Cyanotoxins - new health risk factor in Serbia

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SUMMARY

Cyanobacteria are responsible for poisoning numerous cases of livestock and other animals, but sometimes they induce serious problems in humans. Mass occurrence and blooms of cyanobacteria were studied in rivers, canals, lakes, and accumulations that had been examined in Serbia during period 1980-2002. Because of massive blooming of cyanobacteria, when they produce cyanotoxins, which could be responsible for poisoning, and damage of organs, studies are steered to better understanding cyanotoxins effects on human health. The investigation on microcystins, as one of many cyanotoxins, is increasing due to the related ecological and public health risks. There is evidence that microcystins have a tumor promoting activity and microcystin-LR could be tumor initiator and possibly cancerogenic to humans. Epidemiological studies have suggested that there is connection between primary liver cancer (PLC) and microcystins, as one of risk factor for high incidence rate of PLC in Serbia.

Key words: *Cyanobacteria; Bacterial Toxins; Poisoning; Neoplasms; Risk Factors; Microcystins; Non MeSH Serbia*

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Abbreviations:
PLC, primary liver cancer; OATP, organic anion transport polypeptide; CRC, colorectal carcinoma; Mc-LR, microcystin-LR; PP1, protein phosphatase; PP2A, protein phosphatase 2A; TNF-α, tumor necrosis factor alpha; ROS, reactive oxygen species; CaCo-2, human colon adenocarcinoma cell line; NCNC, B human lymphoblastoid cell line; IPDDC-2A, human astrocytoma cell line; TBARS, thiobarbituric acid reactive substances; MAPK, mitogen activate protein kinase; DAG, diacylglycerol; PLC, phospholipase-C; PKC, protein kinase-C

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INTRODUCTION

Cyanobacteria (blue-green algae) are diverse group of prokaryotes. They appeared some 2.5 billion years ago, and they are common inhabitants of soil, rivers, freshwater lakes, and reservoirs throughout the world. Under favorable conditions, certain cyanobacteria form nuisance "blooms" (1). During the "blooming," some cyanobacteria produce toxins, called cyanotoxins, which are products of their secondary metabolism. In addition, cyanobacteria are known to produce a great variety of bioactive substances (antibacterial, antifungal, insecticidal, antialgal, herbicidal, etc.) (2) (Figure 1a, b).

Figure 1a,b. Cyanobacterial "blooming" in Serbia

The known toxin-producing genera are *Anabaena, Aphanizomenon, Cylindrospermopsis, Cylindrospermum, Lyngbya, Oscillatoria, Planktothrix, Anabaenopsis, Microcystis, Hapalosiphon, Nodularia, Nostoc, Raphidiopsis, and Umezakia* (3). Numerous studies have shown that cyanotoxins could have negative effects on animal and human health, and there are many of epidemiological data and laboratory reports of animal and human poisoning with lethal consequences. Likewise, there are many reports of cyanobacterial blooming in Serbia (4,5). Globally, the most frequently found cyanobacterial toxins in blooms from fresh and brackish waters are the cyclic peptide toxins of the *microcystin* and *nodularin* family (3). The investigation on microcystin topics is increasing due to the related ecological and public health risks. Human exposure to *microcystins* may occur through a direct route such as drinking water, recreational water, or hemodialysis, or through an indirect route such as food (6).

In regard to their chemical structures, cyanotoxins are divided on cyclic peptides, alkaloids, and lipopolysaharides (LPS). According to their mode of action, *cyanotoxins* are classified into hepatotoxins, neurotoxins, skin irritants, and other. Group of hepatotoxins include *microcystins, nodularins* and *cylindrospermopsin*. Neurotoxins are *anatoxin-a, homoanatoxin-a, anatoxin-a(s), saxitoxins (paralytic selfish poisons)*. Contact irritant- dermal toxins: *debromoaplysiatoxin, lungbyatoxin* and *aplysiatoxin.*

Other toxins include *mikroviridin J* and β-*N-metilamino-L-alanin* (3,7).

Cases of acute intoxication by cyanobacteria and other effects on human are known for a long time ago, in USA (1931, 1968, 1975, 1985), Australia (1979, 1981, 1995), China (1993), England (1989), Sweden (1994) and Brazil (1996) (8). It was found a correlation between the occurrence and the severity of symptoms such as diarrhea, vomiting, skin rashes, fevers, or ear irritations and the duration of water contact and cyanobacterial cell density. That epidemiological study was carried out with 852 participants in Australia in 1995. Allergic and toxic reactions have been observed after swimming in several rivers and lakes around Berlin, Germany. Reports from Portugal and USA about pyrogenic reactions after dialysis also mentioned cyanobacteria in the water source before treatment (9). One of the most important is Caruaru syndrome in

Brazil, where was used water for hemodyalisis containing cyanotoxins. In 116 out of 130 patients (89 %) at a dialysis center in Caruaru, visual disturbances, nausea, and vomiting associated with hemodialysis was found. Fifty-six patients died and after autopsy in 16 patients, histopathological picture of liver tissue was uniform: liver cell deformity, extensive necrosis, severe cholestasis, mixed-leukocyte infiltration. Electron microscopy showed intracellular edema, mitochondrial changes, and injuries to the rough and smooth endoplasmic reticule, lipid vacuoles, and residual bodies. Concentration of *microcystin* in water used for hemodyalisis was 19.5μg/l, which is about 20 times more than recommendation secure of microcystin dose of 1μg/l/day by WHO (10).

Animal studies showed that acute intoxication with microcystins causes irreversible hepatotoxic effects, and could at non-lethal exposures, damage other organs, such as intestine, kidney, lung, and brain (11). Many authors accept that hypovolemia and circulatory collapse are probably the cause of early death from microcystin poisoning (12).

MICROCYSTINS

The first chemical structures of cyanobacterial cyclic peptide toxins found in freshwater cyanobacteria were cyclic heptapeptides (that is they contain seven peptide-linked amino acids) with the general structure: cyclo-(D-alanine^1-X^2-D-MeAsp^3-Z^4-Adda^5-D-glutamate^6-Mdha^7) in which X and Z are variable L amino acids, D-MeAsp^3 is *D-erythro*-β-methylaspartic acid, and Mdha is *N*-methyldehydroalanine (Figure 2). The amino acid Adda, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, is the most unusual structure in this group of cyanobacterial cyclic peptide toxins. These compounds were first isolated from the cyanobacterium *Microcystis aeruginosa* and therefore the toxins were named microcystins (13).

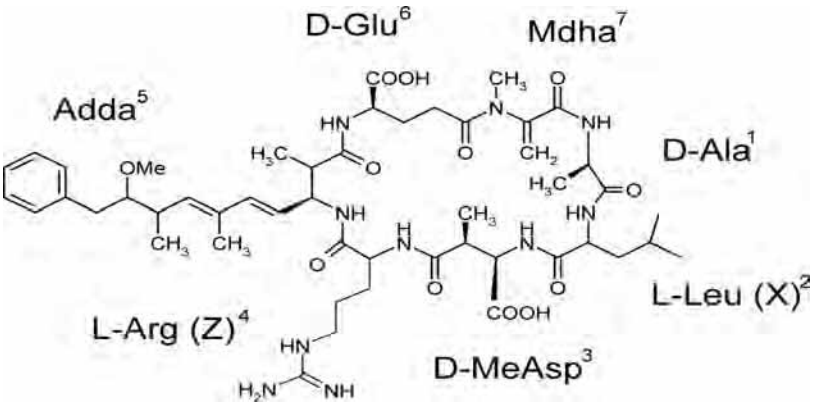

Figure 2. Chemical structure of microcystin-LR (14)

Microcystin-LR cannot readily cross cell membranes, and hence does not enter most tissues. After oral uptake, it is transported across the ileum (3). Small intestine is the main route of microcystin absorption and a large amount of toxin is contained in villi inducing apex erosion (11). In human, after absorption, microcystin penetrate the hepatocyte membrane via two routes: by bile acid transportation system (15) and by the members of the organic anion transporting polypeptide superfamily OATP (OATP1B1, OATP1B3, OATP1B2) (16,17). In addition, the organic anion transporters are expressed in gastrointestinal tract, kidney, and brain, as components of the blood-brain barrier (11) and nine OATPs have been identified in humans (18).

Studies on laboratory animals, employing radiolabeled microcystins have demonstrated that the liver is the main site of toxin accumulation (after oral, intraperitoneal, or intravenous application). Microcystins covalently bind and irreversibly inhibit activity of serin/threonine protein phosphatases, PP1 and PP2A that result in hyperphosphorylation of many cellular proteins in acute exposure. Inhibition of PP1 and PP2A is principally mediated by Adda group of microcystins, although most microcystin variants contain dehydroalanine, which can covalently link to cysteinyl sulphur on the phosphatase. Protein phosphorylation status plays a critical role in cellular signaling and hence all aspects of cellular function (19).

It is evident that the liver plays important role in the detoxification of microcystins. Detoxification products were found in urine, faces and liver cytosol fractions (3). The accepted pathway for microcystin excretion is conjugation to glutathione (GSH). GSH competes for binding at the N-methyl dehydroalanine to form Mc-GSH conjugate of (19). Glutathione is a tripeptide consisting of glutamic acid, cysteine, and glycine and this compound is very important part of the defense mechanism against toxic material, such as some drugs and carcinogens. If the potentially toxic substances were not conjugated to GSH, they would be free to combine covalently with DNA, RNA, or cell protein and could thus lead to serious cell damage. A number of potentially carcinogens are conjugated to the nucleophilic GSH and enzymes and reaction is catalyzed by glutathione S transferases, which are present in high amounts in liver cytosol and in lower amounts in other tissues (20).

Although Mc excretion in mammals has been proposed to be mainly intestinal, the chemical nature of excreted Mc has not been determined. Experimental studies are needed to determine and quantify the metabolism and excretion of Mc and metabolites (19).

MECHANISMS OF CELL TOXICITY AND TUMOR PROMOTING ACTIVITY OF MICROCYSTIN-LR

Inhibition of PP1 and PP2A and phosphorylation of intermediate filaments, cytokeratins 8 and 18, (3) and other intracellular proteins, by Mc-LR lead to cytoskeletal deformation, collapse and aggregation of both cytokeratins and actin filaments around nucleus in liver cells that resulting in loss of cell-cell contact and forming bleb formation and cellular scaffold. That is a consequence of reactive oxygen species generation (ROS), resulting in alteration of mitochondrial membrane permeability, cytochrome-c release, activation of caspase 3 via caspase 9, releasing of calcium ions, and finally lead to apoptosis of hepatocytes (19,21,22). Exposure to single low doses of Mc-LR (50 μg/kg) for 24 hours results in bid-bax-bcl-2 mediated apoptosis. Acute exposure to Mc-LR increase level of ceramide, which are the second best indicator of diverse stress stimuli, suggesting that exposure to Mc-LR results in oxidative stress. While sphingolipids were increased as a result of oxidative stress caused by Mc-LR exposure, the levels of Bcl2 decreased in a dose dependent manner leading to apoptosis (23). High doses of Mc-LR (70 μg/kg) caused apoptosis via a reactive oxygen species (ROS) pathway (17) and causes rapid *in vitro* apoptosis within 2 minutes (16 mM/L) to 30 minutes (1 mM/L) mediated by ROS production and inhibition PP1 and PP2A, activation by caspase 3-dependent apoptosis. Also, caspase 3-depended apoptosis could occur in combination with up-regulation of TNF- α *in vivo* (24).

Mc-LR toxicity is mediated by immunosuppression of host system (23). It is found that Mc-LR induces production of pro-inflammatory cytokines TNF-α

and IL-1β *in vitro*, which can mediate tissue destruction and lethality. The principal cytokine known to injure the liver and lung is TNF-α. Kupffer cell in the liver are responsible for releasing that cytokines which may accelerate liver damage and death (12). Also, it is found that TNF-α induced damage of endothelial and neighboring parenchymal cells and inducts a response that may be linked to the production of ROS (24). Also it is known that microcystin in non-toxic concentration could induce DNA damage. It was observed in human hepatoma cells HepG2, that dose of 1 μg/ml of *microcystin* could induce dose and time dependent DNA strand breaks reaching the maximum level after 4 hours after exposure (25). Exposure of CaCo-2 (human colon adenocarcinoma cell line) to non-cytotoxic doses (0.2, 1 and 5 μg/ml) of Mc-LR induced increasing of intracellular ROS (reactive oxygen species) formation after 30 min, and increase dose and time dependent DNA strand breaks, which is approved by comet assay. Žegura et al. (2008) (11) investigated different sensitivity of B human lymphoblastoid NCNC cell line as model for lymphocytes, human astrocytoma cell IPDDC-2A as model for glia cell line to exposure of Mc-LR. It was shown that Mc-LR did not affect NCNC cells, but induced ROS formation in IPDDC-2A cells. Sicińska at al.(2006) (26) shown that microcystin-LR in concentration of 100 nM, which is minimal toxic dose, could change antioxidative enzymes activities: catalase, superoxide dismutase and glutathione reductase and formation of reactive oxygen species (ROS) in human erytrocytes and that this compound induces increase of the level of thiobarbituric acid reactive substances (TBARS), formation of echinocytes, hemolysis, conversion of oxyhaemoglobin to methaemoglobin, and decrease of membrane fluidity on the level of 16 carbon atom fat acids. It has been shown that the central nervous system (CNS) can also be a target for Mc-LR toxicity (11), where OATP1A2 is found to mediate Mc-LR transport to the blood-brain barrier (23). Maidana et al. (2006) (27) had shown that *in vivo,* Mc-LR induced oxidative stress in rat hippocampal cell and affected the long term and spatial memory. Susceptibility of CNS to oxidative stress is because of its low capacity for antioxidant defense (11).

In vitro and in *vivo* studies, provide evidences that Mc-LR is mutagenic and tumor promoter (28), and that microcystins inhibit DNA repair (19). Studies from China give evidence of a tumor promoting activity of Mc even in concentrations of 0.1 μg/L (9).

Chronic exposures to low concentrations of microcystin contribute to the increased the risk for primary liver and colon cancer development (19, 25). Humpage et al. (2000) (29) reported that microcystins in drinking water stimulated the growth of aberrant crypt foci in the mouse colon (11). Low doses and longer periods of exposure to microcystin affect cell cycle control by suppressing apoptosis and promoting cell division in polyploid hepatocytes *in vitro* by activated MAPK pathway (19). MAPK cascade activate PLC (phospholipase-C) which brakes phosphotidyl ionsitol phosphate (PIP) into inositol phosphate-3 (IP3) and diacylglycerol (DAG). DAG activates PKC (protein kinase- C) which is responsible for activation MAPK cascade that involves ERK1/2, MEK, p38, RAF-1. DAG pathway could be responsible for activation Bcl-2, PKC, which result in tumor promotion, cell survival and proliferation in long-term exposure to microcystin.(23). Active MAP kinases stimulate the synthesis and phosphorylation of transcription factors, such as FOS and JUN. The transcription factors activated by these various signaling cascades in turn stimulate the production of growth factors, receptors for growth factors, and proteins that directly control the entry of cells into the cell cycle (30). Oncogene of *jun* family has a significant role in control of gene expression especially with *fos* oncogene, which encodes nuclear oncoproteines. After regulatory and structural changes, these products of oncogene lead to malignant transformation (31).

Svirčev et al. (32) suggested connection between cyanobacterial blooms in reservoirs for drinking water supply and high incidence rate of PLC in humans in Central Serbia. Incidence of PLC mortality rate, in Central Serbia was 11.6 in period 1980-1990. The PLC incidence was significantly increased (34.7) in 2000 - 2002 in Šumadija, Niš and Toplice (regions affected by heavy cyanobacterial "blooms") while in the rest of Central Serbia incidence was 13.6. Also, epidemiological studies suggested that microcystins are one of the risk factors for the high incidence of primary liver cancer (PLC) in certain areas of China, where people have consumed pond-ditch water contaminated with blue-green algae (33, 34). Also, the presence of aflatoxin B1 and Hepatitis B virus in drinking water could explain the higher rate of observed PLC in China and that combined effects are responsible for endemicity of PLC. In some rural areas of China, contamination of drinking water by microcystin-LR, is consider to be an important external factor in the carcinogenesis of CRC (colorectal carcinoma) (35).

CONCLUSION

This paper adverts that there is evidence of liver injury in people who drink water contaminated by cyanobacterial toxins and evidence that microcystins act as tumor promoter in animals.

After acute intaperitoneal or intravenous injection of microcystins severe liver damage, intrhepatic hemorrhage, heart failure and death were seen in animals. Taking into account that microcystin-LR is mutagenic, this compound could possibly be carcinogenic to humans. Having in mind that PLC incidence could be connected with drinking water quality and that PLC high risk regions in Serbia correspond with cyanobacterial blooming, cyanotoxins as a risk factor should be considered as potential cause of PLC in future.

Conflict of interest

We declare no conflicts of interest.

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