

## LIVER HISTOPATHOLOGIC ALTERATIONS IN THE FROG *Rana (Pelophylax) ridibunda* INDUCE BY THE ACTION OF RELDAN 40EC INSECTICIDE

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**Abstract.** Histological and histochemical alterations in the liver of the frog *Rana ridibunda* induced by the action of the insecticide Reldan 40EC (chlorpyrifos-methyl) were investigated. The animals used in the experiment were treated with 0.01 ml/g body weight Reldan 40EC and kept at 4-6°C, respectively at 22-24°C. The toxic was administrated by intraperitoneal shots (one shot every two days, in a scheme of three weeks). At the end of the experiment we observe an increase in the area occupied by the Kupffer cells as well as an increase in their color intensity. Mild karyomegalia and polyploidy together with accumulation of infiltrates was evident. We also observe a fibrosis around the blood vessels and between hepatocytes.

**Keywords:** *Rana ridibunda*, histopathologic alteration, liver, chlorpyrifos-methyl

### INTRODUCTION

During the last few years, many amphibian populations have been decreasing dramatically, and extinction caused by man-made changes in the environment [2], has occurred in a few species. Frogs are more vulnerable than other vertebrates to environmental contaminants because frog eggs are not protected by semiimpervious shells and frog skin is water permeable [6].

Pesticides are applied throughout the world often with unintended consequences on ecological communities. In some regions, pesticides are associated with declining amphibians, but we have a poor understanding of the underlying mechanisms [26].

Few researchers have worked on the relationship between the frog *Rana ridibunda* and organochlorine pesticides [15, 24, 25] or heavy metals [16, 29].

Reldan 40EC (active substance is chlorpyrifos) is an organophosphate insecticide, acaricide, and nematocide. Its chemical formula is  $C_9H_{11}NO_3PSCl_3$  and its chemical name is O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate. The primary mechanism of toxicity for organophosphorus pesticides, like chlorpyrifos, is cholinesterase (ChE) inhibition. The inhibition of the enzyme acetylcholinesterase (AChE) results in the buildup of acetylcholine (ACh) at choline receptors, causing continual nerve stimulation [12]. Chlorpyrifos is a relatively weak AChE inhibitor compared to its metabolite chlorpyrifos oxon [8], thus toxicity is initiated by the formation of chlorpyrifos oxon by oxidative desulfuration [7, 12]. Factors influencing the toxicity of chlorpyrifos between species and groups include metabolic rate, the number of target sites available for chlorpyrifos metabolism to chlorpyrifos oxon [3], organism surface area, and lifestage [8].

The aim of this study was to assess the several of histological alterations observed in liver frogs that were intoxicated with Reldan 40EC in a dose of 0.01 ml/g body weight and kept at 4-6°C, respectively at 22-24°C.

### MATERIALS AND METHODS

Adult specimens of amphibians (*Rana ridibunda*), of both sexes, captured in spring (April-May) from the surrounding areas of the city Pitești (Romania) were kept unfed in freshwater aquaria for 5 days. 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 22-24°C with no treatment, in running water which was changed everyday, (1) one lot containing animals which were subjected to treatment with Reldan 40EC in a dose of 0.01 ml/g of body weight and kept at 4-6°C, (2) a second lot containing animals which were subjected to treatment with Reldan 40EC in a dose of 0.01 ml/g of body weight and kept at 22-24°C in a thermostatic chamber. 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.

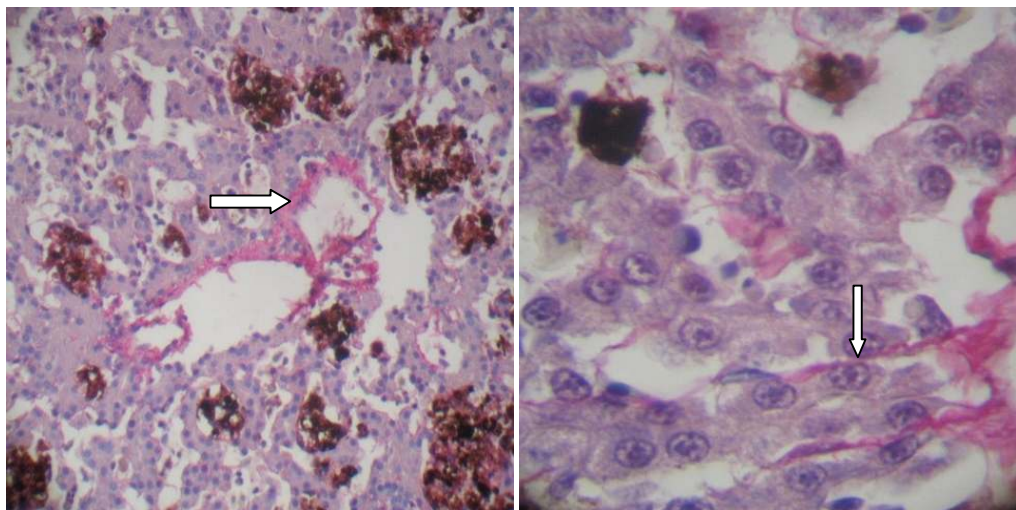
The animals were killed at the end of the treatment under chloroform anesthesia and liver was quickly removed. The pieces were fixed in 8% formalin for poikilotherms and further processed for paraffin wax-embedding using routine protocols. Consecutive 5  $\mu$ m-thick sections were cut using a rotary microtome (Slee Maintz Cut 5062) and a series of sections were stained with H&E, Sirius red for collagen [13] and Perls' staining. To avoid differing intensity of staining in different tissues, all sections to be stained by the same method were stained simultaneously.

### RESULTS

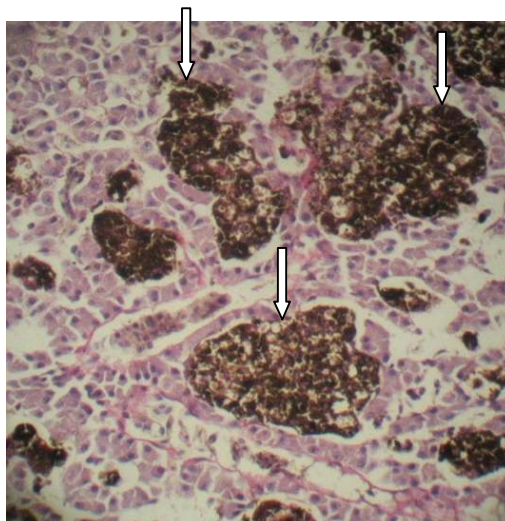
Liver from the animals who treated with Reldan 40EC in a dose of 0.01ml/g body weight and kept at 4-6°C did not exhibit any macroscopic alterations in structure. Light microscopy reveals significant

alteration in the frog's liver. Picro-Sirius red stain indicated the presence of an important amount of collagen situated perisinusoidally (Fig.1). An enlargement of the Disse interspaces was also observed. In the livers of the intoxicated frogs kept at

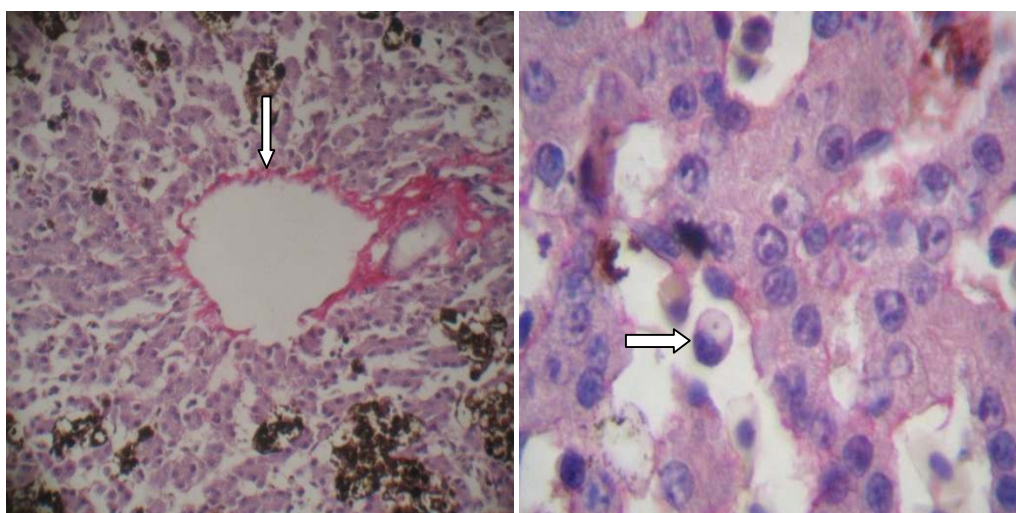
4-6° there was an increase in the area occupied by melanomacrophages as well as an increase in their color intensity (Fig.2). Binucleated hepatocytes were not frequent. Slight cytomegaly and some focal and mainly solitary infiltrates were detected.



**Figure 1.** Liver of frogs (*Rana ridibunda*) intoxicated with Reldan 40EC in a dose of 0.01ml/g body weight and keep at 4-6°C, stained with PSR. The arrow indicates a perivascular (left, 100x) and perisinusoidal fibrosis (right, 400x).



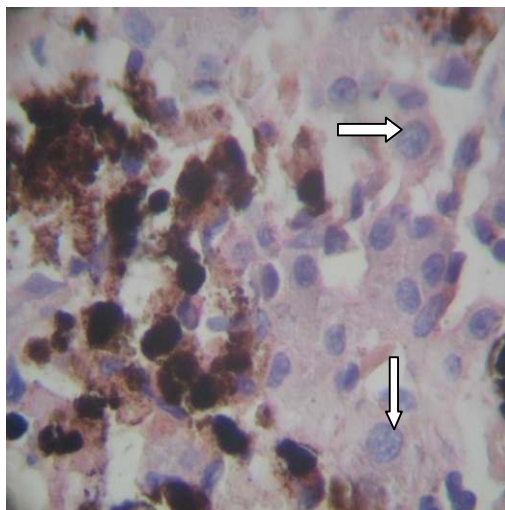
**Figure 2.** Section of frog liver (*Rana ridibunda*) intoxicated with Reldan 40EC in a dose of 0.01ml/g body weight and keep at 4-6°C, stained with PSR. The arrows indicate an enormous area occupied by the melanomacrophages (100x).



**Figure 3.** Liver of frogs (*Rana ridibunda*) intoxicated with Reldan 40EC in a dose of 0.01ml/g body weight and keep at 22-24°C, stained with PSR. The arrow indicates a perivascular fibrosis (left) (100X). Small foci of infiltrates (right) (400X).

In the frogs that were treated with Reldan 40EC in a dose of 0.01mg/l and kept at 22-24°C marked alterations in the liver were detected. Fibrosis around blood vessels and between hepatocytes was lightly prominent (Fig. 3, left). The liver of animals showed a

decrease in the number of melanomacrophagic cells. Diffused infiltrates were evident either singly or in small groups (Fig. 3, right). Karyomegaly, as well as karyomegaly probably related to polyploidy, was evident (Fig. 4).



**Figure 4.** Section of frog liver (*Rana ridibunda*) intoxicated with Reldan 40EC in a dose of 0.01ml/g body weight and kept at 22-24°C. PSR stain. The arrow point to some enlarged nuclei (400X).

## DISCUSSIONS

As a consequence of exposure against Reldan 40EC insecticide, the liver of adult animals undergoes significant changes, mainly of a functional character, involving both hepatocytes and Kupffer-melanomacrophagic cells.

Hepatic fibrosis is regarded as a common response to chronic liver injury and is characterized by excessive deposition of extracellular matrix components [11]. It has been shown that oxidative stress can stimulate fibroblast [22] and hepatic stellate cell proliferation [14] and collagen synthesis [10, 21] both in vitro and in vivo.

The liver of animals who were treated with Reldan 40EC in a dose of 0.01ml/g body weight and kept at 4-6°C, showed an increase in the number of melanomacrophagic cells (MMC). These aspects, also observed in a previous study during the frog's prehibernation phase [1], are possibly supported by two mechanisms, namely, proliferation and hypertrophy. The area of the livers of frogs occupied by the MMCs was roughly similar to that observed in animals exposed to cadmium [18]. The reasons for the increased surface of MMCs are not well known.

In particular, studies performed on the liver of *Rana esculenta* have shown a direct relationship between Kupffer cell differentiation and melanin accumulation; in amphibians, therefore, a large amount of melanin is considered a marker of terminally differentiated Kupffer cells [4, 23].

Melanomacrophages are focal accumulations of pigmented macrophages in liver, spleen and, more rarely, kidney of fishes [5] and also in liver of frogs [17]. These may contain four types of brown to black pigments, namely, melanin, lipofuscin, ceroid, and hemosiderin/ferritin. Melanomacrophages proliferation

has been associated with several natural factors, such as normal aging, starvation, and infectious diseases [31]. Increased density of these has also been observed in experimentally treated fish [5], amphibians [17], and naturally polluted frogs [9]. The ability of melanin to scavenge reactive oxygen species [28] suggests that melanin could protect pigment cells against the oxidative stress that may accompany the formation of reactive oxygen species in these cells. Melanin has also been proposed as a novel biochemical mechanism involving the accumulation, stimulatory action, and eventually detoxification of metal ions [20].

Perls' reaction evidenced some ferric catabolites accumulated within melanomacrophages, possibly due to an increase of red blood cell turnover [9]. Interestingly, the iron stored inside melanomacrophages in the form of hemosiderin and ferritin constitutes a system of intracellular storage and sequestration of metals, in addition to the metallothionein and glutathione systems [27]. An increase in the area, which is stained blue using Perl's method, is an indication of an increase in the Fe content of Kupffer cells. Probably, this pesticide causes rupturing of small blood vessels, resulting in the discharge of erythrocytes to the surrounding tissues.

At 22-24°C, the Reldan 40EC insecticide in same concentrations determined an even more pronounced histological modification of the liver. It should be noted that the surface of frog livers covered by MMCs was much smaller compared with frogs treated with same pesticide and kept at 4-6°C. The existence of an extensive fibrous area with duct-like structures in liver has been described by Tan [30], who noted that these ductules proliferate along the fibrous septa. This description is quite similar to that in our study. Extensive fibrosis is presumed to be attributed to carcinogenesis [19].

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