HISTOPATHOLOGICAL RESPONSES OF THE LIVER TISSUES OF *RANA RIDIBUNDA* TO THE CHAMPIONS 50WP FUNGICIDE

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Abstract
Adult male and female frogs Rana ridibunda were exposed to $0.125x10^{-3}$mg Champion 50WP/g body weight administrated by intraperitoneal injection, 1 injection at 2 days in a scheme of 3 weeks. The animals were kept at 4-6˚C, respectively at 22-24˚C in tap water tank. At the end of the experiment we observe large quantities of Pearls reagent material in Kupffer cells, dilatation of blood vessels, peri-hepatocyte, peri-centrilobular, peri-sinusoidal and periportal fibrosis, an expansion of Disse spaces, presence of leukocyte infiltrates, vacuolated hepatocytes with small pyknotic nuclei, and necrotic areas in the parenchyma. Histological changes were more powerful at animals that were kept at 22-24ºC than 4-6ºC. Under normal conditions, the largest amount of copper accumulates in the cytosol. When the amount of copper is high, it accumulates in organelles: nucleus and lysosomes. Copper accumulation in the lysosomes is associated with the initiation of detoxification process and is the beginning of its biliary excretion.

Keywords: *Rana ridibunda,* liver, copper hydroxide, fibrosis, necrosis, hepatocytes

1. INTRODUCTION

There is an increasing concern due to the decline of amphibian populations and the large number of malformations found in many geographic regions.
Heavy metals enter the aquatic environment naturally through weathering of the earths crust. In addition to geological weathering, human activities have also introduced large quantities of metals to local water bodies, thereby disturbing the natural balance in the ecosystem [6]. Heavy metals contamination has been reported in aquatic organisms [1, 15]. Metals can either increase or decrease hepatic enzyme activities and can lead to histopathological hepatic changes depending on the metal type and concentration [16].
Industrial effluents, agricultural runoffs, transport, burning of fossil fuels, animal and human excretions, and geologic weathering and domestic waste contribute to the heavy metals in the water bodies [8].
Environmental contamination with copper comes from its extensive use in commercial and industrial products, agricultural use in fertilizers and different biocides, animal feed additives and growth promoters, electroplating, textile products, petroleum refining, manufacture of copper compounds [11].
Copper plays a major role as cofactor in hematogenesis [5]. Copper is one of the most critical trace elements in livestock because it is necessary for haemoglobin formation, iron absorption from GI-tract and iron mobilization from tissue stores [14]. Ingestion of large doses of copper salts may result progressively in irritation of the gastrointestinal tract, nausea, vomiting, salivation, gastric pain, hemorrhagic gastritis, diarrhea, capillary damage, liver and kidney damage and central nervous system stimulation followed by depression.
Champion 50WP is a foliar fungicide with protective action. Copper hydroxide witch comprises 77% of this product governs the toxicity of the product. The remaining components have low to negligible toxicity.
The present paper is concerned with histopathological studies, the aim of which was to determine the toxicity of Champion 50WP fungicides towards marshal frog (*Rana ridibunda*) and their effect on the liver structures at two thermic intervals (4-6ºC, respectively 22-24ºC).

2. MATERIAL AND METHODS

The animals examined in this study were adult of *Rana ridibunda*, of both sexes, captured in spring (April-May) from the surrounding areas

of the city Piteşti (South Romania). The animals were kept in laboratory condition in aquaterrarios filled with tap water for five days to test their health and accommodate them for the experiment. The water was changed daily to avoid the accumulation of toxic substances.

After adaptation in the lab, the frogs were separated in lots, which were used separately for the following experiments: two lots of control individuals, containing animals kept in laboratory at 4-6°C, respectively at 22-24ºC with no treatment, in running water which was changed everyday, (1) one lot containing animals which were subjected to treatment with Champion 50WP in a dose of $0.125x10^{-3}$/g of body weight and kept at 4-6ºC, (2) a second lot containing animals which were subjected to treatment with Champion 50WP in a dose of $0.125x10^{-3}$/g of body weight and kept at 22-24ºC in a thermostatic chamber. Ten animals were used for each lot.

The toxic was administered by intraperitoneal shots, one shot every two days, in a scheme of 3 weeks. The administered dosage of insecticide was not lethal as none of the subjects died through the experiment.

We began sacrificing the animals at the end of treatment by decapitation, under chloroform anesthesia, and liver fragments, approximately 1 cm^2, were quickly removed. The pieces were fixed in 8% formalin for poikilotherms and further processed for paraffin wax-embedding using routine protocols. Consecutive 5 µm-thick sections were cut using a rotary microtome (Slee Maintz Cut 5062) and a series of sections were stained with Hemalaun Mayer and Sirius red for collagen [12] and Perls staining for iron. To avoid differing intensity of staining in different tissues, all sections to be stained by the same method were stained simultaneously.

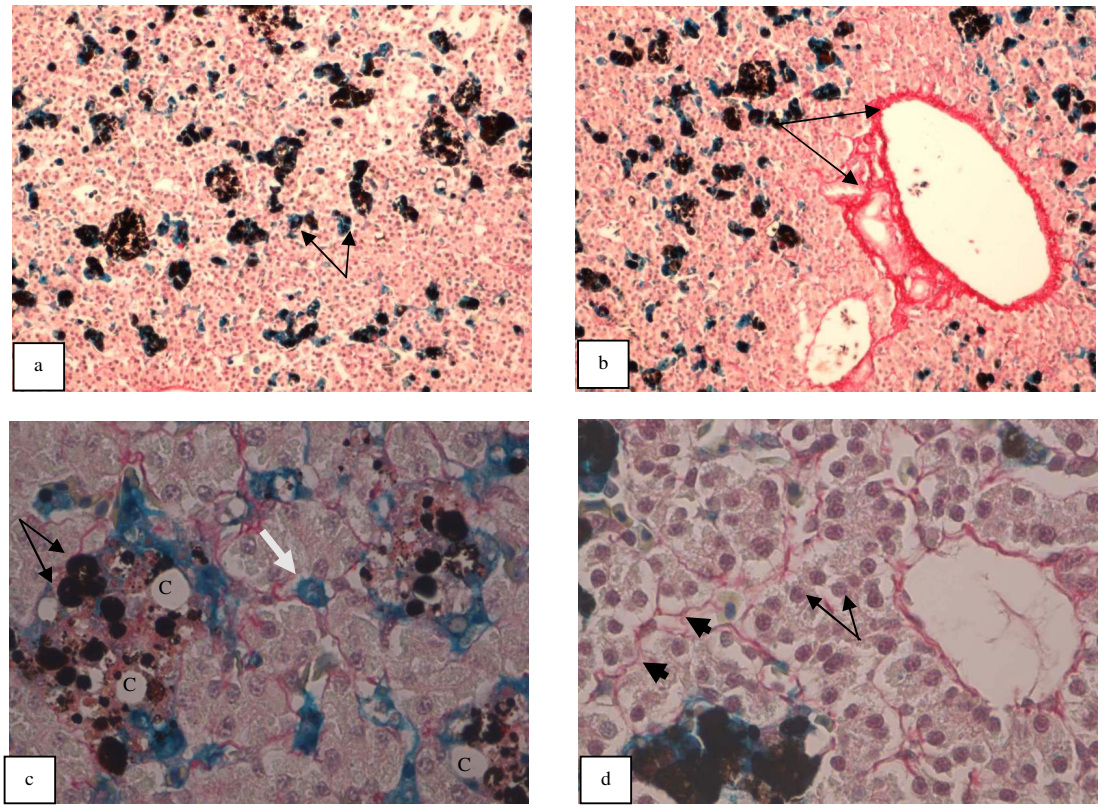

Figure 1. ***Rana ridibunda*** **liver treated with Champion 50WP fungicide and kept at a temperature of 4-6˚C: a- liver parenchyma with Perls-positive material; b- dilation and fibrosis of blood vessels. 100×; c- deposits of melanin pigment (black arrows), ceroid (C) and iron (white arrow); d- peri-hepatocyte fibrosis (arrowhead), vacuolated hepatocytes with small pyknotic nuclei, (black arrow). 400×. Perls' stain, H-Sirius red.**

3. RESULTS AND DISCUSSION

Administration of Champion 50WP fungicide for *Rana ridibunda* specimens kept at a temperature of 4-6˚C determined the appearance of large quantities of Perls reagent material in Kupffer cells in the liver (Fig. 1a). Its presence is due to intense hemolysis of red cells expressed at this level and iron accumulation in hemosiderin form. Along with hemosiderin deposits one can observe numerous accumulations of melanin in the form of small deposits, ceroid and lipofuscin (Figure 1c). Another effect of copper accumulation in the liver is the dilation of blood vessels (Figure 1b).

The same concentration of fungicide determines more pronounced hepatotoxic effects if animals are kept at a temperature of 22-24˚C. Liver parenchyma stores large amounts of iron, which characterize installation of mesenchymal hemosiderosis, because iron is stored in Kupffer cells (Figure 2a). Lipofuscin, melanin (in large quantity) and ceroid (lipid-like pigment) are found along with hemosiderin deposits. Histological changes in melano-macrophages, suggest the involvement of these cells in the detoxification process.

Toxic administration is associated with centrilobular veins (Figure 2c) and blood vessels dilation (Figure 2b) to ensure effective infusion of hepatocytes. In this respect, there are thin-walled collateral blood vessels (Figure 2b) necessary for additional feeding of hepatocytes.

Liver parenchymal fibrosis is more advanced with peri-hepatocyte (Figure 3b) peri-centrilobular (Figure 3c), peri-sinusoidal (Figure 3b) and periportal fibrosis (Figure 3a). In the liver lobules there is an expansion of Disse spaces filled with fibrillary material. Hepatocytes are vacuolated and provided with small pyknotic nuclei, in process of degeneration. Hepatocytes were observed to have polyploid nuclei (Figure 3c). The presence of leukocyte infiltrates is the body's inflammatory response to the copper action.

Histological changes in melano-macrophages were obtained by Loumbourdis and Vogiatzis [13] in cadmium poisoning of *Rana ridibunda* and Boncompagni et al., [2] in chromium and heptachlor-epoxide poisoning of *Rana aesculenta*. These results show the role of melano-macrophages in the defense mechanisms by means of melanin synthesis (which has substantially increased in intoxicated animals) and by increasing the catalase activity.

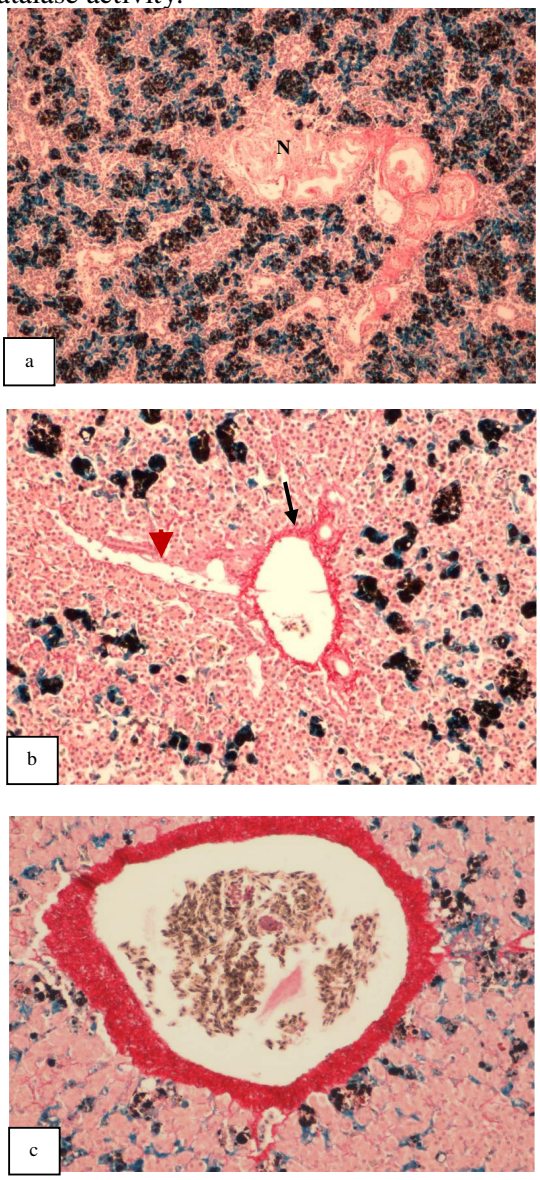

Figure 2 *Rana ridibunda* liver treated with Champion 50WP fungicide and kept at a temperature of 22-24˚C: a- liver parenchyma with abundant Perls-positive material; necrotic areas in the parenchyma (N); b- dilated blood vessels (black arrow); collateral blood vessels (red arrow); c- dilated centrilobular vein. 100×. Perls' stain, H-Sirius red.

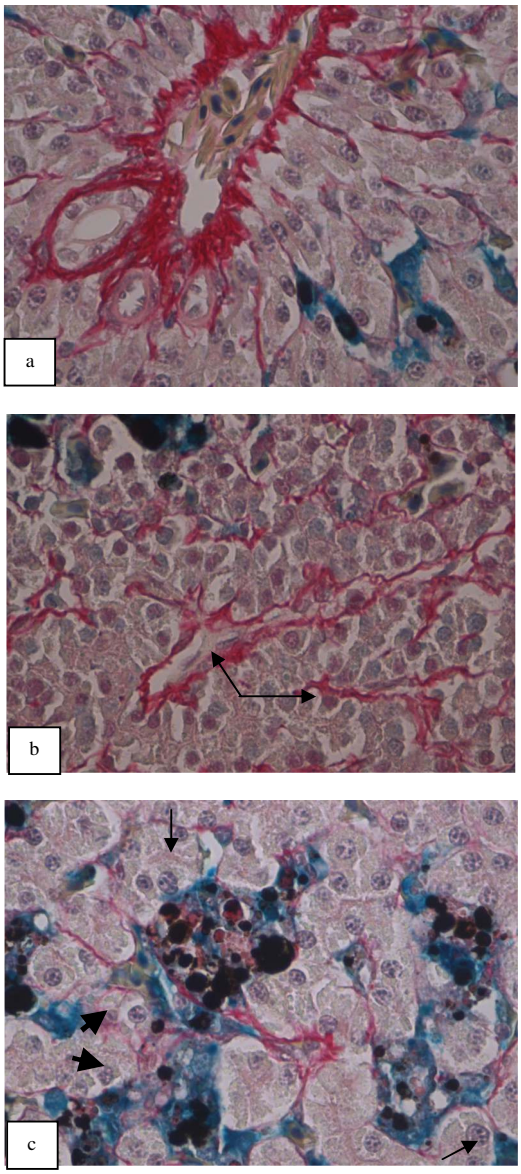

Figure 3. *Rana ridibunda* liver treated with Champion 50WP fungicide and kept at a temperature of 22-24˚C: a - periportal fibrosis; b- perisinusoidal fibrosis (black arrow); c- vacuolated hepatocytes with pyknotic nuclei (arrow head); the presence of polypolid nuclei (black arrow). 100×. Perls' stain, H-Sirius red.

Histological and histochemical alterations in the liver of the frog *Rana ridibunda* induced by the action of the insecticide Reldan 40EC (chlorpyrifos-methil) were observed by Păunescu et al [19]. Chlorpyryfos-methil induces an increase in the area occupied by the Kuppfer cells as well as an increase in their color intensity, mild karyomegalia and polyploidy together with accumulation of infiltrates a fibrosis around the blood vessels and between hepatocytes.

Ebara et al. [7] observed an increased accumulation of copper in the form of copper metallothionein in areas occupied by hepatocarcinomas in rats, suggesting that this nutrient would be involved in the development of liver cancer.

Similar liver changes, following the administration of copper in copper sulphate form have been observed by Osman et al. [16] in *Oreochromis niloticus* fish species. The authors observed the liver vessels dilation, hepatocyte degeneration even their necrosis after 6 weeks of toxic administration in water. At the same fish species, Figueiredo-Fernandes et al [9] observed vacuolation and hepatocyte necrosis installation after 21 days of treatment. Impairment of hepatocyte nucleus size under the toxic action of copper was reported by Paris-Palacios et al. [18], in *Brachydanio rerio* species. Changes in shape and size of the nuclei are the result of increased cellular metabolic activity [3].

Higher level of Cu accumulation might have damaged the liver to increase these enzymes [21]. The liver which accumulated the largest amount of copper is involved in achieving homeostatic balance of metal concentration. It is the place of metalloenzymes and metal storage. It can also achieve biliary metal excretion [17].

Under normal conditions, the largest amount of copper accumulates in the cytosol [4]. When the amount of copper is high, it accumulates in organelles: nucleus and lysosomes [10]. Copper accumulation in the lysosomes is associated with the initiation of detoxification process and is the beginning of its biliary excretion. Another mechanism for adaptation of hepatocytes to a high concentration of copper is the copper limitation by cytoplasmic metallothionein [17] or by other proteins with high or low molecular weight, such as ceruloplasmins. Metallothionein (MT) is a low molecular weight cytoplasmic protein that has an affinity for heavy metals such as copper (Cu), zinc (Zn) and cadmium (Cd). This

protein was identified as two isoforms in both invertebrates and vertebrates, including humans. MT has the ability to reduce the toxicity of heavy metals by binding them, thereby participating in the detoxification process [17]. In amphibians, it was identified in liver and kidney in a single isoform [20].

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