Bull. Environ. Contam. Toxicol. (2006) 76:522–528

DOI: 10.1007/s00128-006-0951-9

Bulletin of Environmental Contamination and Toxicology

Copper Kinetics and Hepatic Glutathione Levels in the Copper Exposed Frog *Rana ridibunda* after Tetrathiomolybdate Treatment

N. S. Loumbourdis

Department of Zoology, University of Thessaloniki, GR 54124 Thessaloniki, Greece

Received: 15 July 2005/Accepted: 28 December 2005

As a transition metal, copper is an essential element and constitutes part of the active site of many enzymes associated with oxidative metabolism and transport proteins. These enzymes include those such as superoxide dismutase, catalase, peroxidase, cytochrome c oxidase, superoxide dismutase, alcohol dehydrogenase and alkaline phosphatase.

Excess concentration of copper in tissues is a potential toxicant, resulting in various disorders such as Wilson's disease (Brewer et al.1991). A disease similar to Wilson's disease has been described in Long-Evans Cinnamon (LEC) rats, suffering from spontaneous hepatitis due to gross accumulation of Cu in the liver (Li et al. 1991).

One chelator for Wilson's disease currently used clinically is tetrathiomolybdate (TTM), which rapidly reduces hepatic Cu in patients with Wilson's disease. TTM can remove copper directly from the liver, and it is highly selective with regard to Cu among transitional metals in the body and can be used without disruption of the metabolism of the essential metals such as Zn and Fe (Ogra et al. 1996).

Glutathione (GSH) is a tripeptide containing glutamyl, cysteinyl and glycyl amino acid residues. Due to the presence of the free sulphydryl group on the cysteinyl residue, GSH has a great propensity for forming complexes with ions of metals that have strong electrophilic characteristics, such as mercury (Zalups and Lash, 1996). In biological systems, the sulphydryl group on the cysteinyl residue of GSH is one of the most important sites when it comes to the formation of complexes between metals and GSH. A number of metals such as cadmium and mercury form bonds with the sulphydryl group of GSH.

MATERIALS AND METHODS

Forty adult female frogs, *Rana ridibunda,* were obtained from a local dealer who collected them from a relatively unpolluted area of Northern Greece. Before processing, the frogs were acclimatized for 6-7 days in plastic aquaria (35x23x23.5 cm) containing dechlorinated tap water. Mean tap water quality parameters were: hardness 288 mg $CaCO_3$/L, pH 7.40, conductivity 650-700 μS/cm, nitrites <0.025 mg/L, phosphates <0.10 mg/L. ammonium <0.05 mg/L,

and copper below the detection limit (0.05mg/L). The water was changed every 2 days and the aquaria were cleaned thoroughly, first with detergent, then with 1% nitric acid and finally with deionized water. Before changing the water, the frogs were fed larvae of *Tenebrio molitor* raised in our laboratory for many generations. The uneaten food was removed from the tray.

Thirty-two animals, chosen arbitrarily, were placed in two plastic aquaria (120x65x60 cm), filled with dechlorinated tap water, with 16 frogs in each aquarium. Eight frogs, which served as controls (day 0), were placed in a third, similar aquarium. In the first aquarium, the experimental frogs were exposed to 20ppm Cu dissolved in tap water for 7 days (eight frogs) and 14 days (the remaining 8 frogs). In the second aquarium, the 16 frogs, similarly exposed to 20ppm Cu, were injected daily intraperitoneally with 2mg/100gr body weight TTM In the third aquarium, the 8 frogs were injected with saline. Twenty-four hours after the last injection, the animals were killed by immersing them in 0.5% MSS 222 (tricaine methanosulfonate).

Portions of the liver, kidneys, large intestine rinsed thoroughly to remove all its contents, ventral skin as well as the whole gall bladder were removed immediately and weighed to the nearest milligram. Tissues were handled with plastic forceps, chilled in liquid nitrogen and kept in plastic boxes in a freezer (-25^{0}C). All the glassware and plasticware used in Cu determination were presoaked in 10% HNO_3 (analytical grade).

Tissues under study were cut into small pieces, dried in an oven at 80^{0}C for 48h (to constant weight), and powdered using a mortar and pestle. About 0.5g of the tissue was used. Tissues were digested in 10ml HNO_3 (analytical grade) over a hot plate at about 120-150^{0}C under a reflux cap. Cu was analyzed using a Perkin-Elmer 403 atomic absorption spectrophotometer with an oxygen-acetylene flame. The spike/recovery tests were always in the range of 95-102%. The Cu concentration was expressed as ppm (μg/g) dry weight.

The amount of hepatic reduced glutathione (GSH) was determined using the Richardson and Murphy (1975) method with slight modifications. 200-300 mg of frozen liver tissue was thawed and immediately homogenized, using a glass homogenizer and Teflon pestle. The homogenate was centrifuged at 5000 g for 15

min. Forty microliters of the supernatant were mixed with 50μl of 0.01 M DTNB [5.5-dithiobis-(2-nitrobenzoic acid)] and 910 μl of PBS buffer (pH 8.0) and incubated at room temperature, in the dark, for 15 min. OD was measured at 412 nm, while GSH concentration was expressed as μg/g of wet weight tissue.

The normality of 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 was used for correlation. Differences were deemed significant at $p<0.05$. Statistical analyses were carried out with SPSS 8.0 for Windows.

RESULTS AND DISCUSSION

This work is an attempt to study the effect of treatment with TTM on one lower vertebrate, the frog *Rana ridibunda,* exposed to aqueous solutions of copper. This appears to be the first study concerning a lower vertebrate and its reaction to this treatment would be helpful in understanding the way of copper removal on a lower vertebrate not suffering from the above described disorders. Thus, this animal could be used as an animal model in studying the impact of copper toxicosis due to its exposure in an environment burdened by this metal and the removal of copper by a chelator such as TTM.

Exposure of the animals to copper alone for 7 days resulted in increased copper concentrations in all the tissue samples examined (Table 1). After 14 days of exposure to copper alone, there was a variable change in copper concentration (Table 1). There were further increases in the kidneys, liver and skin, but, compared with samples exposed for 7 days, copper concentrations in the large intestine and gall bladder had decreased. Exposure of animals to Cu+TTM for 7days showed an increase in Cu concentrations in all the organs studied (Table 1).

In the animals exposed to Cu+TTM for 14 days there was a reduction in Cu concentrations, in comparison with the animals exposed to Cu+TTM for 7 days in the large intestine, skin and gall bladder. On the contrary, increases in Cu concentration (similar to that of animals exposed to 7 days Cu+TTM) were observed in the kidney and liver.

Regarding GSH, there was a continuous increase in its concentration, except in the case of 14 days' Cu alone exposure (Table 2).

As was expected, exposure of frogs to copper for 7 days resulted in increases in Cu concentrations in all the organs studied, a situation which was also observed in an earlier study (Papadimitriou and Loumbourdis, 2003). Exposure for 14 days resulted in further increases in the kidneys and liver, but no change in the large intestine and a decrease in the gall bladder. The same intestinal Cu concentrations in the animals exposed for 7 and 14 days indicates that copper moves continuously from the intestine to other tissues. The simultaneous increase in the liver Cu concentration may indicate that part of this copper from the intestine moves to the liver.

One factor in the absorption of copper from the intestine is the de novo synthesis of metallothionein. The other protein known to have a role in copper metabolism in the enterocyte is ATPase 7A (Thornburg, 2000). Absorbed copper is then bound to albumin to form an albumin-copper complex and transferred to the liver. The low copper concentrations in the kidney of frogs exposed for 7 days was expected, since it is well known that this organ is not the preferred site for copper

Table 1. Copper concentration (mean±SD) (μg/g dry wt) in various organs of the frog *Rana ridibunda* after exposure to Cu alone or to Cu+TTM. Sample size in parenthesis.

	Days of exposure		
	0 (control)	7	14
		Cu alone	
Liver	9.83±8.67 (5)	296.62±89.99 (8)[a]	572.29±378.25 (7)[a]
Kidney	4.20±0.84 (5)	20.37±10.72 (8)[a]	98.28±150.70 (7)[a]
Intestine	10.02±4.47 (5)	107.75±41.53 (8)[a]	71.28±39.53 (7)[a]
Skin	4.46±1.14 (5)	24.37±4.63 (8)	104.57±81.12 (7)
Gall bladder	23.01±9.48 (5)	119.25±45.46 (8)	23.04±9.48 (7)
		Cu+TTM	
Liver		58.25±43.59 (8)[b]	71.12±19.20 (6)[c]
Kidney		217.75±68.43 (8)[b]	348.08±35.08 (6)[c]
Intestine		47.37±26.57 (8)	45.02±10.75 (6)
Skin		71.12±19.20 (8)	47.02±13.11(6)
Gall bladder		191.37±135.64 (8)	65.66±26.07 (6)[c]

a=statistically significant difference between control and 7d and 14d in Cu alone exposure.
b=statistically significant difference between 7d Cu+TTM and 14d Cu+TTM
c=statistically significant difference between 14d Cu+TTM and 7 and 14d Cu alone

deposition (Papadimitriou and Loumbourdis, 2003) and that renal Cu excretion seems to be of minor importance compared with hepatic Cu excretion, a situation also observed in the fish *Oncorhynchus mykiss* (Grossel et al. 1998).

Once copper reaches the liver, it has three distinct pathways for discharging excess copper. The first involves ceruloplasmin, an important transport protein for copper in hepatocytes; about 90% of copper in plasma is bound to ceruloplasmin (Cousins, 1985). A second and more important route for hepatic copper excretion is the bile. It is generally believed that most biliary copper originates from lysosomes in hepatocytes (Thornburg, 2000). The third pathway is its binding with metallothioneins or other low or high molecular weight proteins (Papadimitriou and Loumbourdis, 2003).

The decline in gall bladder Cu concentrations at 14 days' exposure indicates that, most probably, a large part of copper sequestration changes direction from the gall bladder to the kidneys and/or other parts of the body. This decrease in copper concentrations in the gall bladder is accompanied by an increase in copper in the kidneys. Interestingly, as is shown in Table 1, the decrease in copper concentrations in the gall bladder at 14 days' exposure equals the increase in copper concentrations in the kidneys.

Comparison of animals exposed to Cu alone with those of Cu+TTM treated animals, showed essential differences in the first 7 days of exposure; there was a significant increase in copper concentrations in the kidneys, gall bladder and skin

Table 2. Glutathione (μg/g wet wt) (mean±SD) concentration in the liver of the frog *Rana ridibunda* exposed to Cu alone or to Cu+TTM for 7 and 14 days. Sample size in parenthesis.

Days of exposure		
0 (control)	7	14
	Cu alone	
1.02±0.19 (5)	1.72± 0.56 (8)[a]	1.56±0.28 (7)[b]
	Cu+TTM	
	1.81±0.42 (8)[a]	2.65±0.50 (6)

a=statistically significant difference between control and 7d in Cu alone, 7d in Cu+TTM and 14d in Cu+TTM exposure.
b=statistically significant difference between 7d and 14d in Cu alone

of Cu+TTM exposed animals, accompanied by a decrease in copper concentrations in the liver and large intestine. At 14 days of exposure to Cu+TTM there was a further significant increase in the kidneys, no increase in the liver and a decrease in all other organs.

These results show that TTM can remove deposited hepatic Cu, thus resulting in a reduction in hepatic Cu. According to Sugawara et al (1995), TTM first acts on Cu other than MT-bound Cu. TTM has also been used in Cu poisoned sheep (Gooneratne et al, 1989). In that study, intravenous administration of TTM prevented the development of chronic Cu poisoning in the sheep, reducing the rate of accumulation of Cu in the liver in Cu-dosed animals but increasing the Cu content of kidneys. The results of the present study, particularly those of Cu+TTM and seven days exposure, are very similar, suggesting that the mechanism for Cu removing from the liver and accumulating in the kidneys may be similar to that of sheep. In a previous study (Papadimitriou and Loumbourdis, 2003), it was found that, at 15 days exposure, the concentration of kidney Cu similarly increased, suggesting that a second routing of Cu detoxification might took place.

The removal of large quantities of copper by bile is a phenomenon observed in the animals exposed to Cu alone, as well as in the animals exposed to Cu+TTM. In the second case, the values are much greater, suggesting that TTM can remove more quantities of copper, most probably that bound to metallothionein. In spite of the findings of Komatsu et al (2000), that the Cu excreted by bile in Cu exposed Wistar rats is negligible, we found that particularly in the first seven days of exposure to Cu and Cu+TTM, large quantities of Cu are excreted by bile. Grossel et al (1998), working with the fish *Oncorhynchus mykiss*, also came to the same conclusion. Cu elimination from the liver and excretion by bile is an important route for Cu detoxification.

GSH is used as a biomarker of pollution of organisms by heavy metals. It is

capable of creating complexes and of detoxifying cations of heavy metals as soon as they enter the cells, thus constituting the first line of defense against the cytotoxicity of heavy metals (Singhal et al., 1987). The increase in GSH concentration observed after the first 7 days of exposure to Cu alone, shows that GSH is formed as a result of a reaction to the accumulation of copper, similarly as a first line of defense. The decrease in GSH concentration after 14 days' exposure, probably shows that the mechanism of its synthesis has been disturbed. Indeed, in a previous study (Papadimitriou and Loumbourdis, 2002) we found that exposure of frogs to high copper concentrations resulted in a decrease in GSH concentration and an accompanying increase in MDA. It is well-known that MDA is the result of lipid peroxidation with the destruction of cell membranes as a final consequence. This decrease in GSH may be the consequence of destruction of the system of GSH synthesis. Thus the continuous increase in GSH in the animals exposed to Cu+TTM, most probably is indicative of the decrease in Cu load in the liver, thus resulting in the undisturbed removal of copper.

REFERENCES

Brewer GJ, Dick RD, Yusbasiyan-Gurkin V,. Tankanow R, Young AB, Kluin KJ (1991) Initial therapy of patients with Wilson's disease with tetrathiomolybdate. Arch Neurol 48: 42-47.

Gooneratne SR,. Howell JMc.C,. Gawthorne JM, Kumaratilake JM (1989) Subcellular distribution of copper in the kidney of normal, copper-poisoned, and thiomolybdate-treated sheep. J Inorg Biochem 35: 23-36.

Grossel MH, Hogstrand C, Wood CM (1998) Renal Cu and Na excretion and hepatic Cu metabolism in both Cu acclimated and non acclimated rainbow trout (*Oncorhynchus mykiss).* Aquat Toxicol 40: 275-291.

Komatsu Y, Sadakata I, Ogra Y, Suzuki KT (2000) Excretion of copper complexed with thiomolybdate into the bile and blood in LEC rats. Chem Biol Interact 124: 217-231.

Li Y, Togashi Y, Sato S, Emoto T, Kang J.-H Takeichi N, Kobayashi H.,. Kojima Y,. Une Y &. Uchino J (1991) Spontaneous hepatitis copper accumulation in Long-Evans Cinnamon rats with hereditary hepatitis. A model of Wilson's disease. J Clin Invest 87: 1858-1861.

Ogra Y, Ohmichi M, Suzuki KT (1996) Mechanisms of selective copper removal by tetrathiomolybdate from metallothionein in LEC rats. Toxicology 106: 75-83.

Papadimitriou E, Loumbourdis NS (2002) Exposure of the frog *Rana ridibunda* to copper: Impact on two biomarkers, lipid peroxidation, and glutathione. Bull Environ Contam Toxicol 69: 885-891.

Papadimitriou E, Loumbourdis NS (2003) Copper kinetics and hepatic metallothionein levels in the frog *Rana ridibunda* after exposure to $CuCl_2$. Biometals 16: 271-277.

Richardson, R.J,. Murphy S.D (1975) Effect of glutathione on tissue deposition of methylmercury in rats. Toxicol Appl Pharmacol 31: 505-519.

Singhal RK, Anderson M.E, Meister A (1987) Glutathione, a first line of defense against cadmium toxicity. FASEB J 1: 220-223.

Sugawara N, Motoyuki Y, Sugawara C (1995) Excretion of copper in copper-overloaded Fisher and Long-Evans cinnamon rats treated with tetrathiomolybdate. J Trace Elem Exp Med 8: 1-10.

Suzuki K.T, Yamamoto KY, Ogra Y, Kano S, Aoki Y (1994) Mechanisms for removal of copper from metallothionein by tetrathiomolybdate. J. Inorg. Biochem, 54, 157-165.

Thornburg LP (2000) A perspective on copper and liver disease in the dog. J Vet Diagn Invest 12: 101-110.

Zalups RK, Lash LH (1996) Interactions between glutathione and mercury in the kidney, liver and blood. In: Toxicology of metals. Ed: Chang L.W. Lewis publishers. New York,. pp 145-163.

Ziche, M, Jones J, Gullino PM (1982) Role of prostaglsndin E and copper in angiogenesis. J Nat Cancer Inst 69: 475-482.