Histopathology of Carp (*Cyprinus carpio* L.) Larvae Exposed to Cyanobacteria Extract

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Abstract

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The aim of this study was to examine histological changes of tissues of carp embryos and larvae exposed for a short-term (8 days) or a long-term (30 days) to the crude extract of cyanobacteria with the cumulative concentration of 130.0 $\mu g \cdot l^{-1}$ (high concentration of the extract), 13.0 $\mu g \cdot l^{-1}$ (medium concentration of the extract) and 1.3 $\mu g \cdot l^{-1}$ (low concentration of the extract) of microcystins LR, RR and YR. The concentration of 130.0 $\mu g \cdot l^{-1}$ was used only for the short-term exposure. Tissue sections were stained with haematoxylin-eosin and PAS and examined using light microscopy. Apoptotic cells were detected by TUNEL test. Changes of liver and kidney, in particular, were examined. No histopathological changes were found in control or experimental groups after the short-term exposure except for non-resorbed yolk sacks in the group exposed to the high concentration of the extract. The development had been retarded in this group. Vacuolar dystrophy of hepatocytes accompanied by damage of nuclei (pyknosis, karyolysis) were found after the long-term exposure in the group exposed to the low concentration of the extract. Focal necroses and dystrophic changes of hepatocytes with vacuolization and nuclei damage (pyknosis, karyolysis, hyperchromatosis, karyorrhexis) were found in the group exposed to the medium concentration of the extract for a long term. Apoptotic cells were detected in the liver. No changes were found in the kidney. The results documented the damage of liver tissue in larval stages of carp exposed to the crude extract of cyanobacteria in early life stages of development. The degree of damage depended on the concentration of the extract.

Microcystins, embryo-larval test, liver damage, fish, apoptotic cells

Cyanobacteria are a common and natural component of most water ecosystems. Mass development of cyanobacteria closely correlates with eutrophication of waters. The development of cyanobacterial water blooms decreases water quality from viewpoint of water management, hygiene and fishery.

Cyanobacteria can produce and incidentally release in to their environment substances having a biological activity such as enzymes, vitamins, toxins, extracellular polysaccharides, attractants, amino acids and other organic acids, antibiotics and hormones. These substances can influence growth and development of other water organisms. Toxins of the blue-green algae may be divided according to various points of view. Carmichael (1992) divides the cyanotoxins according to methods of detection into cytotoxins and biotoxins. The biotoxins may be classified according to their biological activities as neurotoxins, hepatotoxins, cytotoxins, genotoxins, immunotoxins and embryotoxins. Population of a single species of blue-green algae may produce several toxins (Maršálek and Turánek 1996). Hepatotoxins are most common and frequently involved in acute toxicoses. They damage the structure and function of liver. They are structurally cyclic and

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heat-resistant peptides. Well known hepatotoxins are microcystins, microviridins, nodularin and cylindrospermopsin (Maršálek and Turánek 1996). Microcystin LR is the most common and most often studied hepatotoxin. The mechanism of its influence is on cellular level (Eriksson 1990). More than 60 congeners of microcystins are known at present (Fischer et al. 2001). Microcystins are cyclic heptapeptides. They have hepatotoxic effect. Many authors examined the histopathological findings and the mechanism of influence of microcystins. Deformation of hepatocytes is the most pronounced effect (Runnegar and Falconer 1986; Falconer and Yeung 1992). Falconer and Yeung (1992) concluded that the mechanism of microcystin toxicity to the hepatocyte is through cytoskeletal damage leading to loss of cell morphology, cell to cell adhesion and finally cellular necrosis. This structural damage manifests as intrahepatal haemorrhages (Falconer et al. 1983). Liver insufficiency develops in long-term surviving individuals (Beasley et al. 1989). The organotropism of microcystin LR manifests clearly on the cellular level and is specific for hepatocytes. Toxicity of microcystin LR *in vivo* primarily consists in the hepatocellular deformation inducing degenerative changes of the tissue (Eriksson et al. 1987, 1988ab, 1989).

Recently, research into this area has also been aimed at the evaluation of effects of cyanotoxins to the early life stages of organisms. The effect of microcystins and the crude extract of cyanobacteria on the development of fish and amphibians were studied (Oberemm et al. 1997, 1999; Wiegand et al. 1999) without description of histopathology of fish embryos and larvae. Ultrastructural changes in hepatocytes of post-hatching loach larvae after exposure to microcystin LR were described by Liu et al. (2002). These authors described deformation of nucleus, moving of nuclei to the side of nuclear membrane, convolution of the nuclear membrane, vesiculation of the rough endoplasmic reticulum, reduction and transformation of RER into concentric membrane whorls. Ultrastructural alteration also occurred in the heart – fibrillation of the heart muscle and deformation of erythrocytes.

The level of dissolved microcystins in the Czech Republic measured in drinking water reservoirs, recreational reservoirs and fish pond with *Microcystis ichthyoblabe* dominance amounted to 0-45 $\mu g \cdot l^{-1}$, 0-180 $\mu g \cdot l^{-1}$ and 225 $\mu g \cdot l^{-1}$, respectively (Bláha and Maršálek 2001). The concentrations of microcystins in the cyanobacterial biomass from Czech water bodies vary from 0 to 4450 $\mu g \cdot g^{-1}$ of dry weight (Maršálek et al. 2001).

Histopathology of carp larvae exposed to the crude extract of cyanobacteria after embryonal (short-term) and embryo-larval (long-term) tests are presented in this study. The concentrations of microcystins were chosen by comparison with literature and with the level of dissolved microcystins in natural waters in the Czech Republic.

Materials and Methods

The carp eggs were obtained by stripping at the fishery in Oslavany (Czech Republic). Fertilised and unsticked carp eggs were divided into eight groups, each containing two hundred eggs. The eggs were incubated in glass vials containing 0.5 l of water. The bath was changed every 8 hours. The conditions in baths were following: water temperature 21.5 - 22.5 °C, dissolved oxygen 65 - 113% (i.e., 5.5 - 10.1 $mg \cdot l^{-1}$) and pH 7.9 - 8.9.

The larvae were been fed by nauplii of the brine shrimp *Artemia salina* (commercially delivered as Artemia PREMIUM) since the 5th day. Feeding was performed 20-30 minutes before every bath change.

The tests were performed with the crude cell extract obtained from terrain samples of water bloom (Brno reservoir, Czech Republic). The sample contained the planktonic species of *M. aeruginosa* (85%), *Microcystis ichthyoblabe* (5%) and *Aphanizomenon flos-aquae* (3%). The sample was collected from the surface water bloom (0 to 0.3m depth) and concentrated by a plankton net of 22 µm. The sample was stored frozen at –20 °C. The concentration of microcystins was determined by HPLC according to the method described by Lawton et al. (1994). The total microcystin concentration (MC) was 1129 $\mu g \cdot g^{-1}$ of dry weight in the biomass. To obtain the crude extract, the material was ultrasonicated for 7 minutes and centrifuged for 20 minutes at 5000 rpm. Re-extraction was done twice by standard water. The final concentration of hepatotoxic microcystins in the crude extract used for the exposure was 15.7 $\mu g \cdot l^{-1}$ (9.6 $\mu g \cdot l^{-1}$ of microcystin YR, 6.0 $\mu g \cdot l^{-1}$ of microcystin LR, 0.1 $\mu g \cdot l^{-1}$ of microcystin RR). The

amount of the biomass was 22.1 $\mu g \cdot g^{-1}$ of dry weight. These biomass concentrations commonly occur in the Brno reservoir (Czech Republic).

The number of eggs in each group was 200. The crude extract of cyanobacteria was added to the water at three concentrations: 0.5 $\mu g \cdot l^{-1}$ of microcystin LR (low concentration of the extract), 5.0 $\mu g \cdot l^{-1}$ of microcystin LR (medium concentration of the extract) and 50.0 $\mu g \cdot l^{-1}$ of microcystin LR (high concentration of the extract). Control eggs were incubated in toxin-free water. The cumulative concentration of microcystins was 1.3, 13.0 and 130.0 $\mu g \cdot l^{-1}$, respectively. The tests were finished after 8 days (short-term exposure) and after 30 days (long-term exposure). The tests experiments with high concentration were finished after 8 days because very high mortality of embryos.

Five fish from each group were killed, immediately fixed in Bodian solution and processed using standard methods for histology. Tissue sections (5 μm) were stained with haematoxylin-eosin and PAS. Apoptotic cells were detected with TUNEL test. All sections were examined using light microscopy. Liver and kidney tissues were examined, in particular.

Results

Tests with short-term exposure

No histopathological changes were found both in fish from control and experimental groups except for non-resorbed yolk sacks in the larvae from group exposed to the high concentration of the extract. It means that the development has been retarded in this group (Plate I, Fig. 1).

Tests with long-term exposure

No changes in liver and kidney were found in the control group. Vacuolar dystrophy of hepatocytes (Plate I, Fig. 2) with damage of nuclei (pyknosis, karyolysis) was found in the group exposed to low concentration of the extract. These changes were found in all sampled larvae. No changes were found in the kidney.

Focal necroses (mainly perivascular) and dystrophic changes of hepatocytes with vacuolisation and nucleic damage (pyknosis, karyolysis, hyperchromatosis, karyorrhexis) were found in the group exposed to the medium concentration of the extract (Plate II, Fig. 3). These changes were found in all sampled larvae. Using TUNEL test for detection of apoptosis apoptotic cells were detected in the liver, mainly in perivascular and interstitial liver tissue (Plate II, Fig. 4). No changes were found in the kidney.

Discussion

High mortality and retarded development in tests with short-term exposure may be due to encreased energy demand of detoxication processes, as described by Wiegand et al. (1999). The histopathological changes of liver in our study were similar to the changes described in various papers in young and adult fish. Rodger et al. (1994) described the histopathological changes of brown trout (*Salmo trutta*) associated with the death of water blooms of *Anabaena flos-aquae*. The changes in liver were characterised by confluent necrosis showing cellular degeneration and loss of obvious cell boundaries. Pyknosis and karyorrhexis of hepatocytes was obvious. Similar changes in liver have been described in different fish species by other authors, e.g. Garcia (1989), Råbergh et al. (1991), Tencalla et al. (1994). Råbergh et al. (1991) described degeneration of kidney tubuli after intraperitoneal application of the lethal dosis of microcystin LR to the carp. Carbis et al. (1996) detected histopathological changes in the gills, in liver and kidney of carp exposed to microcystins by gavage, immersion and intraperitoneal administration. Intraperitoneal inoculation caused necrosis or dose-depended degeneration. Gavaging caused changes in the histopathology of the liver and gills. Cellular degeneration and necrosis occurred in the liver, gills and kidneys when carp were introduced to a tank containing 1.7 $\mu g \cdot ml^{-1}$ of microcystins. Carbis et al. (1997) studied carps exposed to *Microcystis aeruginosa* at Lake Mokoan (Australia). The total concentration of the microcystins was approximately 4.0 $\mu g \cdot g^{-1}$ of the lyophilised scum material. During

February, March and April the liver histology was characterised by cytoskeletal collapse, cytoplasmic vacuolization, pyknosis, chromatin margination, eosinophilia and widespread hepatocyte atrophy, particularly in areas close to the arterial blood supply in about 66% of the carp examined. During February and March gill samples were characterised by necrosis, folder lamellar tips and mild epithelial ballooning in about 30% of the carp examined. Fischer et al. (2000) indicate that hepatocyte necrosis represents primary events in microcystin induced hepatotoxicity in the rainbow trout and that apoptotic cell death seems to be of only secondary nature. Fischer and Dietrich (2000) suggest that, in comparison to the pathological events in salmonids exposed to microcystin, in which a slower development of pathology and primarily necrotic cell death prevails, the pathology in the carp develops rapidly and at lower toxin concentrations. According to them, this is most likely due to a more efficient uptake of toxins, while the mechanism of cell death is primarily apoptosis.

We detected damage of liver in in fish from tests with long term exposure. The degree of damage depended on the concentration of the extract. We did not observe any damage of kidney.

Histopatologie larev kapra (*Cyprinus carpio* L.) vystavených extraktu sinic

Cílem práce bylo zjistit histologické změny kapřích embryí a larev vystavených krátkodobě (8 dní) a dlouhodobě (30 dní) hrubému extraktu cyanobakterií s kumulativní koncentrací 130.0 $\mu g \cdot l^{-1}$ (vysoká koncentrace extraktu) 13.0 $\mu g \cdot l^{-1}$ (střední koncentrace extraktu) a 1.3 $\mu g \cdot l^{-1}$ (nízká koncentrace extraktu)) mikrocystinů LR, RR a YR. Koncentraci 130.0 $\mu g \cdot l^{-1}$ jsme použili pouze krátkodobě. Tkáňové řezy jsme barvili hematoxylin-eosinem a barvením PAS a vyhodnotili pomocí světelné mikroskopie. Apoptotické buňky byly detekovány TUNEL testem. Zejména jsme sledovali jaterní a ledvinnou tkáň. Nenalezli jsme žádné histologické změny u kontrolních a pokusných skupin po krátkodobé expozici, s výjimkou nevstřebaného žloutkového váčku ve skupině s vysokou koncentrací extraktu. Tento nález naznačuje retardovaný vývoj v této skupině. Ve skupině s nízkou koncentrací extraktu a dlouhou expozicí jsme zjistili vakuolární dystrofii hepatocytů s poškozením jader (pyknóza, karyolýza). Fokální nekrózy a dystrofické změny hepatocytů s vakuolizací a poškozením jader (pyknóza, karyolýza, hyperchromatóza, karyorexe) jsme zjistili po dlouhodobé expozici ve skupině vystavené střední koncentraci extraktu. V jaterní tkáni jsme zaznamenali přítomnost apoptotických buněk. Nenalezli jsme žádné změny v ledvinné tkáni. Ze sledování vyplývá, že jsme zjistili postižení jater u larvárních stádií kapra obecného vystaveného působení extraktu cyanobakterií v ranných fázích vývoje. Stupeň poškození závisel na koncentraci extraktu.

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Plate I
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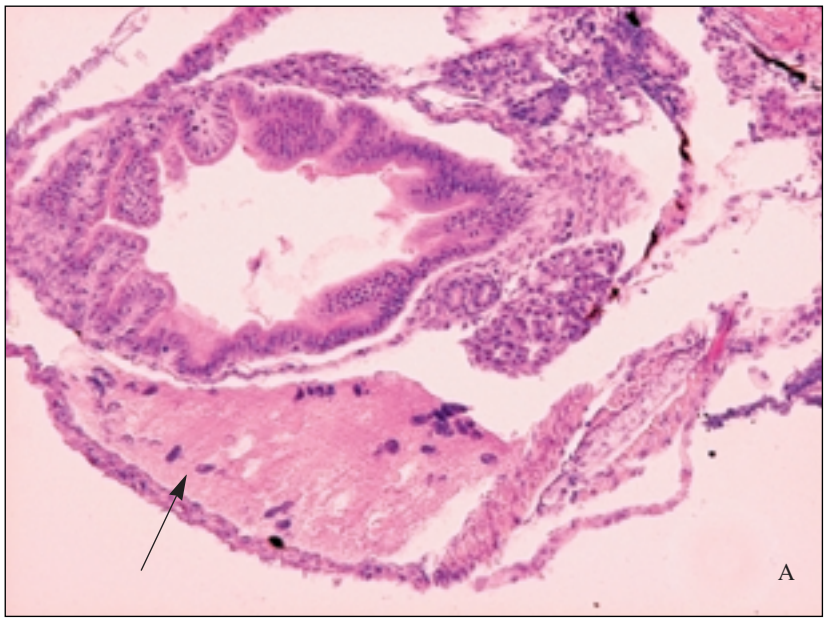


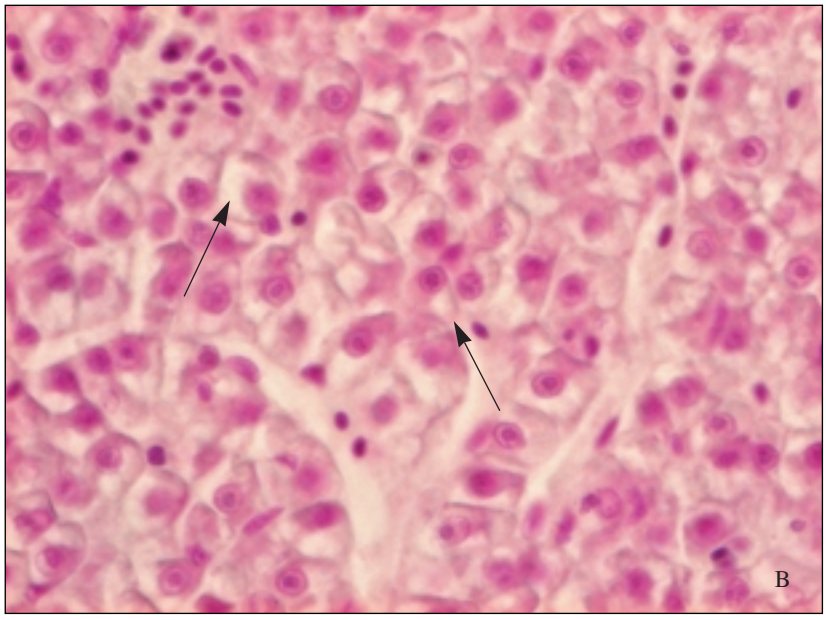
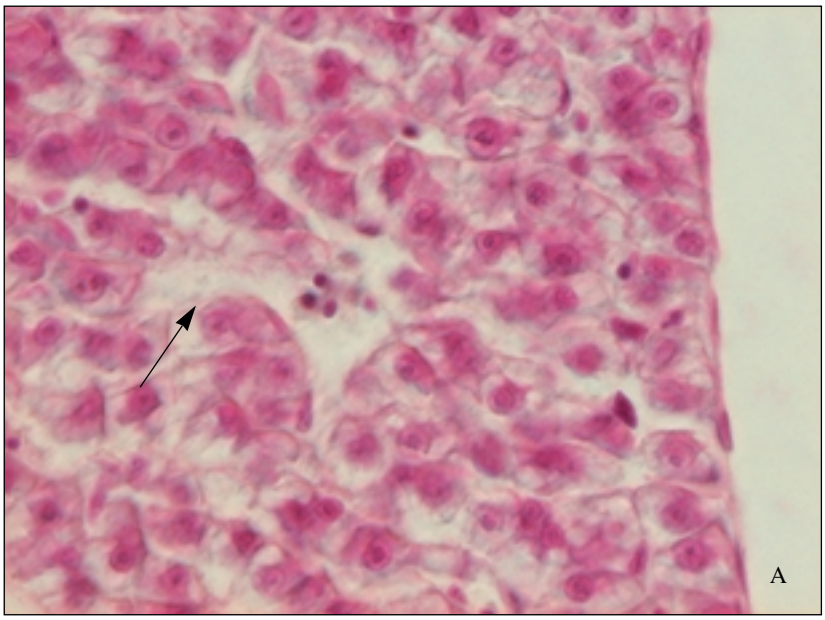


Fig. 1. A. The larva of carp from short exposure. Retardation of development of fish exposed to the high concentration of the extract, non resorbed yolk sack (→H&E × 100).
B. Vacuolar dystrophy of hepatocytes with pyknosis and karyolysis of nuclei in fish with low concentration of the extract and long exposure (→H&E × 400).

Plate II



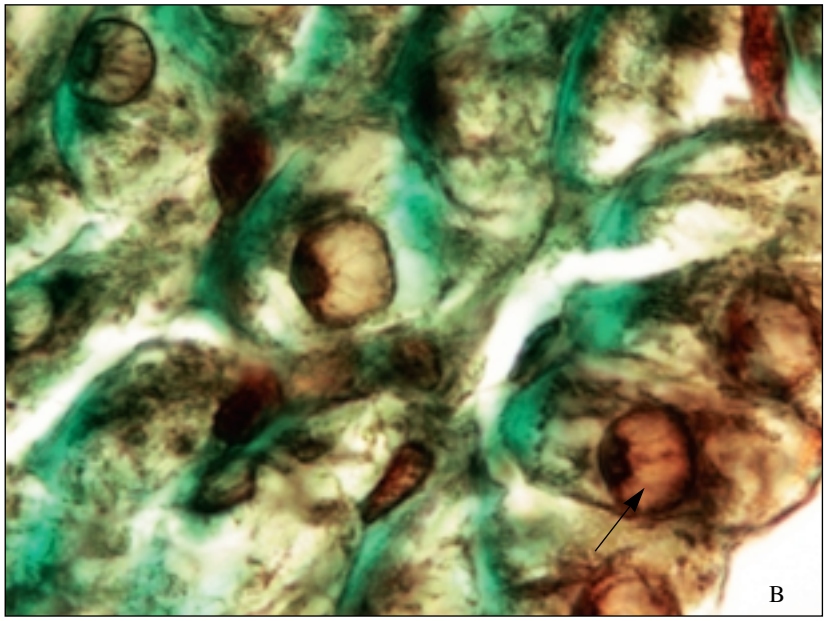


Fig. 2. The liver of carp from long exposure and medium concentration of the extract.
A. Perivascular focal necrosis (→H&E × 400). B. Apoptotic cells (→TUMEL × 1000).