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Aquatic Toxicology 50 (2000) 109–124

AQUATIC TOXICOLOGY

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Biochemical and (ultra)structural hepatic perturbations of *Brachydanio rerio* (Teleostei, Cyprinidae) exposed to two sublethal concentrations of copper sulfate

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Received 3 April 1999; received in revised form 5 October 1999; accepted 12 October 1999

Abstract

The aim of this study was to compare biochemical and (ultra)structural perturbations induced by Cu in the liver of *Brachydanio rerio* exposed for 14 days to sublethal concentrations of copper and then replaced for 14 days to clean water. Toxicity of Cu was clearly demonstrated: simultaneously to Cu accumulation, the liver developed large lysed areas and hepatocytic alterations. However, the majority of the parenchyma was composed of functional dark hepatocytes displaying typical feature of increased metabolism: development of rough reticulum, increase in size of nucleus and nucleolus, glycogenic depletion. Increase in hepatic protein content and of anti-oxidative defences (glutathione content, catalase and glutathione-*S*-transferase (GST) activities) indicated that the overall response of the liver was adaptative. In all hepatocytes a cord-like structure of the nucleoli was suspected to be associated to metal deposition. After 14 days depuration, the liver still contained high Cu concentrations and the hepatic alterations were not reversed. Such complementary studies are necessary for a better understanding of the deleterious effects of pollutants and for the development of biomarkers for metal toxicity.

Keywords: Cu; Liver; Oxidative stress; Cytology; Fish

1. Introduction

Copper pollution appears in the aquatic environment from natural and anthropogenic sources such as mine washing, agricultural leaching (GERBE, 1998) and direct application as algicide and molluscicide (Dueck et al., 1987). Cu is frequently used for aquatic vegetation control in fish culture systems; usually it is applied in concentration between 0.3 and 2.0 mg l^{-1} (Nor, 1987; Segner and Braunbeck, 1990a). Although Cu is a trace element essential to life it is one of the most toxic heavy metals (Nor, 1987; Tòth et al., 1996). Fish are an invaluable test model in environmental toxicology for the determination of lethal and

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0166-445X/00/$ - see front matter
PII: S0166-445X(99)00090-9

sublethal effects of aquatic pollutants. Monitoring biochemical and histo-cytological changes in fish liver is a highly sensitive and accurate way to assess the effects of xenobiotic compounds in laboratory and field studies (reviews of Hinton and Laurén, 1990; Biagianti-Risbourg, 1997). Liver is known to be the primary organ for copper storage in fish (Tòth et al., 1996; Perkins et al., 1997). Data on metal effects on hepatic metabolism in fish are rather numerous (Viarengo, 1985; Grosell et al., 1998). Metals can either increase or decrease hepatic enzyme activities (e.g. catalase, superoxyde dismutase, glutathion peroxydase, glutathion reductase) and protein content depending on the metal type and concentration, fish species, water hardness and length of exposure (Radi and Matkovics, 1988; Rodriguez-Arriza et al., 1993; Tòth et al., 1996; Furuno et al., 1996; Shimizu et al., 1997; De Boeck et al., 1997). Although the toxicological effects of copper on fish are well documented, the variability of the reported results are large (Hodson et al., 1979; Saxena et al., 1982; Segner and Braunbeck, 1990a). Histo-cytological studies have shown an important variability in hepatic responses to variable Cu concentrations and exposure duration. The reported changes are either adaptive as lysosomal proliferation, and reticulum development (Segner and Braunbeck, 1990a) or degenerative as losses in integrity of mitochondrial, plasma or nuclear membranes, fragmentation of endoplasmique reticulum and development of autophagic vacuoles (Leland, 1983; Bunton et al., 1987; Roncero et al., 1992). Nevertheless, the simultaneous analysis of both biochemical and ultrastructural responses of fish liver to metallic stress are particularly scant. Thus it is difficult to assess if the ultrastructural events interpreted as adaptive responses of the liver are really associated to a development of antioxydative protective systems in relation with an enhanced protein synthesis. In this study we presented a chronological analysis of the oxidative stress and the structural perturbations in the liver of the female of *Brachydanio rerio* exposed to sublethal concentrations of Cu.

2. Materials and methods

2.1. Fish maintenance

B. rerio (250 females total weight, 0.20 g, S.D. 0.07 g and total lenght, 3.24 cm, S.D. 0.52 cm) were purchased from a pisciculture centre of tropical fish in Reims. Histological analysis of ovaries showed that the liver of these females were not involved in exogenous vitellogenesis since about 90% of the ovocytes were at stage V1 (Bruslé, 1982). Prior to experiments fish were acclimatized to laboratory conditions for 3 weeks in bottled water from a Champagne spring (eau 'Cristaline', source des Grands Bois, 51170 Fismes, France); pH ,8 $\pm$ 0.5; temperature, 20°C $\pm$ 1; hardness, 300 mg l^{-1}; [Ca^{2+}], 124 mg l^{-1}; [Mg^{2+}], 25 mg l^{-1}; [Na^{2+}], 11 mg l^{-1}; [K^{+}], 3.5 mg l^{-1}; [HCO_{3-}], 399 mg l^{-1}; [Cl^{-}], 16 mg l^{-1}; [NO_{2-}] $<$ 0.01 mg l^{-1}; and [SO_4^{2-}], 60 mg l^{-1}. Two series of three lots of fish (control, fish exposed to two different Cu concentrations), each including 40 individuals, were kept in aquaria with a density of four fish l^{-1}. Fish were submitted to actual concentrations of 40 $\pm$ 5 or 140 $\pm$ 30 μg Cu l^{-1}as $CuSO_4$. Copper sulfate is frequently used in agricultural and viticultural treatments for its antifungus properties, it is present in many fertilizers and pesticides and thus can contaminate aquatic ecosystems (Jurado, 1983; GERBE, 1998). Cu concentrations in aquaria were adjusted by addition of aliquots of a 0.5 g l^{-1} $CuSO_4$ solution. Water Cu concentrations in each aquarium were controlled every 5 h by atomic absorption spectrometry with electrothermical atomisation (Spectrometer Varian AA20). Tests were conducted in semi-static conditions (water renewed every day). After 14 days exposure, 25 fishes were placed in clean water. After 7 days, 14 days exposure and 14 days exposure followed by 14 days depuration, fish were killed by decapitation without being anaesthetised and their livers were used for

1. Histo-cytology studies (for each sampling five livers were examined in histology and three in cytology).
2. Determination of Cu content (two lots of five livers pooled).

3. Biochemical analysis (three lots of three livers pooled).

Histo-cytological procedures were immediately performed. Samples used for biochemical analysis were stored for a week at −80°C.

2.2. Histo-cytology

The liver of five fish from each treatment were used for histology. Livers were immersed in Bouin's fluid for 24 h, rinsed in distilled water, dehydrated and embedded in paraffin. Sections of 6 μm were stained with nuclear fast red and picro-indigo carmine. For cytology studies, liver samples were fixed by immersion (3 h at 4°C) in 3% glutaraldehyde buffered to pH 7.2 with 0.1 M sodium cacodylate. Specimens were washed three times in buffer and post fixed in 1% buffered osmium tetroxide (1 h at 4°C). They were rinsed in buffer, dehydrated in a graded acetone serie and embedded in spurr. Semi-thin sections were stained with methylene blue-azur II (Richardson et al., 1960). Ultrathin sections were stained with uranyl acetate and lead citrate according to Reynolds (1963) and examined in a Philips 201 electron microscope (CIME, Jussieu Paris). Morphometric parameters were measured on electron micrographs (minimum of measures: 20 nuclei, 15 nucleoli and 200 mitochondria of each group).

2.3. Cu hepatic content

For each sampling, two pools of five livers were dried and their organic matter was extruded using 200 μl of pure nitric acid (10 h at 70°C) diluted with 2 ml of water. The content of Cu was determined in duplicate (number of measures: $n=4$) by atomic absorption spectroscopy with electrothermical atomisation (Varian spectra AA20). Hepatic concentrations of Cu were expressed as μg g^{-1} d.w. (dry weight) ± S.D. Since Cu is an essential element, the Cu content in control fish liver have been taken into consideration. Therefore the bioaccumulation factor (BF) was calculated with the following formula: $BF = ([Cu]_{liver} - [Cu]_{liver\ of\ control\ group})/[Cu]_{water}$, expressed as mg Cu kg^{-1} of liver (d.w.) per mg Cu l^{-1} in water.

2.4. Hepatic protein, glutathione content, catalase and GST activities

Three pools of three livers were homogenised in cold 0.1 M sodium phosphate buffer (pH 7) with a Potter homogeneiser (Servodyne wiser head, Cole Parmes, Nilen IC) centrifuged 15 min at $2300 \times g$ (4°C) and the total protein content of supernatants was determined according to Bradford (1976) using bovine albumin (Sigma) for calibration ($n=6$). Protein was expressed in mg g^{-1} fresh weight (f.w.) ± S.D. Catalase activity of supernatants was determined according to Teisseire et al. (1998) ($n=6$). Catalase activity was expressed as millimoles of hydrogen peroxide consumed per mg of protein and per min ± S.D.

A volume of 40 μl of the supernatants were used for the determination of total glutathione content. Tests were realised ($n=6$) in phosphate buffer (50 mM, pH 7.5) with 2.5 mM of ethylenediaminetetraacetic acid (EDTA), 0.5 mM of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 0.1 mM of NADPH and 0.5 unit of glutathione reductase for a final volume of 1 ml. Evolution of 412 nm absorbance was followed during an incubation of 3 min and the total glutathione content was obtained using a glutathione reduced (ICN) calibration procedure (Akerboom and Sies, 1981).

A volume of 50 μl of supernatants were used to measure ($n=6$) the GST activity by following the complexation of reduced glutathione (GSH) with 1-chloro 2,4-ditrinobenzene (CDNB) during 3 min according to Habig et al. (1974). The evolution of the optical density (OD: 340 nm) is a linear function of the enzymatic content as much as its slope was inferior to 0.05 unit of OD per min. The measure was thus made during 3 min with 1 mM of GSH (ICN) and 1 mM of CDNB in phosphate buffer (0.1 M, pH 6.5) for a final volume of 2 ml. The activity of GST was expressed as nanomoles of CDNB conjugated per min per mg of protein ± S.D.

Table 1
Cu hepatic concentration in zebrafish exposed to Cu

Exposure	Liver [Cu] in control group	Liver [Cu] in fish exposed to 40 μg Cu l^{-1}		Liver [Cu] in fish exposed to 140 μg Cu l^{-1}	
	μg g^{-1} dw ± S.D.	μg g^{-1} dw ± S.D.	BF	μg g^{-1} dw ± S.D.	BF
7 days	12.0 ± 0.3	18.5 ± 0.3	162	35.1 ± 0.35	154
14 days	11.9 ± 0.3	19.6 ± 0.3	192	46.5 ± 0.4	231
14 days depuration	11.7 ± 0.3	18.7 ± 0.3		45.2 ± 0.4	

3. Results

3.1. Liver Cu content

Control fish presented an hepatic Cu concentration of about 11.9 ± 0.7 μg Cu g^{-1} (Table 1), this value is in accordance with the values of normal hepatic Cu content in fish liver (Segner, 1987; Roncero et al., 1992). At both water Cu concentrations, the metal accumulated rapidly in liver during the first 7 days of the test (+50 or +200% in livers of fish respectively exposed to 40 or 140 μg Cu l^{-1}). After this period, bioaccumulation increased but only slightly until the end of the experiments (+10 or +30% in livers of fish exposed to 40 or 140 μg Cu l^{-1}, respectively). About the same bioconcentration factors were measured at both Cu water concentrations (Table 1). No significant decrease of liver Cu content was detected when fish were replaced for 14 days in clean water: the decrease was about 6% in fish previously exposed to 40 and 140 μg Cu l^{-1}.

3.2. Liver structure and ultrastructure

3.2.1. Control fish

The homogenous parenchyma of *B. rerio* liver, was composed of hepatocytes arranged in typical tubular architecture, the liver structure and ultrastructure (Figs. 1 and 2) was quite similar to that described by Braunbeck et al. (1990) and Hinton and Couch (1998). The hepatocytes contained extensive glycogen fields enclosing few α-glycogen particles (G) and rare small lipid droplets (L). Stacks of cisternae of rough endoplasmic reticulum (R) occupied the perinuclear and pericanalicular areas of the cell and smooth endoplasmic reticulum was never observed. The mitochondria were of appreciable morphological diversity; regular spherical and oval forms were by far the most common. Mitochondrial bodies, relatively few in number (Table 3), were easily perceptible as amorphous grains of rather high electron-density. Typically with Cyprinidea, bile canaliculi (Fig. 2) were intercellular, mostly located between two hepatocytes, and also intracellular (c), invaginating the hepatocyte towards the nucleus area. The centrally-located and spherical nucleus (diameter: 5.5 ± 0.5 μm) contained little marginal heterochromatin and a relatively compact and dense nucleolus of about 1.4 μm in diameter (Fig. 2).

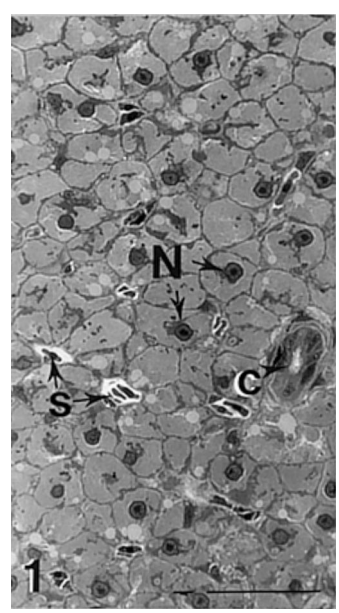

Fig. 1. Section of paraffin-embedded liver in control zebrafish. C, bile canal; N, nucleus; S, sinusoide (bar = 50 μm).

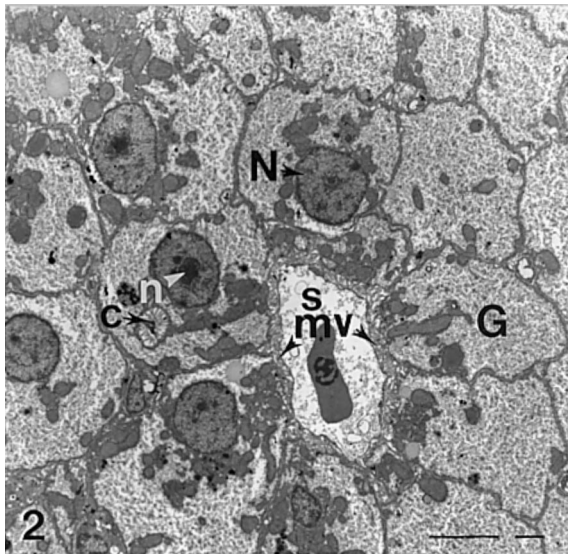

Fig. 2. Typical cord-like arrangement of hepatocytes around a sinusoide in control fish liver. c, bile canaliculus; G, glycogen; li, lipid; m, mitochondria; mv, microvilies; N, nucleus; n, nucleolus; s, sinusoide (bar = 10 μm).

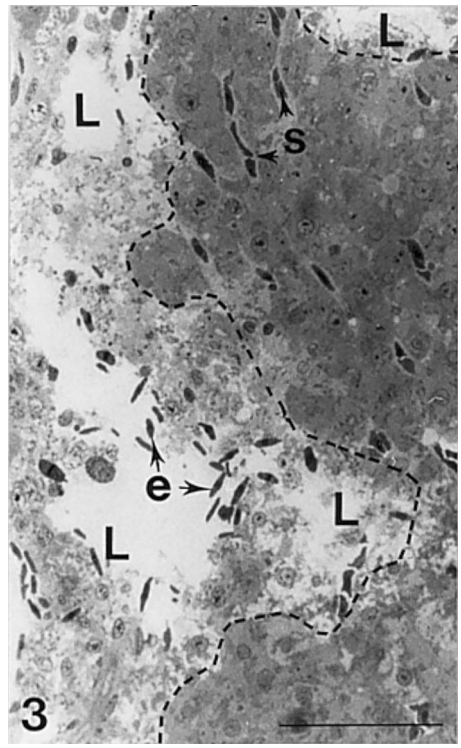

Fig. 3. Hepatic parenchyma of zebrafish exposed 7 days to 40 μgCu. l^{-1} showing large lysed areas. E, erytrocyte; L, lysed area; s, sinusoide; - - -, limite of lysed area (bar = 70 μm).

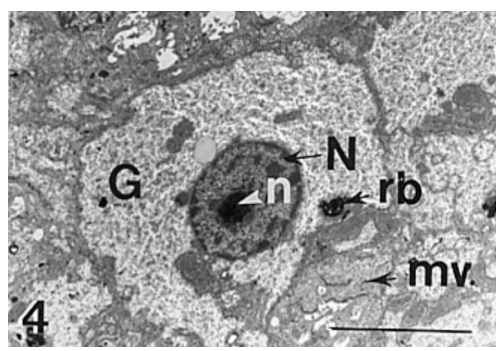

Fig. 4. Ultrastructure of clear hepatocytes in fish exposed for 14 days to 40 μg Cu l^{-1}. The cytoplasm contained glycogen pools with slightly reduced number of particles and few membranous organelles. G, glycogen; mv, microvillies; n, nucleolus; rd, residual body (bar = 8 μm).

3.2.2. Treated fish

After 7 days exposure to both Cu concentrations, areas of liver parenchyma near hepatic veins were completely lysed (Fig. 3). Whatever the duration of exposure to Cu these areas devoid of cell were more extensive and numerous after treatment of the fish by 140 μg Cu l^{-1} than by 40 μg Cu l^{-1}. For example, on paraffin sections, these lysed areas covered about 50% of the total liver surface of fish treated 14 days by 140 μg Cu l^{-1}, whereas it never exceeded 40% in fish exposed 14 days to 40 μg Cu l^{-1}. At the ultrastructural level, the lysing hepatocytes (Fig. 5a) contained nuclei at various stages of pycnosis, disrupted plasma membranes, cytoplasmic membrane residues, few lysosomes, residual bodies and swollen mitochondria with electron-dense matrix exhibiting typical cristae (Fig. 5b). Concentric arrangements of hepatocytes (Fig. 6), scant in control livers, increased in number and size during Cu exposure. In these structures vascularization (veins, sinusoids) was highly reduced, almost absent.

After 7 days exposure to the low concentration of 40 μg Cu l^{-1} the remainder of the parenchyma was composed of hepatocytes similar in size, and glycogen and granular reticulum (R) content to those described in control hepatocytes (Tables 2

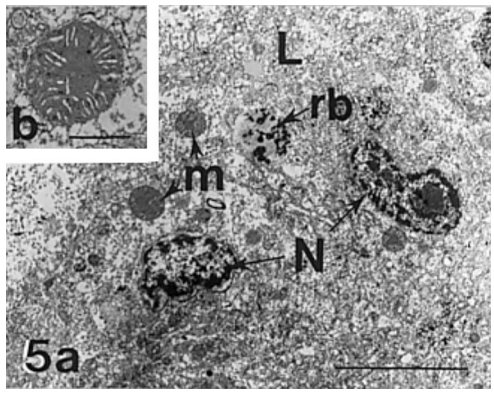

Fig. 5. Zebrafish exposed for 14 days to 40 μg Cu l^{-1}. The lysing hepatocytes (a) displayed picnotic nucleus, residual bodies and altered mitochondria (b) with electron-dense matrix, numerous mitochondrial bodies but typical cristae. L, lysed area; m, mitochondria; N, nucleus; rb, residual body (a, bar = 5 μm; b, bar = 2 μm).

and 3). However, nearly all the mitochondria were affected (Table 3) but only slightly (increase in length, few disrupted cristae, slightly increased electron-density of the matrix). After 14 days exposure to 40 μg Cu l^{-1}, 7 or 14 days exposure to 140 μg Cu l^{-1} as well as during the depuration period, the unlysed part of the parenchyma was heterogeneous and composed of two types of hepatocytes:

- Few large and clear hepatocytes (Table 2), mostly located in the periphery of the lysed areas. They often presented large glycogen pools containing few glycogen particles, altered mitochondria (broken or containing disrupted cristea) and very reduced granular reticulum (Figs. 4, 11 and 12, Table 3).
- Very numerous small basophilic hepatocytes (Table 2). They were roundish in outline (Figs. 7 and 10) and characterised by developed rough endoplasmic reticulum which occupied large parts of the cell as long and numerous parallel arrays of cisternae (Figs. 8 and 9, Table 3).

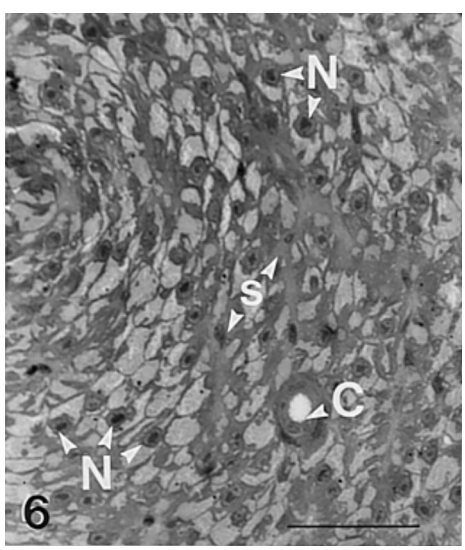

Fig. 6. Fish exposed for 7 days to 140 μg Cu l^{-1}. Concentric arrangement of hepatocytes containing nuclei with high electron-dense nucleolus. C, bile canal; N, nucleus; s, sinusoide (bar = 50 μm).

During the exposure period, whereas the density of glycogen particles did not vary much, glycogen depletion was essentially detectable by the clear surface reduction of the glycogen pools (Figs. 8 and 9). This event was concentration- and time-dependent (Table 3). The increase in number and size of lipid inclusions was observed only after exposure to 140 μg Cu l^{-1} and was comparable to the lipidic inclusions observed after a 14 day depuration (Figs. 13 and 14). However the lipidic content of hepatocytes was very different within adjacent cells and within individuals. The chondriom of the basophilic hepatocytes was also affected (Table 3). Mitochondria were frequently pleïomorphic and often displayed swollen electron-lucent areas between the outer and the inner mitochondrial membrane (Fig. 9), or showed an increase in their matrix electron-density (Fig. 8,Table 3). Additionally, the number of intramitochondrial bodies significatively increased with time and Cu concentrations (Table 3). The number of peroxisomes were not increased neither in basophilic nor in clear hepatocytes.

The most original feature was revealed in the nuclear structures. On semi-thin sections, nuclei appeared more basophiles (Figs. 6 and 7). This increased basophilia was revealed especially for the highest Cu water concentration and for the longest duration of exposure (14 days). Ultra-

Table 2
Morphometric parameters of hepatocytes from Cu treated zebrafish

Group of Danios		Clear hepatocytes	Dark hepatocytes	Nucleus surface μm²	Nucleolus diameter μm
		Surface μm²	Surface μm²		
Control		225 ± 4		30 ± 5	1.4 ± 0.4
40 μg Cu l^{-1}	7 days	236 ± 10		45 ± 5	2.1 ± 0.5
	14 days	243 ± 20	202 ± 20	41 ± 5	1.8 ± 0.4
	14 days depuration	250 ± 20	192 ± 25	44 ± 5	1.7 ± 0.4
140 μg Cu l^{-1}	7 days	240 ± 20	200 ± 20	53 ± 8	2.8 ± 0.5
	14 days	250 ± 20	190 ± 25	57 ± 7	3.1 ± 0.6
	14 days depuration	250 ± 20	180 ± 25	58 ± 5	2.2 ± 0.4

structure changes of the nuclear constituents were obvious: increase in size of the nucleus and nucleolus (Table 2), accumulation of heterochromatin (Fig. 8) and electron-dense cord-like arrangement of the nucleolus (Figs. 4, 8 and 9, Table 3).

3.2.3. Fish exposed for 14 days to Cu and replaced for 14 days in clean water

At both Cu concentrations, Cu contained in the fish liver did not decrease during the depuration period, thus no significant hepatic decontamination was revealed and hepatic lysis was still observable, but did not increase. At day 14 of depuration, concentric arrangement of hepatocytes remained similar in aspect and size to those described after 14 days exposure. The parenchyma was still composed of two types of hepatocytes: few clear and large hepatocytes and numerous small basophile hepatocytes (Fig. 10). Clear hepatocytes, located near the lysed areas, showed large glycogen pools with rarefied particles and roundish mitochondria with electron-lucent matrix and lysed cristea; their nucleus displayed a cord-like nucleoli (Figs. 11 and 12). The basophile hepatocytes were characterised by a more developed granular reticulum, large lipid inclusions often associated with pleïomorphic mitochondria (Fig. 14) and large and strongly stainable nuclei (Fig. 13). Parallel stacks of granular endoplasmic reticulum cisternae occupied large parts of the hepatocytes and the glycogen pools have a more reduced surface (Fig. 14). The nuclei of both types of hepatocytes displayed a prominent nucleolus of high electron-density (Table 3) with a clear cord-like structure (Figs. 11 and 14).

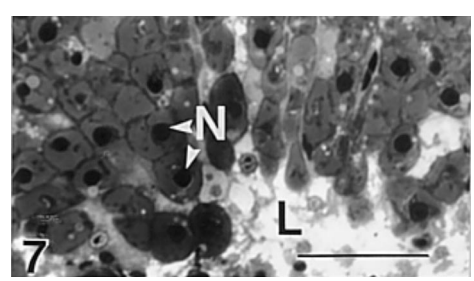

Fig. 7. Parenchyma lysis in the liver of zebrafish exposed for 14 days to 140 μg Cu l^{-1}. At the periphery of the lysed areas dark hepatocytes showed roundish outline and nuclei of high electron-density. L, lysis; N, nucleus (bar = 40 μm).

Table 3
Semi-quantitative analysis of hepatocytic perturbations in Cu treated zebrafish[a]

Concentration of Cu (μg l^{-1})	0	40					140					
Duration of exposure	Control	7 days	14 days		Depuration		7 days		14 days		Depuration	
Clear (C) or dark (D) hepatocytes	−	−	C	D	C	D	C	D	C	D	C	D
Lysosomes	+	+	+	+	++	++	+	+	++	++	++	++
Storage material												
Glycogen	+++	+++	+	++	+	++	+	++	+/−	+	+/−	+/−
Lipid	+/−	+/−	+/−	+	+/−	++		+	−	++	−	+++
Endoplasmic reticulum												
Parallel stacks of cisternae	+	+	−	+	−	++	−	++	−	+++	−	+++
Dilation of cisternae	−	−		−		−		−		−		−
Mitochondria												
Altered mitochondria	+/−	+	+++	+	+++	++	+++	+	+++	++	+++	++
Mitochondrial body:	+	++		++		++		++		+++		+++
Number per mitochondria	5 ± 1.5	8 ± 2	10 ± 2	8 ± 2	12 ± 2	9 ± 2	10 ± 2	8 ± 2	15 ± 2	12 ± 3	16 ± 2	15 ± 3
Nucleus												
Nucleus size	+	+	++	+	++	+	++	++	+++	++	+++	++
Nucleolus size		+	++	+	++	+	+++	+++	+++	+++	+++	++
Cord-like nucleolus	−	++	+++	++	+++	+++	+++	++	+++	+++	+++	+++

[a] The relative development of intra-cellular elements were coded from − (absent) to +++ (important development).

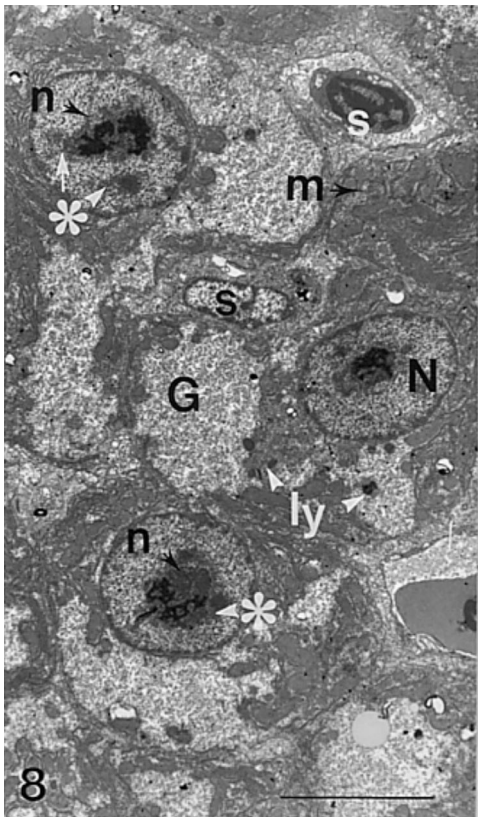

Fig. 8. Ultrastructure of dark hepatocytes in fish exposed to 140 μg Cu l^{-1}. After 7 days exposure dark hepatocytes displayed developed rough endoplasmic reticulum, reduced glycogen deposite, heterochromatin-rich nucleus (asterisk) and electron dense nucleolus with a cord-like structure. (bar = 7 μm) G, glycogen; li, lipid; ly, lysosome; mb, mitochondrial bodies; m, mitochondria; mv, peri-sinusoidal microvilies; N, nucleus; n, nucleolus; R, rough endoplasmic reticulum; s, sinusoide.

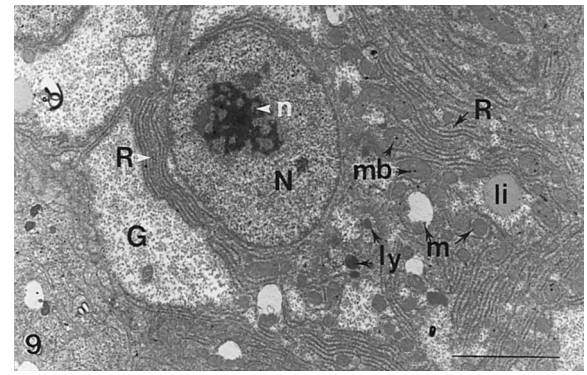

Fig. 9. Ultrastructure of dark hepatocytes in fish exposed to 140 μg Cu l^{-1}. After 14 days exposure, reticulum in association with altered mitochondria occupied a large part of the cytoplasm and the cord-like structure of nucleolus was clear (bar = 4 μm). G, glycogen; li, lipid; ly, lysosme; mb, mitochondrial bodies; m, mitochondria; mv, peri-sinusoidal microvilies; N, nucleus; n, necleolus; R, rough endoplasmic reticulum.

3.3. Biochemical perturbations

3.3.1. Total protein content

The liver of control fish contained a stable proteic content of 120 ± 10 mg protein g^{-1} (f.w.). The increase of hepatic total protein was Cu concentration- and time-dependent (Fig. 15a). After 14 days of exposure the protein level reached + 64 and + 136% in fish exposed respectively to 40 and 140 μg Cu l^{-1}. Regardless to Cu concentrations this high protein level persisted after 14 days depuration.

3.3.2. Catalase activity

Catalase activity in control fish was stable (6 ± 1 mmol H_2O_2 mg protein $^{-1}$ min^{-1}). This enzyme activity increased (Fig. 15b) with the Cu concentration and the duration of exposure. It reached

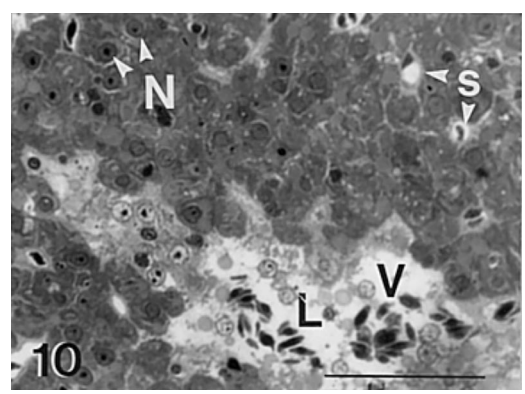

Fig. 10. Fish previously exposed for 14 days to 40 μg Cu l^{-1} then replaced for 14 days in clean water. The hepatic parenchyma showed perivascular lysis, few large and clear hepatocytes and contained numerous dark hepatocytes. (bar = 70 μm). L, lyse; N, nucleus; s, sinusoides; V, vein.

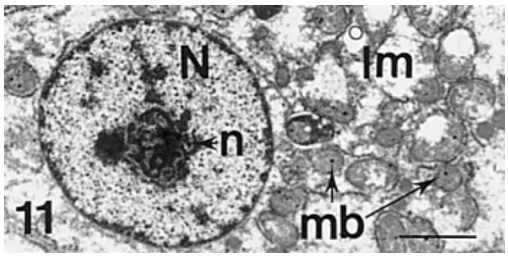

Fig. 11. Fish previously exposed for 14 days to 40 μg Cu l^{-1} then replaced for 14 days in clean water. Ultrastructure of clear hepatocyte displaying altered mitochondria with numerous mitochondrial bodies, poorly developed reticulum and cord-like nucleolus. (bar = 2 μm). lm lysed mitochondria; mb, mitochondrial bodies; N, nucleus; n, nucleolus; s, sinusoides; V, vein.

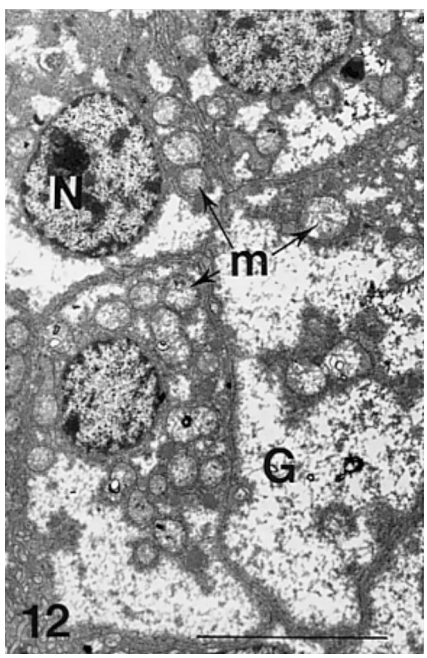

Fig. 12. Fish previously exposed for 14 days to 140 μg Cu l^{-1} then replaced 14 days in clean water. Clear hepatocytes showed very altered mitochondria with lysed cristae, reduced glycogen content and few endoplasmic reticulum cisternae. (bar = 8 μm). G, glycogen; m, itochondria; N, nucleus; n, nucleolus;

+ 830 or + 3400% in fish exposed 14 days to 40 or 140 μg Cu l^{-1}, respectively. After 14 days in clean water the catalase activity still increased in fish previously exposed to 140 μg Cu l^{-1} (+ 79%) but was lowered of − 58% in fish previously exposed to 40 μg Cu l^{-1} (Fig. 15b).

3.3.3. Total glutathione content

Glutathione content was rather stable in control group (30 ± 5 mg g^{-1} protein). During the exposure period, the quantity of total glutathione increased to about 60 or 80% in fish exposed for 14 days to 40 or 140 μg Cu l^{-1}, respectively. Whereas in fish exposed to 40 μg Cu l^{-1} the glutathione concentration increased with time, it reached a steady-state at day 7 in fish exposed to 140 μg Cu l^{-1}. At the end of depuration period, higher glutathione levels were measured (Fig. 15d).

3.3.4. GST activity

Liver GST activity was stable in control group (191 ± 14 nmol CDNB min^{-1} mg $protein^{-1}$) and increased with time and Cu concentration in treated fish (Fig. 15c). The hight values of GST

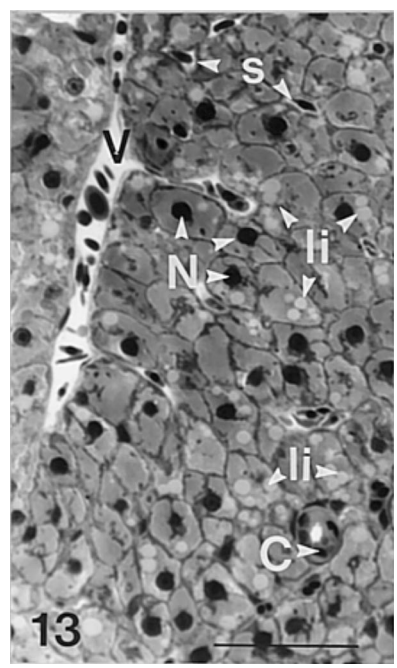

Fig. 13. Fish previously exposed for 14 days to 140 μg Cu l^{-1} then replaced for 14 days in clean water. Numerous dark hepatocytes displaying important lipid inclusions and very electron-dense nucleus (bar = 50 μm). C, bile canal; li, lipid; N, nucleus; V, vein.

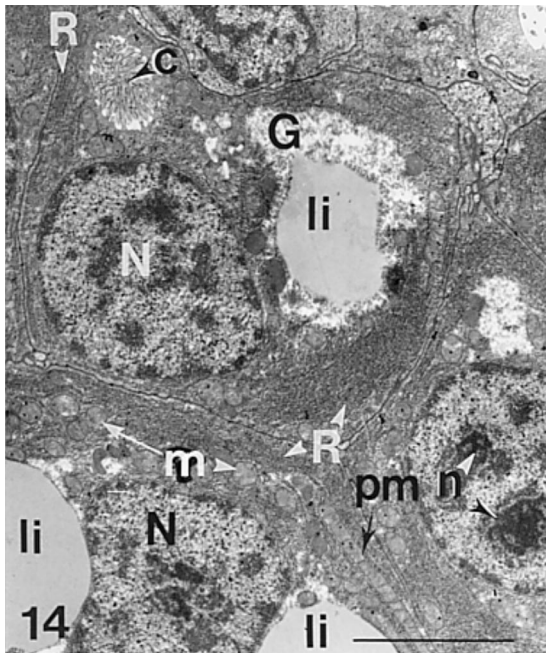

Fig. 14. Fish previously exposed for 14 days to 140 μg Cu l^{-1} then replaced 14 days in clean water. Ultrastructure of dark hepatocytes showing huge lipid droplets, numerous stacks of rough endoplasmic reticulum cisternae associated with pleiomorphic mitochondria and large nuclei with cord-like nucleolus. (bar = 4 μm). C, bile canal; c, canaliculus; G, glycogen; li, lipid; m, mitochondria; N, nucleus; n, nucleolus; pm, pleiomorphic mitochondria; R, rough endoplasmic reticulum.

activity measured after 14 days of exposure (+60% in fish exposed to 40 μg Cu l^{-1}, +190% in individuals treated by 140 μg Cu l^{-1}) persisted until the end of the depuration period (Fig. 15c).

4. Discussion

The present study shows obvious biochemical and (ultra)structural perturbations in livers of *B. rerio* exposed to sublethal concentrations of Cu. Copper is present as a cofactor in various enzymes (e.g. Cu, Zn-superoxide dismutase, cytochrome oxidase...) but as a toxic substance is able to bind the cysteine side chains of proteins and to form multidentate complexes with histidine and tryptophan side chains of proteins. According to Tòth et al. (1996) this is the molecular basis of the toxicity of copper ions. As previously shown in numerous vertebrates (Tòth et al., 1996; Grosell et al., 1998) copper mainly accumulates in liver where its biological effects are important. In fish exposed to 140 μg Cu l^{-1} B.F. was only slightly higher than that of fish exposed to 40 μg Cu l^{-1}. The increase of BF with Cu water concentration was not linear. The Cu hepatic accumulation was biphasic, important during the first 7 days exposure but moderate after. This variation could be partially explained by a delayed increase of hepatic Cu excretion (Grosell et al., 1998). In *Chanos chanos* and in *Fundulus heteroclitus* individual BF decrease with increasing water copper or other metal concentrations (Chermoff and Dooley, 1979; Segner and Braunbeck, 1990a). According to these authors fish do not develop regulation of metal burden below a certain threshold and low metal water concentrations can result in high bioaccumulation.

The liver parenchyma of treated zebrafish presented lysed areas. Although initially located near veins, they enlarged and became massive at the end of the exposure period. Cu is known to induce the formation of reactive form of oxygen which can produce enzymatic deactivation, lipidic peroxidation and DNA damage (Regoli et al., 1997; Segner and Braunbeck, 1998). Cu have a great capacity to alter membrane structural lipids and could provoke membranous disruption (Roncero et al., 1992). The lysis distribution may reflect a heterogeneous distribution of Cu in the parenchyma. This heterogeneous response of the liver has been previously observed in some vertebrates exposed to Cu (Jurado, 1983; Bunton et al., 1987). Perturbations of cell size and basophilia revealed in our study have been previously correlated to hemodillution, peroxidative alteration of the membrane and/or impairment of osmoregulation caused by heavy metals (Bouquegneau and Gilles, 1979; Leland, 1983; Roncero et al., 1992). In the treated zebrafish liver there is a mix of degenerative (lyses, mitochondria alterations, reduction of granular endoplasmic reticulum in clear hepatocytes) and adaptative changes occurring (development of granular endoplasmic reticulum, increased number of mitochondria, nuclear and nucleolar enlargement in dark hepatocytes)

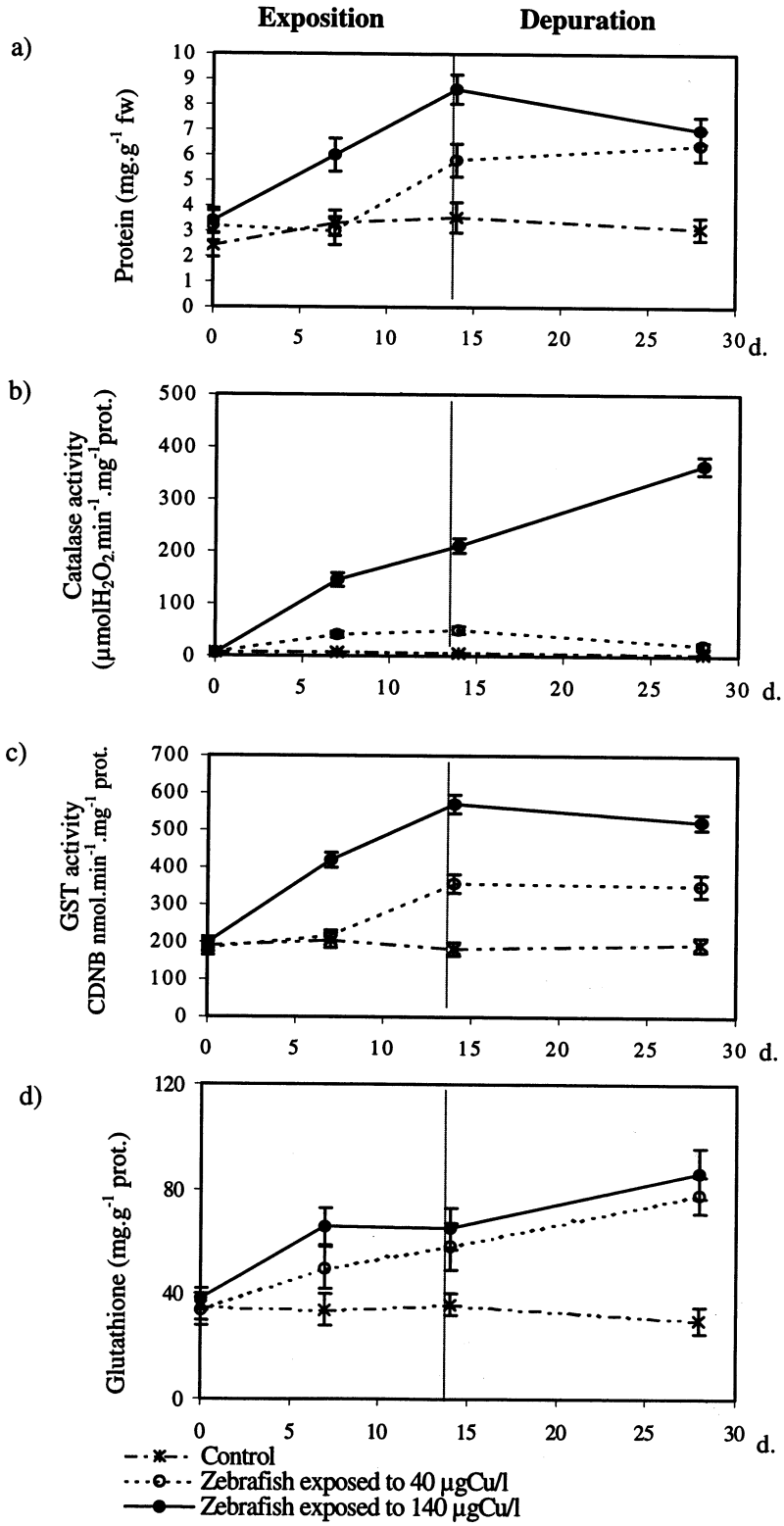

Fig. 15. Evolution of hepatic biochemical parameters in zebrafish exposed to two sublethal Cu concentrations; (a), protein content; (b), catalase activity (c), GST activity; (d), glutathione content.

which makes difficult the interpretation. However, dark hepatocytes were much more numerous than clear hepatocytes. Thus the overall response of the liver seemed to be adaptive.

Treated zebrafish showed a clear increase in protein content. This event could be correlated to nucleoli enlargement and development of granular reticulum in dark hepatocytes; signs of increased protein synthesis. Nuclear cytoplasmic ratios and relative nuclear volumes were significantly higher in Cu-treated zebrafish. Alterations in the volume and shape of the nucleus have often been regarded as signs of increased metabolic activity but may be of pathological origin (Braunbeck et al., 1990). In the present study, elevated metabolic activity of hepatocytes was documented by the increase of the protein content and the stimulation of antioxidative responses (increased catalase and glutathione content) and was correlated to morphological changes. This is not surprising since Cu is known as a promotor of oxidative stress (Cossu et al., 1997; Regoli et al., 1997; Segner and Braunbeck, 1998). GST is a well known enzyme of phase II of the metabolism of detoxication which conjugates glutathione to certain xenobiotics compounds or to their metabolites (Almar et al., 1998). Nevertheless it has been recently demonstrated that GST shows peroxydase activities and may act in the antioxydative defence (Bartling et al., 1993; Berhane et al., 1994; Almar et al., 1998). Although Cu is not conjugated to glutathione by GST, the increase of hepatic GST activity may be explained by its antioxydative activity. GST induction may be also related to decontamination processes of Cu-altered constitutive molecules of the cell (e.g. lipid or protein peroxides). However these induced defences (catalase, GST, total glutathione) did not appear efficient enough to entirely subdue the deleterious effects of copper toxicity since lysis occured and alterations were observable in clear hepatocytes.

No development in the number of peroxisomes was observed in treated zebrafish. Neither Leland (1983), Segner and Braunbeck (1990a) or Roncero et al. (1992) reported an increased number of peroxisomes in Cu exposed *Salmo trutta*, *Chanos chanos* and *Tinca tinca*. Braunbeck (1998) reported that the level of catalase activity was corelated with the number of hepatocytic peroxisomes where the great part of this enzyme activity is located. However we observed an important induction of catalase activity during Cu contamination without any increase of the peroxisomal compartment. Catalase possess two cytosolic and peroxisomal isoformes: CAT1 and CAT2, and one mitochondrial isoforme: CAT3 (Koolman and Roöhm, 1994; Scandalious, 1997). With regards to the clear increase in the number of mitochondria observed in liver of the treated zebrafish, an induction of the catalase activity due to CAT3 is suspected. However this hypothesis needs to be further studied.

The most surprising observation, in regard to membrane lysis, was the absence of myelinic figures in altered hepatocytes since they represent a common feature in fish hepatocytes exposed to a variety of chemicals (Phillips et al., 1987). The explanation could be that hepatocytes contained relatively few membranous organelles at the begining of the experiments. Mitochondrial alterations were common in Cu intoxication as previously shown by Leland (1983), Segner (1987) and Roncero et al. (1992). They were probably due to metal accumulation in mitochondrial dense bodies and matrix (Buck and Osweiler, 1981). In treated zebrafish the mitochondrial intermembrane space shows extensive balloon-type changes. When Braunbeck (1998) uses intracardiac introduction of fixative, mitochondrial alterations are seen but they do not include this type of alteration which may be due to the technique of fixation by immersion. Nevertheless, this perturbation was found in nearly all the hepatocytes of Cu exposed fish and was never observed in control fish. Thus this change is suspected to be induced by Cu exposure of the fish.

Treated zebrafish showed glycogen depletion which has been correlated with enhanced levels of glycemia required in fish submitted to various stress conditions (Hinton et al., 1978; Biagianti-Risbourg, 1997). An interesting feature in Cu-treated fish was the presence of electron-dense, cord-like nucleoli. Similar perturbations have been reported but not explained in hepatocytes of Arctic chars from acidic high alpine lakes contaminated by trace metals (Hofer et al., 1997). Intranuclear contamination by metals has been shown in differ-

ent vertebrates following exposure to metals such as As, Pb, Bi and Se (Carmichael and Fowler, 1975; Fowler, 1987). Leland (1983) has observed intranuclear electron-dense particles in hepatocytes of juvenile rainbow trout exposed to Cu and postulated that it was a metal deposite. However nucleolus was not concerned by this pertubation. So in zebrafish hepatocyte, Cu deposition in cord-like nucleoli, is suspected and is under analysis in our laboratory. Since cord-like nucleoli were observable in clear altered hepatocytes as well as in dark functional ones, we can not deduce if this perturbation was pathologic or not. However it did not inhibit the protein synthesis.

Frequent and huge particular concentric arrangements of hepatocytes in liver of treated fish have been previously observed in *B. rerio* intoxicated with procymidone, a dicarboximide fungicide (Paris-Palacios et al., 1999). This unspecific perturbation, with no perivascular fibrosis, could not be clearly related to pathologic process induced by chemicals or metals. Moreover in both cases concentric arrangements of hepatocytes were still observable during the depuration period.

According to Hinton et al. (1978), Hinton (1993), Segner and Braunbeck (1990b), Braunbeck (1998), Lackner (1998) and Biagianti-Risbourg et al. (1999) the observed perturbations (development of nucleus, nucleolus and rough endoplasmic reticulum, increase in protein synthesis, activation of antioxidative defences, glycogenic depletion) characterized an unspecific adaptive response of the liver to stress. However, at both Cu concentrations, these events were accompanied by degenerative features (hepatocyte lysis, alterations of mitochondria, decrease in organelle number in clear hepatocytes). After 14 days depuration, the propagation of lysis was stopped and functional dark hepatocytes were predominant. Additionally, antioxidative defences (glutathione and GST) and protein synthesis were still strongly developed. During the depuration period, zebrafish liver still underwent oxidative stress but its overall response was of adaptive nature; this is confirmed by the fact that none of the fish died.

Although liver lysis and alterations of clear hepatocytes were clearly a degenerative phenomena, the changes observed in the numerous dark and functional hepatocytes were adaptive. The hepatic responses were unspecific and did not appear reversible even 14 days after the end of the exposure period since Cu was remnant in liver of zebrafish. In this work biochemical changes have been correlated to ultrastructural perturbations. Such complementary studies are necessary for a better understanding of the deleterious effects of pollutants. These hepatic perturbations, as a result of heavy metal exposition, imply that fish liver may serve as a sensitive biomarker for the toxicity of sublethal concentrations of metals as well as other pollutants (Hinton, 1993; Biagianti-Risbourg, 1997; Braunbeck, 1998; Hinton and Couch, 1998; Lackner, 1998).

Acknowledgements

This study was supported by grants through the 'Unité de Recherche Vigne et Vin de Champagne', UPRES-EA 2069 and 'Toxicologie et Ecotoxicologie des pesticides et des Métaux lourds': Europol'Agro. The English used in this manuscript was revised by M.P. Eullaffroy.

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