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Exposure of the Frog *Rana ridibunda* to Copper: Impact on Two Biomarkers, Lipid Peroxidation, and Glutathione

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Copper (Cu) is an essential trace element and it is not a potent liver toxin except in certain cases of genetic defects resulting in the inability to excrete Cu. However very high Cu intake can result in liver damage (Sternleib 1980). The effects of the metal overload prompted research into the mechanisms of its toxicity. When in excess "free" Cu can catalyse the production of the highly toxic hydroxyl radicals from intracellularly generated hydrogen peroxide. Oxidative stress may produce DNA damage, enzymatic inactivation and preoccupation of cell constituents, especially lipid peroxidation (Halliwell and Gutteridge 1989).

Glutathione (GSH), the most abundant cellular thiol, is involved in metabolic and transport processes and in the protection of cells against the toxic effects (Meister and Anderson 1983). GSH has been shown to form GS-Me complexes with various metals, through its thiolate sulfur atom (Rabestein et al. 1985). In light of these studies, it has been proposed that GSH is capable of complexing and detoxifying heavy metal cations soon after they enter the cell, thus representing a first line of defence against heavy metal cytotoxicity (Singhal et al. 1987; Freedman et al. 1989; Naganuma et al. 1990).

In this study, the levels of malondialdehyde (which is a lipid peroxidation product), GSH and Cu concentration in the liver of the frog R. *ridibunda* were measured. The aim of this study was to examine some effects of Cu on the liver, enriching our knowledge on the impact of Cu on two biomarkers of exposure, lipid peroxidation and glutathione.

MATERIALS AND METHODS

Adult female frogs, R. *ridibunda* were purchased from a local dealer, who collected them from unpolluted areas of Macedonia, Northern Greece. The mean values (±SD) of body length and body weight of the frogs were 257±9 mm and 104.6±11.3 respectively. Prior to the experiment, frogs were kept in plastic boxes (35×23×23.5 cm) in 2-3 cm dechlorinated tap water for acclimatization. Water was changed every 3 days and boxes were cleaned thoroughly. Frogs were fed larvae of *Tenebrio molitor*, raised in the laboratory under controlled conditions.

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Ninety frogs were placed in three plastic tanks (120×65×60 cm) presoaked overnight in 10% HNO_3 (analytical grade). Experimental animals were exposed to 50 and 100 ppm of the metal. $CuCl_2$ was prepared as a stock solution in deionized water. Control animals were kept in clean water throughout the experiment. At the end of the 5th, 15th and 30th days of Cu exposure, three samplings (10 animals) in each group (experimental and control) were made.

Animals were sacrificed by immersing them in MS 222 (tricainae methanosulfate). All the weights were expressed to the nearest milligram. Liver samples were chilled in liquid nitrogen crushed into small pieces with plastic forceps and then placed in plastic boxes and kept in a freezer (-25°C) until analyses.

The amount of hepatic GSH was determined by the method of Richardson and Murphy (1975) with slight modifications. GSH concentration was expressed as μm/g of wet weight tissue. For the measurement of the extent of lipid peroxidation we used the method of thiobarbituric acid (TBA) reaction to measure malondialdehyde (MDA). The MDA concentration of the samples were calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}, and expressed as nmole/g of wet tissue.

About 0.5 g of the liver was cut into small pieces, dried in an oven at 80^0C for 48h (to constant weight), powdered with a pestle and mortar and was used for quantitative Cu determination. The difference between wet and dry weight was the water content. This tissue was digested in 10 ml HNO_3 (analytical grade) over a hot plate, at about 120-150^{0}C under a reflux cap. Cu was analysed using a Perkin-Elmer 403 atomic absorption spectrophotometer with oxygen-acetylene flame. The Cu concentration was expressed as ppm (μg/g) dry weight.

The normality of the parameters was checked with the Kolmogorov-Smirnov test, and since all followed normal distribution, statistical analyses were based on parametric tests. One-way analysis of variance (ANOVA) and Dunnett's comparison tests were used to compare the means and Pearson's test for correlation. Differences were deemed statistically significant at $p<0.05$. Statistical analyses were carried out with SPPS 8.0 for Windows.

RESULTS AND DISCUSSION

Regarding the gross morphological characteristics of the frogs (body length, body weight, liver weight), there were no statistically significant differences between the groups.

The amount of lipid peroxidation, a marker of oxidative damage of lipid in liver homogenates was assayed as MDA concentration. The latter was increased with the increase of Cu concentration (Table 1). No statistically significant difference was observed during the first five days of exposure, the increase becoming

Table 1. Mean values (±SD) of GSH and MDA in the liver of adult control and exposed frog *Rana ridibunda* to 50 and 100 ppm of Cu for 5, 15 and 30 days.

	5 days	15 days	30 days
GSH (umole/g)			
control (N=10)	1.8±0.16	0.997±0.18[c]	1.042±0.12 [c]
50 ppm (N=10)	0.96±0.22[c]	1.802±0.57	1.806±0.51
100 ppm (N=10)	0.97±0.16[c]	1.55±0.32	1.57±0.46
MDA(nmole/g)			
control (N=10)	22.97±7.61[a]	22.14±6.9[b]	24.43±7.6[a]
50 ppm (N=10)	22.42±7.95[a]	39.87±12.9	38.7±9.11
100 ppm (N=10)	24.94±4.98[c]	37.90±11.9	46.35±13.3

[a] Values differed statistically significantly from values of 100 ppm 15d and all 100 ppm 30d.
[b] Values differed statistically significantly from values of 100 ppm 15d and 50 and 100 ppm 30d.
[c] Values differed statistically significantly from values of 100 ppm 15d.

statistically significant after that day. The value of liver MDA concentration of controls was significantly different from values of 100 ppm for both 15th and 30th day exposures. The same differences were found for the concentration of 50 ppm statistically significant after that day. The value of liver MDA concentration of controls was significantly different from values of 100 ppm for both 15th and 30th day exposures. The same differences were found for the concentration of 50 ppm at the 5th day. At the end of the experiment the MDA content at 100 ppm was higher and exhibited an almost 1.5-fold and 2-fold increase compared to the values of the same concentration at 5th and 15th day but the difference was not statistically significant.

At the beginning of the experiment, the concentration of hepatic GSH remained unchanged for both 50 and 100 ppm of copper concentration. By the end of 15th day GSH concentration increased in both 50 and 100 ppm compared to the control value as well as the values of the 5th day (statistically significant differences between the group of 100 ppm and the control group on 15th day and the group of 100 ppm of the 5^{th} day of exposure). The values of hepatic GSH at the 30^{th} day of exposure were almost the same with those of the 15th day.

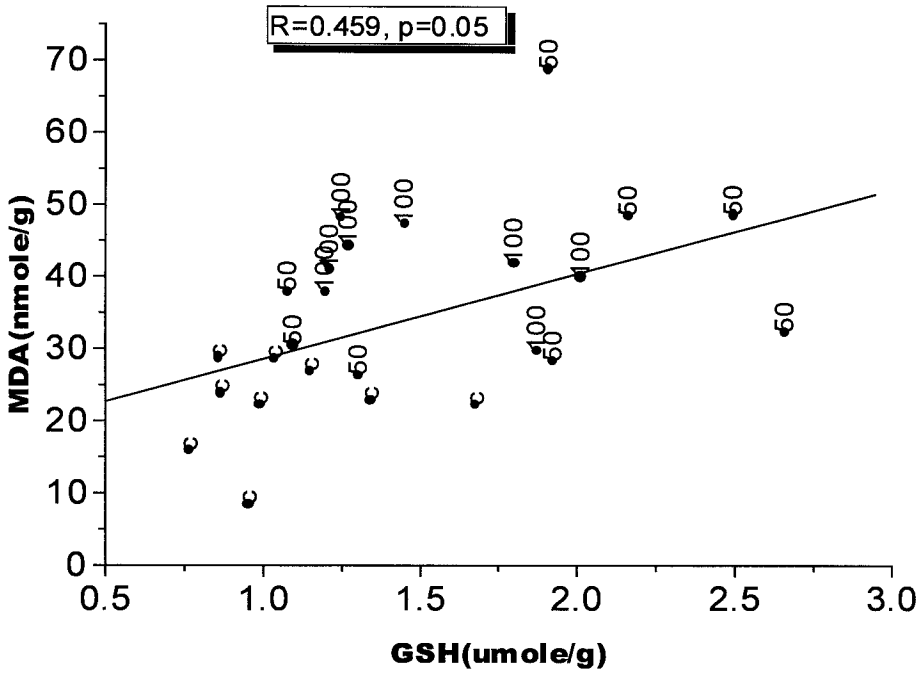

Figure 1. Positive correlation between GSH and MDA concentration in the liver of adult frog *Rana ridibunda* exposed to 50 and 100 ppm of Cu for 15 days (c:control, 50:50 ppm, 100:100 ppm).

The concentration of MDA was positively correlated with the time of exposure (r=0.424, p=0.01) contrary to GSH concentration which was negatively correlated with time (r= -0.359, p=0.01).
In the group of 15 days of exposure, GSH concentration was positively correlated with MDA concentration in the liver (r=0.459, p=0.01) (Figure 1).

Cu concentration increased in the liver during the exposure of animals (Figure 2). The concentration of the metal was positively correlated with the concentration of the MDA at the 30th day of exposure (r=0.481, p=0.05).

All groups of animals were quite homogenous since we did not observe any statistically significant differences in the gross morphological characteristics among the different exposure groups throughout the experimental procedure. Since we didn't notice any deaths or changes in the animals' behavior we can hypothesize that all the differences in the studied parameters were due to the presence of the copper.

Many of the pathologic effects of the metals overloaded are consistent with oxidative damage to membranes or macromolecules. Further they could have an indirect effect mediated by the formation of oxyradicals. These reactive species

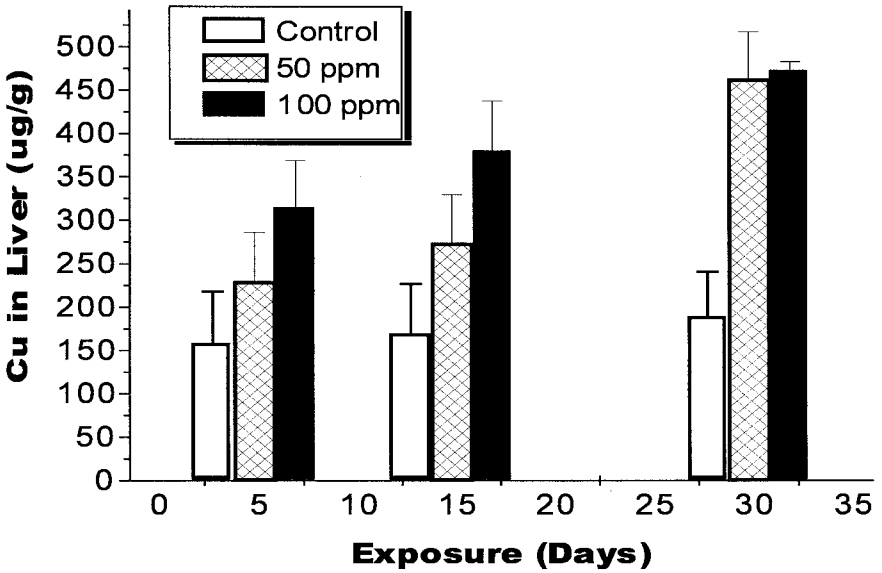

Figure 2. Cu accumulation in the liver of the frog *Rana ridibunda* exposed to 50 and 100 ppm of the metal for 5, 15 and 30 days.

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). Not unexpectedly, Cu ions are able to catalyze the formation of hydroxyl radicals. A common consequence of Cu-induced production of reactive oxygen species is increased lipid peroxidation (Bremner, 1998). MDA is an end product of lipid peroxidation. The major change in the studied parameter took place after 15 days exposure to the metal. For both 50 and 100 ppm, MDA concentration increased compared to the control groups and remained almost in the same levels at the end of the experiment.

Yamada et al. (1992) noticed that excessively accumulated copper provoked hepatic injury through initiating lipid peroxidation. Zhang et al. (2000) also found that Cu overloaded in the rats with copper sulfate given orally (500 mg Cu/Kg w.) for 8w, increased MDA concentrations in serum and liver homogenates. MDA levels in the liver of rats fed with 150-600 ppm Cu for 2 months were increased with the increasing dose of the metal (Hwang et al. 1998). Our results did not prove that the mechanism of Cu injury is by lipid peroxidation but it is strongly suggestive that it plays an important role.

The oxyradicals formed as a result of presence of metals, could not only enhance lysosomal damage by promoting the peroxidation of the membranes but could also reduce the antioxidant cellular defenses. Metals such as Cu and mercury can increase the production of oxyradicals by stimulating their formation and/or

inhibiting the normal pathway by which these reactive species are usually removed (Christie and Costa 1984). GSH functions in the mobilization and delivery of metals between ligands, in the transport of metals across cell membranes, as a source of cystein of metal binding and as a reductant or cofactor in redox reactions involving metals (Ballatori 1994).

The highest concentrations of MDA observed in the group of 100 ppm at the end of the experiment were paralleled with the same concentrations of GSH observed for 15 days of the exposure. It seemed that after the defensive action of SH compounds the lysosomal damage reduce the antioxidant cellular defenses. This was probably the reason of negative correlation observed between GSH production in the liver and the time.

Heavy metal accumulation in the cells can result in decreased availability of reduced GSH, due to both GSH binding and oxidation. On the other hand, heavy metals such as Hg^{2+} and Pb^{2+} have also been demonstrated to increase the concentration of GSH in both mammalian (Woods and Ellis 1995; Lash and Zalups 1996) and fish tissues (Thomas and Wofford 1984; Thomas and Juedes 1992), suggesting that in vivo metal treatment could also interfere with GSH metabolism. Exposure to Cd caused elevation in GSH levels in liver of the frog Rana *ridibunda* (Vogiatzis and Loumbourdis, 1998). Regoli et al. (1998), found that laboratory exposure to Cu in the Antarctic scallop *Adamussium colbeki*, depleted the levels of total GSH. Standeven and Wetterhahn (1991) have shown the increased hepatic GSH levels in rats treated by a moderately toxic dose of hexavalent chromium.

To conclude, the frog seemed to face an oxidative stress over the first 15 days of Cu exposure, resulting in both lysosomal and antioxidant responses (increasing levels for both MDA and GSH in the liver of the frog). At the 30^{th} day of the Cu exposure, the highest concentrations of MDA resulted in an inhibition of further increased GSH levels.

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