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Glycogen, Proteins, and Aminotransferase (GOT, GPT) Changes in the Frog *Rana ridibunda* Exposed to High Concentrations of Copper

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The expansion of industrial activity in the last few decades has led to a remarkable increase in the presence of heavy metals in the environment (Falahi-Ardakani, 1984). Pollutants such as heavy metals enter living organisms with facility and accumulate in many tissues (Medveder et al., 1997, Kargin F, 1998). Copper (Cu) is a trace element which plays an important role in the cellular metabolism, although it becomes toxic if elevated concentrations are introduced into the environment. Among the many causes of Cu environmental pollution are mainly mining, industrial discharge, sewage sludge disposal, pesticide application and fertilizers (Nor, 1987). Chronic Cu toxicosis, leading mainly to hepato-biliary damage, is the most common form in both humans and animals.

In our first study concerning Cu, we examined Cu bioaccumulation in various tissues of the frog *Rana ridibunda* (Papadimitriou and Loumbourdis, 2003). This frog is widely distributed in Europe and is very common in the Balkans, with a wide distribution in Greece. For this reason, this frog serves as a suitable bioindicator of environmental pollution since it meets all the criteria listed by Lower and Kendall (1990). The main aim was to examine the effects of Cu on the liver metabolism of *Rana ridibunda*, thus enriching our knowledge about the impact of Cu on the energy metabolism of the frog.

MATERIALS AND METHODS

In this study, the levels of glycogen, protein, and the activities of aspartate aminotransferase (GOT) and alanin aminotransferase (GPT) in the serum of the frog *R. ridibunda* were measured.

Adult female frogs *Rana ridibunda*, were purchased from a local dealer, who had collected them from unpolluted areas of northern Greece. To ensure acclimatization, frogs were kept in plastic aquaria (35×23×23.5) in 2-3 cm of dechlorinated tap water, for 5-7 days prior to the experiments. The water was changed every 2 days and the aquariums were cleaned thoroughly, first with detergent, then with 1% nitric acid, and finally with deionized water. The frogs were fed with laboratory raised *Tenebrio molitor* at libidum.

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A total of 90 frogs were used in this study; 60 experimental and 30 control animals were selected at random. Each group (experimental and control) was placed in plastic aquaria (120×65×60 cm) in a room, under seminatural conditions. 30 frogs were exposed to 50 ppm of dissolved Cu, while another 30 frogs were exposed to 100 ppm of dissolved Cu, respectively. High copper concentrations were used, to achieve acute effects. $CuCl_2$ was prepared as a stock solution in deionized water. The control animals were kept in clean water throughout the experiment. Mean water quality parameters during tests were: hardness 288 mg $CaCO_3$/lt, pH 7.40, conductivity 650-700 μs/cm, nitrites <0.025 mg/l, phosphates <0.10 mg/lt, ammonium< 0.05 mg/l, with copper below the detection limits. At the end of the 5^{th}, 15^{th} and 30^{th} days of exposure to Cu, samplings of 10 animals from each of the three groups (experimental and control) were made.

Animals were sacrificed by immersing them in MS222. They were weighed to the nearest milligram and snout to vent length was measured to the nearest millimeter. Liver samples were handled with plastic forceps. The samples were crushed into small pieces, chilled in liquid nitrogen and kept in plastic boxes in a freezer (-25°C).

Small liver samples were thawed rapidly and boiled in 3 ml of 30% KOH for 30 min. 1 ml samples from each liver were analyzed for glycogen with the anthrone reagent (Seifter et al., 1950) using saturated Na_2SO_4 that acted as a co-precipitant to improve glycogen recovery (Zwann and Zandee, 1972) against multiple glucose standards. Glycogen concentration was expressed as mg/g of wet tissue.

The estimation of activities of aminotrasferases was performed using the following procedures: Liver samples were homogenized on ice with the addition of phosphate buffer (5:1 v/w) at pH 7.00. The homogenate was then centrifuged at 15000g at 4^0C for 30 min. In the supernatant, the activities of GOT and GPT were estimated using a GOT-GPT testing kit (ELITECH diagnostics). The activities were expressed as Units/g of wet tissue. For protein determination, about 300 mg of liver was used and liver proteins were determined using the method of Bradford et al. (1976).

The normality of the parameters under examination was checked using the Kolmogorov-Smirnov test and since they all followed normal distribution, parametric analyses were then applied. Analysis of variance (ANOVA) with repeated measures and Scheffe and Dunnett's comparison tests were used to compare the means. Differences were deemed statistically significant at $p<0.05$. Statistical analyses were carried out with SPSS 10.0 for Windows.

RESULTS AND DISCUSSION

The only statistically significant difference in the glycogen concentration was that between controls and 100ppm and 30 days' exposure (Table 1).

Table 1. Mean values (±SE) of glycogen (mg/g), proteins (mg/g) and aminotransferases (U/g) in the liver of adult *Rana ridibunda* exposed to 50 and 100 ppm of Cu for 5, 15 and 30 days.

	5 days in Cu	15 days in Cu	30 days in Cu
Glycogen(mg/g)			
Control (N=10)	33.73±16.75[a]	37.35±18.28	44.40±7.62
50 ppm (N=10)	24.59±13.85[a]	29.68±14.08	35.47±12.45
100 ppm (N=10)	24.18±13.26[a]	35.95±19.06	54.06±20.6
Proteins(mg/g)			
Control (N=10)	77.26±5.27[α]	69.09±8.2	68.69±5.98[b]
50 ppm (N=10)	86.72±10.10[α]	87.46±18.7[α]	55.02±15.28[b]
100 ppm (N=10)	105.40±16.12[a,c]	68.58±8.06	55.04±9.06
GOT (U/g)			
Control (N=10)	0.064±0.02	0.083±0.05	0.061±0.02
50 ppm (N=10)	0.057±0.01	0.078±0.02	0.078±0.02
100 ppm (N=10)	0.071±0.02	0.093±0.03	0.098±0.05
GPT (U/g)			
Control (N=10)	2.33±0.81[α]	2.22±0.23[α]	2.27±0.14[α]
50 ppm (N=10)	2.01±0.69[a,c]	2.73±0.59	2.76±1.27
100 ppm (N=10)	2.02±0.54[a,c]	3.86±1.01	4.46±0.57

a: Values differed significantly from values of 100 ppm 30d.
b: Values differed significantly from values of 50 and 100 ppm 5d.
c: Values differed significantly from values of 100 ppm 15d

The concentration of proteins in the liver increased for both 50 and 100 ppm, compared with the controls at the beginning (Table1). The concentration of proteins on the 15th day of exposure to Cu was almost the same as the values of the control animals, but at 100 ppm a decline was noted, compared with the values of 100 ppm at the beginning of the experiment. By the 30th day of exposure to Cu, a decline was observed in protein concentration for both 50 and 100 ppm, which was significant compared with the values on the 5th day of exposure to Cu (Table 1).

Regarding transaminases, only the activity of GPT increased, while the activity of GOT remained constant nearly at the same levels as the control animals, but a trend of increase, especially at 100 ppm and 30 days exposure, was apparent (Table 1). GPT activity increased on the 15th and 30th days of exposure for both 50 and 100 ppm groups. This increase was statistically significant at 100 ppm by the end of the 30th day of exposure to Cu compared with the values at 5 days and the values of the controls

Due to the high permeability of the skin of frogs, heavy metals can enter the body through this route and are then distributed via the blood circulation to the soft tissues of the frog, such as the liver. Cu ions mimic the action of other divalent ions of the cell such as Ca^{+2} which seems to affect Cu entrance to the cell. On the other hand, membrane proteins, mobile vesicles,

and Cu-binding peptides comprise the integrated network that maintains cellular Cu homeostasis. Vesicles with Cu-transporting ATPase enzymes work in conjunction with mobile Cu carriers to displace excess cytoplasmic Cu or activate enzymes that protect the cell from oxidative damage. These mechanisms come into play in response to a high influx of Cu (Harris, 2001).

In the present study, exposure to Cu resulted in an increase in liver glycogen content by the end of the experiment, and, in the group of animals exposed to 100 ppm of the metal, with a concomitant decrease in liver protein content. We can speculate that gluconeogenesis may take place, although alternatives such as protein loss, as a result of liver damage, could not be excluded The effects of Cu^{+2} on isolated rat hepatic parenchymal cells indicated gluconeogenesis from lactate (Tolbert, 1981). Srivastava (1982) found a decline in the glycogen level in the liver of the catfish *Heteropneustes fossilis,* after exposure to 12.5 mg/l of Cu. The effects of $CuSO_4$ in concentrations of 5, 10, 25 and 50 ppm for 24h in carp hepatopancreas (liver) (*Cyprinous caprio morpha L.*), showed a significant increase in protein content (Radi and Matkovics, 1988).

A decrease in the concentration of proteins in the liver was also recorded in different species, as a result of pathological kidney alterations, leading to a condition known as proteinuria. The most common kidney alterations are those of Bowman's capsules, and proximal and distal convoluted tubules. The lost proteins are usually albumin and β2-microglobulin, the latter being a good biomarker of kidney pathology (Mueller et al., 1998). The decrease in liver proteins in the frog *Rana ridibunda* as a possible impact of cadmium (Vogiatzis and Loumbourdis, 1999.) and lead (Vogiatzis and Loumbourdis, 2001.) is well-known.

Many of the pathological effects of metal overload are consistent with oxidative damage to membranes or macromolecules. Furthermore, they could have an indirect effect mediated by the formation of oxyradicals. These reactive species could enhance lysosomal damage by promoting the peroxidation of membranes and, in the meantime would further reduce the antioxidant cellular defenses (Regoli et al., 1998). A common consequence of the Cu-induced production of reactive oxygen species is increased lipid peroxidation (Bremner, 1998). MDA is the end product of lipid peroxidation. The level of MDA in the liver increased correspondingly with the augmentation in the dose of Cu and exposure time, especially after 15 days of exposure (Papadimitriou and Loumbourdis, 2002). Yamada et al. (1992a) noted that excessive accumulation of Cu provoked hepatic injury through initiating lipid peroxidation. Zhang et al. (2000) also found that Cu overload in rats with copper sulphate given orally (500 mg/kg w) for 8w, increased MDA concentrations in serum and liver homogenates.

The activities of functional enzymes GOT and GPT were also determined in liver homogenates. These enzymes catalyze the transfer of amino acids. A statistically significant increase was recorded only for GPT on the 15^{th}

day of exposure to the metal. At 100ppm, GPT activity was found to be about twice as high at the end of the experiment as compared with the activity of this enzyme at the beginning of exposure (Table 2). The increase in GPT and the increase in MDA observed in another study (Papadimitriou and Loumbourdis, 2002) may indicate copper-related injury to the liver. Karan et al. (1988) found that after a 14 day period of exposure to five concentrations of copper sulphate (0.25-4.0 mg/L $CuSO_4$) in carp (*Cyprinus caprio L.*), activity of GOT and GPT in blood serum and gills increased after the exposure period. Fischer rats fed a diet containing Cu as $CuCl_2$ (150-600 ppm) for 60d, showed increased activity for both GOT and GPT in serum (Sugawara et al., 1995). Hwang et al. (1998) found that rats fed diets with different concentrations of Cu for 2 months showed that both aminotransaminases increased in activity as the dose of Cu in the plasma was augmented.

Copper does not seem to play essential role in glycogen concentration, while its role in protein depletion and transaminase activity seems to be more critical. More thorough biochemical and and histopathological studies are needed to clarify the possible role of copper in protein loss and transaminase activity as a result of liver and kidney damage in frogs.

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