water samples (cells were treated with water samples at a final concentration of 10% - 20 µL of water sample were added to 180 µL RPMI 1640 or DMEM).

The results of our *in vitro* toxicity study using Neuro-2a cell line suggest that September samples from Ludos and Palic are toxic. Palic is a big tourist center, with its lake being used for various recreational purposes (swimming and particularly sailing). It is clear that the monitoring of cyanobacteria and cyanotoxins is obligatory due to well known risks on human health after the inhalation of neurotoxins.

Neuroblastoma cells in culture were used to detect sodium channel-specific marine toxins based on an end-point determination of mitochondrial dehydrogenase activity. The assay responds in a dose-dependent manner to ciguatoxins, brevetoxins, and saxitoxins, and delineates the toxic activity as either sodium channel enhancing or sodium channel blocking. The assay responds rapidly to sodium channel activating toxins, allowing dose dependent detection in 4-6 h. Brevetoxins can be detected at 250 pg, and purified ciguatoxins are detected in the low picogram and subpicogram levels. Sodium channel blocking toxins can also be detected with an approximate sensitivity of 20 pg in 24-48 h [18]. This technique is simple and sensitive and demonstrates potential as an alternative to animal testing for sodium channel activating and blocking toxins [11,12,19]. Also, the results obtained from cell bioassay of ciguatoxic finfish extracts correlated with those obtained from mouse bioassays [18]. It can be assumed that the toxicity of the examined water samples is owed to neurotoxic cyanotoxins present, but more studies are needed for the confirmation of these results.

Our *in vitro* toxicity study using MRC-5 cell line suggests that September samples from Ludos, Palic,

Krivaja and Zobnatica are toxic. Certain human diploid fibroblast (HDF) cell lines such as MRC-5 have been validated as safe cell substrates for the manufacture of vaccines and have led the way for acceptance of animal cell lines for the production of recombinant therapeutic proteins. HDFs are capable of multiple passages and may achieve more than 70 population doublings prior the "Hayflick limit" (maximum passage limit at which division ceases). HDFs are remarkably stable and retain the predominantly diploid normal karyotype of the original tissue cells [20]. It can be assumed that the toxicity of the examined water samples is owed to cytotoxic cyanotoxins present. More studies are needed for the confirmation of these results.

Analytical methods such as gas chromatography-mass spectrometry or high pressure liquid chromatography-mass spectrometry have been developed for the identification and characterization of already recognized toxic constituents in water. Although these studies have a high sensitivity offering very good information, they are often powerless, particularly in the case of unknown substances, incidental contaminants or degradation products. Often, chemical analysis requires concentration procedures of the samples to be tested. Even then the toxicity of different compounds is not comparable at the same concentration. Therefore, it is essential to rapidly, sensitively and reliably screen the effects of toxic compounds in water instead of analyzing large numbers of compounds, which could be time and cost consuming, particularly if the chemical controlling the toxicity is unknown.

In our study distinct responses depending on the time (season) of collecting the water samples were detected. The majority of May samples generated proliferative response in MRC-5 cell line, while large number of samples from both examined time periods generated proliferative response in Neuro-2a cell line. September samples proved to be more toxic than samples collected in May and also showed no stimulatory effect in MRC-5 cell line.

There is evidence that biologically active compounds, including cyanotoxins, are produced during secondary metabolism of cyanobacteria [21,22]. In our ecological studies regarding the same samples and localities [23] the mass growth and even blooms of cyanobacteria species were detected in the majority of samples that exhibited toxic activity on the examined cell lines. On the contrary, in the water bodies with lower cyanobacterial cell growth, where primary metabolism is firmly established, the production of primary metabolites like vitamins might be the reason for the proliferation of the examined cell lines.

The ability of microcystin to act as a tumor initiator requires it to induce damage at the DNA level in addition to inhibiting the PPs to promote cellular proliferation. Baby Hamster kidney cells (BHK-21) and mouse embryo fibroblast (MEF) cells exposed to microcystin-containing extract or pure microcystin exhibited severe fragmentation of DNA at the higher dose levels of 100 μg/ml [24]. A microcystin-containing extract induced DNA damage in hepatocytes and showed strong mutagenicity in the AMES test [25]. Microcystin-LR induced DNA strand breaks in the human hepatoma cell line HEPG2 occurring in a time- and dose-dependent manner [26]. It is reasonable to suppose that the presence of microcystins, and their proliferative action, in the September samples may be the cause for the proliferative response in Neuro-2a cell line.

Different cell lines can be used for the uncovering of toxins that can not be detected with various analytical methods that use standards. In that way analytical methods confirm the presence of known toxins with the use of the standard, whereas cell lines can detect the presence of unknown toxins. Nevertheless, it is necessary to confirm, compare and correlate results obtained on cell lines with analytical methods and evaluate their strengths.

Humans can be exposed to cyanotoxins orally *via* drinking water, dermally *via* recreational use of lakes and rivers [27] or after inhalation exposure from the use of showers [28]. Although there were no response in the tested cell lines affected by samples from Gruza (the only reservoir for water supply), further analysis on hepatocytes would be strongly advisable because of the role of microcystin as a tumor promoting factor and its cumulative effect in human body caused by intake of microcystin-contaminated drinking water.

In conclusion, our preliminary research confirmed that Neuro-2a and MRC-5 cell lines have responded to the presence of secondary metabolites of cyanobacteria. Cytotoxic effects were detected in samples from lakes (Ludos and Palic), reservoirs (Zobnatica) and rivers (Krivaja).

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