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Histochemical investigations of NADPH- and NADH-tetrazolium reductase activity in the liver of selenium and copper treated carp (*Cyprinus carpio* L.)

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ABSTRACT

The effects of long-term intoxication with low concentrations of selenium and copper on NADPH- or NADH-tetrazolium reductase activity was examined. Carp were separately treated with selenium or copper in concentrations of 0.1 mg/L for 52 days. A non-treated group served as control. Activity of NADPH- and NADH-tetrazolium reductase was demonstrated in the liver tissue using histochemical methods according to Scarpelli et al. NADPH- and NADH-tetrazolium reductase activities were located in intracellular granules. NADH-tetrazolium reductase also showed diffuse distribution in the cytoplasm. Granules possessing the activity of both enzymes represented mitochondria. Results showed that selenium and copper in concentrations of 0.1 mg/L did not induce alterations in NADPH-TR activity over a 52-day period. Treatment with both elements induced oscillations of NADH-TR activity in granules, while copper also induced a significant increase of enzyme activity in the cytoplasm. Prolonged intoxication (after 49 days) with both selenium and copper led to damage to liver tissue and to reduced activity of both enzymes.

Key words: carp, liver, selenium, copper, histochemistry, NADPH- and NADH-tetrazolium reductase

Introduction

Selenium and copper are essential trace elements (HARTIKAINEN et al., 2000; VIARENGO and NOTT, 1993) which, as a result of their presence in various proteins (selenoproteins and cuproproteins), play an important role in numerous cell processes, making them indispensable in the normal functioning of the organism.

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NADPH- and NADH-tetrazolium reductase (NADPH- and NADH-TR) are enzymes which have the property of transferring electrons from the reduced nicotinamide adenine dinucleotide phosphate (NADPH) or the reduced nicotinamide adenine dinucleotide (NADH) to various electron acceptors. In this transfer, electron transport systems such as that of the mitochondria participate. Early research showed that selenium and copper affect various organelles in the hepatocytes, such as lysosomes (KRALJ-KLOBUČAR et al., 1996). Also, COUTURE and KUMAR (2003) suggest that mitochondrial enzymes are targets for inhibition by copper. Therefore, alterations in the activity of NADPH- and NADH-TR following intoxication by selenium or copper could serve as indicators of these two elements' impacts on mitochondrial populations in the liver. With this research we could also provide information on NADPH- and NADH-TR activity in conditions of long-term intoxication with low concentrations of selenium or copper, since it is well known that both elements can be toxic when allowed to accumulate in excess of cellular needs.

Concentration of 0.1 mg/L Se or Cu, although 10 times higher than the permitted concentrations in water, is used in this research because it can be easily reached in freshwaters and subsequently affect wildlife in an aquatic environment. Namely, copper is ubiquitously present in elevated concentrations in freshwaters as a result of agricultural use (as fungicide in viticulture, etc.) and industrial processes (MAZON et al., 2002) such as mining and smelting activities (COUTURE and KUMAR, 2003). Similarly, selenium concentration is often elevated in freshwaters as a result of anthropogenic processes like coal combustion, agricultural drainage, mining of phosphates and metal ores, etc. (HOFFMAN, 2002).

The aim of this study was to determine the effects of long-term exposure with low concentrations of selenium and copper, which can be easily reached in freshwaters, on enzyme activities in carp liver. Alterations in enzyme activities induced by metals or semi-metals may serve as indicators of early changes that in most cases appear before histomorphological alterations.

Materials and methods

One-year-old carp (*Cyprinus carpio* L.) approximately 10 cm long were used in this study. Prior to beginning the experiment, the fish were acclimatised to laboratory conditions by holding them in aquaria containing aerated city tap water for 6 days. Water in aquaria was aerated and changed daily during the experiment;

water temperature was 16 °C before, and 19 °C after, the water was changed; pH was a constant 7.0. Animals were divided into three groups. Two groups of 40 animals each were separately treated with selenium (as $Na_2SeO_3 \times 5\ H_2O$) or copper (as $CuSO_4 \times 5H_2O$) in final concentrations of 0.1 mg/L for 52 days. A third non-treated group of 10 animals was used as control. On the first four days the carp were sacrificed daily, and later at intervals of 2 or 7 days. Unfixed samples of liver tissue were cut on a cryostat at -20 °C and the activity of NADPH- and NADH-TR was demonstrated on the sections according to the method of SCARPELLI et al. (1958). Briefly, the non-fixed tissue sections were rinsed in acetone and then incubated for 30 minutes at room temperature in reaction mixture containing 0.1% NADH (for NADH-TR) or NADPH (for NADPH-TR) as substrate, 0.2% tetrazolium salt (NBT), 0.1 M $NaNO_3$, and 0.2 M TRIS pH 7.4. The sections were then rinsed in distilled water, formol saline and distilled water once more and then mounted in glycerine jelly. The locations of blue–purple diformazan pigment in sections, which match the locations of enzyme activities, were analyzed by light microscope.

Results

Histochemical staining in the control group showed NADPH-TR activity bound exclusively to the granules (Fig. 1a) but the activity of NADH-TR was present

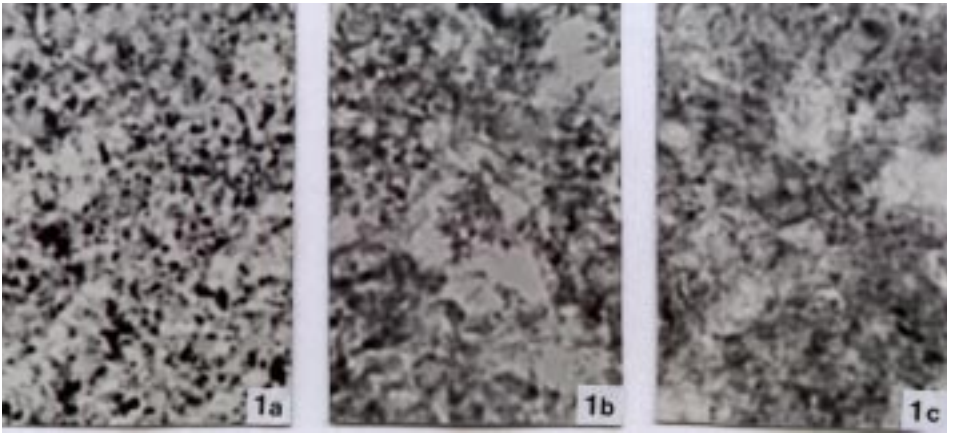

Fig. 1. NADPH-tetrazolium reductase activity in the liver of: a) control carp and carp treated for 52 days with: b) copper, c) selenium (×1280).

both in the granules (Fig. 2a) and was diffusely distributed throughout the cytoplasm (Fig. 2b).

In treatment with selenium or copper the activity of NADPH was demonstrated in the granules. During most of the treatment (52 days) with selenium or copper no changes were noted in the activity of NADPH-TR compared to the control. In both treatments the enzyme activity in granules was reduced compared to the control only after 52 days of treatment (Figs 1b, 1c). In a great number of cells the mitochondrial membranes were disrupted, which demonstrated histomorphological alterations in addition to reduced activity in the cytoplasmic granules.

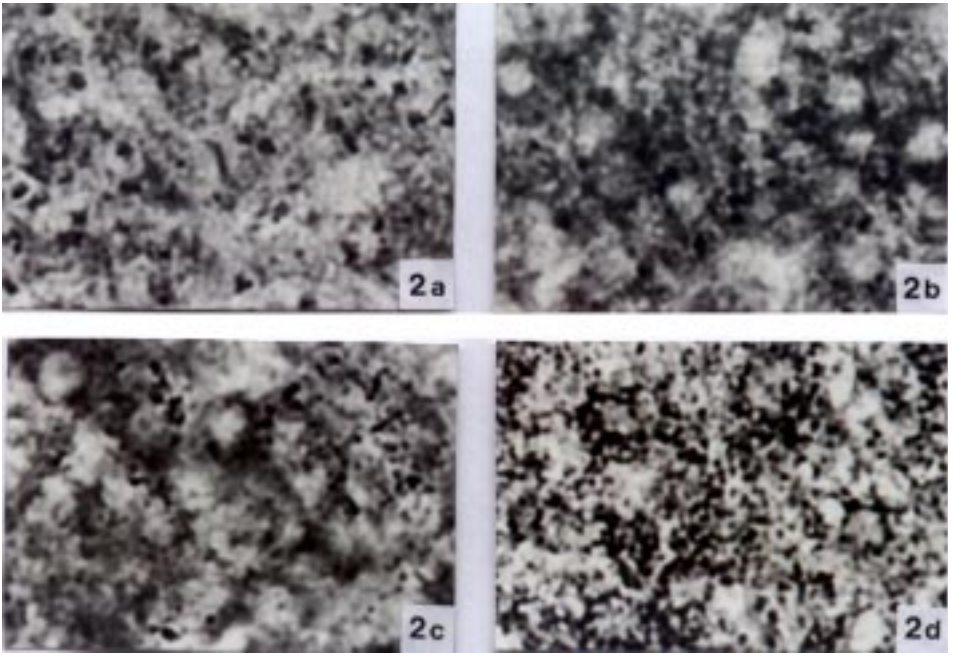

Fig. 2. NADH-tetrazolium reductase activity in the liver of control carp: a) enzyme activity in granules, b) enzyme activity in granules and cytoplasm. Liver of carp treated with selenium during: c) 7 days, d) 11 days (×1280).

The activity of NADH-TR in treated animals was located in both the granules and the cytoplasm and was, in general, stronger than the activity of NADPH-TR. During the first four days of treatment both elements caused an increase in NADH-

TR activity in the granules. In continued treatment with selenium, oscillations in enzyme activity compared to the control were noted. On the 7th day of treatment, enzyme activity rapidly decreased (Fig. 2c) and significantly increased again on the 11th day of treatment (Fig. 2d). After this period, NADH-TR activity was gradually reduced to the end of the treatment period, with the exception of the 49th day when the enzyme activity in the granules was similar to control. Enzyme activity in the cytoplasm during treatment was not significantly changed compared to the control, with the exception of the 4th and 11th days of treatment (Fig. 2d) when changes were negligible.

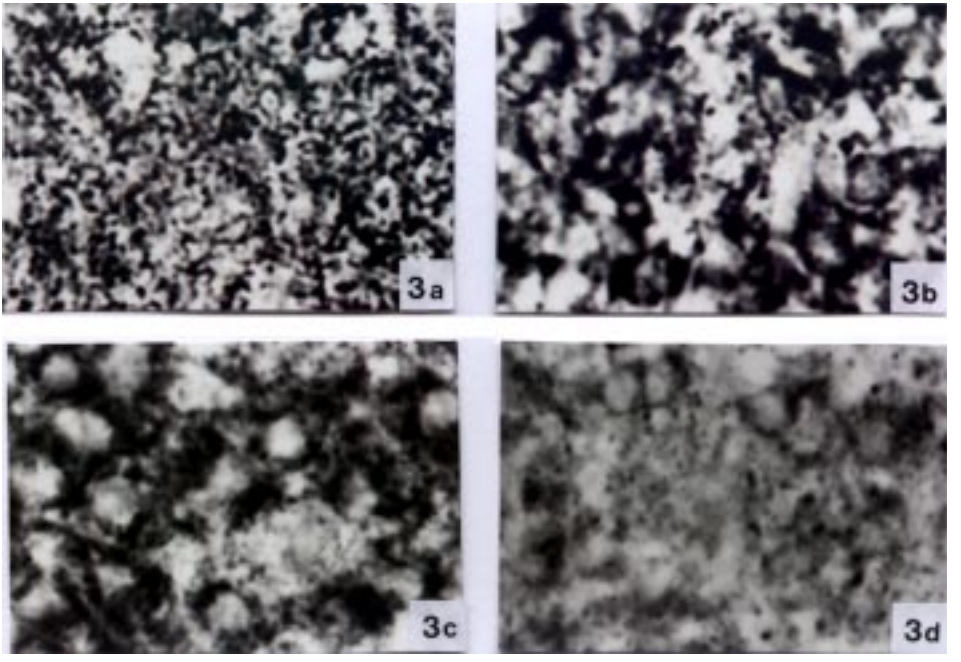

Fig. 3. NADH-tetrazolium reductase activity in the liver of carp treated with copper during: a) 7 days, b) 14 days, c) 42 days and d) 52 days (×1280). Activity of NADH-TR in the liver of control carp is shown in Figs 2a and 2b.

Intoxication with copper also induced oscillations in enzyme activity in granules following the initial increases. On the 7th day of treatment very strong NADH-TR activity in granules was noted, which then decreased and was at its lowest on the 11th day. Following that, enzyme activity again increased to the 21st day of treatment,

equalizing with the peak from the 7th day of treatment. After this period, enzyme activity again decreased rapidly and remained at levels slightly higher, or at the same level, until the end of treatment. During intoxication with copper a significant increase in the diffuse activity of NADH-TR in the cytoplasm was noted (Figs 3b, 3c) with the exception of the 7th day of treatment when enzyme activity was negligible (Fig. 3a).

Treatment with copper and selenium after 52 days resulted in reduced activity of NADH-TR in granules (Fig. 3d) compared to control. In pancreatic tissue of all samples, which represents part of the hepatopancreas of fish, there was no activity of NADPH- or NADH-TR was recorded.

Discussion

This study provides information regarding the hepatocellular distribution of NADPH- and NADH-TR activity. The deposit of diformazan was recorded in granules. It is well known that NADPH- and NADH-TR activity is bound to the mitochondria (ROTHE et al., 1999; KHANNA and PORTER., 2001), therefore the marked granules represent mitochondria. The diffuse distribution of NADH-TR in the cytoplasm is in agreement with the fact by which NADH-TR activity is represented in the endoplasmic reticulum in addition to the mitochondria (MALIK et al., 2000).

The unaltered NADPH-TR activity throughout the 52 days of treatment with selenium and copper suggests that the target organelle for these elements are not the mitochondria. However, reduced activity after 52 days of treatment led to the conclusion that after long-period of treatment even mitohondria became affected with selenium or copper. Thus, in order to impair this enzyme system with low concentrations of selenium and copper, a longer time period is required. This agrees with the fact that both selenium and copper are essential trace elements, which can be incorporated into the appropriate proteins, thereby eliminating all excesses of those elements in the cell. However, a long-term influx of these elements leads to their accumulation and results in damaging impacts, which relate to the reduction of NADPH-TR activity. It is well known that the liver is the prime organ in the accumulation of copper and selenium (PANDEY et al., 2001; FAN et al., 2002).

Short-term exposure to copper and selenium (to the 7th day of treatment) resulted in an increase of NADH-TR activity in granules. It is well known that NADH-TR

marks activity of Complex I in the respiratory chain of the mitochondria (MALIK et al., 2000) in which NADH donates electrons in order to generate energy (ATP). It is possible that selenium and copper induce certain biochemical processes that require energy and thus indirectly affect the oxidative metabolism of the hepatocytes. Such processes could include the defence reactions in which these elements are transformed into less toxic compounds or excretory products. With copper intoxication, these intoxication processes can relate to binding copper to the apothioneins, the synthesis of which requires energy. It is well known that small concentrations of copper induce the synthesis of metallothioneins (VIARENGO and NOTT, 1993; PARK et al., 2001). Increased energy demand in the case of selenium intoxication can reflect the increased metabolic reactions, in which selenium transforms into metabolites that can be excreted from the hepatocytes. It is known that exposure to selenite increases the formation of methylated metabolites, which are excreted via urine (KIM and MILNER, 2001).

Prolonged exposure to low concentrations of selenium and copper resulted in reduced NADH-TR activity. It is known that selenium and copper can act pro-oxidatively (HARTIKAINEN et al., 2000; POURAHMAD and O´BRIEN, 2000) forming free oxygen radicals, which can incite the peroxidation of lipids, thus damaging membranes. Mitochondrial membranes are particularly sensitive to the peroxidation of lipids (SHAMBERGER, 1986). Selenium and copper can damage mitochondrial membranes, thus affecting the functioning of NADH-TR and disrupting intracellular oxidative processes. This agrees with the inhibition of mitochondrial respiration demonstrated during long-term exposure to low concentrations of cadmium and lead (MEYER et al., 1991).

With copper intoxication, the reduction of NADH-TR activity in the mitochondria occurs simultaneously with increased activity in the cytoplasm. This indicates that the electron transport processes via the system that includes the endoplasmic reticulum intensifies in the cytoplasm. It is known that NADH-TR activity is also present in the endoplasmic reticulum, bound to NADH-cytochrome-b5 reductase (MALIK et al., 2000). It is possible that electron transport is bound to the cycling between the cuprous and cupric redox states. The resulting cuprous ions can form highly reactive oxygen species, which in turn cause membrane lipid peroxidation and membrane disruption (LUZA and SPEISKY, 1996). In selenium intoxication, NADH-TR activity in the cytoplasm is unaltered, which suggests that it accumulates in the hepatocytes in smaller concentrations than copper.

This study demonstrates that selenium and copper have a selective effect upon different enzyme systems. The effects of selenium and copper are more strongly expressed on NADH-TR activity than on NADPH-TR activity. Similarly, changes in NADH-TR activity induced by copper are more pronounced that in selenium intoxication, which suggests that in the same concentrations, copper is potentially more hazardous than selenium.

From these results it can be concluded that selenium and copper cause hepatotoxic effects following prolonged intoxication. Such effects are obvious in the reduction of enzyme activity and in histomorphological changes. Histomorphological changes in the liver in the form of lyzed parenhim were noted in the roach following exposure to copper (PARIS-PALACIOS et al., 2000).

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KRALJ-KLOBUČAR, N., M. GORUP: Histokemijska istraživanja aktivnosti NADPH- i NADH-tetrazol reduktaze u jetri šarana (*Cyprinus carpio* L.) nakon davanja selena i bakra. Vet. arhiv 74, 331-340, 2004.

SAŽETAK

Istraživani su učinci dugotrajne intoksikacije niskim koncentracijama selena i bakra na aktivnost NADPH- i NADH-tetrazol reduktaze. Šarani su odvojeno dobivali selen i bakar u koncentraciji od 0,1 mg/L tijekom 52 dana. Kontrolna skupina nije dobivala selen i bakar. Aktivnost NADPH- i NADH-tetrazol reduktaze određena je u tkivu jetre histokemijskom metodom. Njihova ativnost ustanovljena je u unutarstaničnim zrncima, ali je također bila i difuzno raspoređena u citoplazmi. Zrnca u kojima je utvrđena aktivnost oba enzima predstavljaju

mitohondrije. Rezultati su pokazali da selen i bakar tijekom 49 dana davanja u koncentraciji 0,1 mg/L ne izazivaju promjene u aktivnosti NADPH-tetrazol reduktaze. Davanje oba elementa izazvalo je kolebanje u aktivnosti NADH-tetrazol reduktaze u zrncima, a bakar je osim toga značajno potaknuo enzimsku aktivnost u citoplazmi. Produljeno davanje (nakon 49 dana) selena i bakra dovodi do oštećenja tkiva jetre te smanjenja aktivnosti oba enzima.

Ključne riječi: šaran, jetra, selen, bakar, histokemija, NADPH- i NADH-tetrazol reduktaza