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LUNG TOXICITY INDUCED BY THE ACTION OF CHAMPION 50WP FUNGICIDE IN MARSH FROG (*PELOPHYLAX RIDIBUNDUS*)

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ABSTRACT: The histopathology of Champion 50WP on the lung tissues in marsh frog (*Pelophylax ridibundus*), was determined by light microscopy. The frogs were experimentally exposed to sub-lethal concentrations ($0.125x10^{-3}$/g of body weight) of Champion 50WP for 3 weeks at two thermic levels (4-6ºC and 22-24ºC). Tissues were normal in the control group. Histological examinations showed in treated samples a loss of elasticity in the lung walls, dilated blood capillaries, numerous aggregate erythrocytes around the pneumocytes, and existence of a greater number of goblet cells. In parallel, there is a hyperplasia of pseudostratificated epithelium and inflammatory reaction to toxic is evidenced by the presence of leukocyte infiltrates. The present study proves its toxic potential in terms of the damages induced by Champion 50WP fungicide in lung level. These changes were more powerful at animals that were treated and kept at 22-24ºC.
Key words: lung, pneumocytes, goblet cell, respiratory epithelium, *Pelophylax ridibundus*

INTRODUCTION

Agricultural chemicals are receiving increasing attention as a potential cause of amphibian declines, acting singly or in combination with other stressors (Relyea and Mills, 2001). Surveys of natural populations have shown correlations between population declines and proximity to agricultural lands (Bishop et al, 1999; LeNoir et al, 1999; Davidson et al, 2002; Houlahan and Findlay, 2003; Davidson, 2004). Also, many malformed amphibians have been reported to occur in agricultural areas where pesticides and fertilizers are applied extensively (Ouellet et al, 1997; Taylor et al, 2005).

Champion 50 WP is a contact fungicide based on copper, used in preventing and combating mildew and bacterial burns in vines, fruit trees, vegetables, potatoes. It contains 50% Cu as $Cu\ (OH)_2$. Fungicides based on copper compounds are produced to prevent mold growth (anticriptogamic action).

Cu plays a major role as cofactor in hematogenesis (Chiou et al, 1999). Cu 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 (Mpofu et al, 1999). 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.

The toxic action of copper is shown by blocking biochemical reactions acting on -SH group protein or by eliminating trace elements which are active centers of enzymes. These changes induce reduction of cell metabolism, stimulation of lipid peroxidation, inhibition of oxidative phosphorylation, disruption of calcium homeostasis and changes in cell membrane structure and permeability (Farkas et al, 2003).

The purpose of this study is to examine the histopathological changes in lung tissues induces by the action of Champion 50WP fungicide in marsh frog (*Pelophylax ridibundus*).

MATERIALS AND METHODS

In this study, adult male and female *Pelophylax ridibundus* (Anura – Amphibia) specimens were used. The frogs were captured in spring (April-May) from the surrounding areas of the city Piteşti (South Romania) and 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.

The individuals were separated in lots, which were used separately for the following experiments: two lots of control individuals, containing animals kept in laboratory at 4-6ºC, 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 toxic was not lethal as none of the subjects died through the experiment. All frogs were handled in accordance with the standard guide for care and use of laboratory animals.

At the end of 3rd week of treatment, the frogs in each lot were sacrificed after chloroform anesthesia and lungs were taken to assess histological changes via light microscope examination. Tissues samples were fixed in 8% neutral formalin for 24h. Samples were then processed using a graded ethanol series and embedded in paraffin. Paraffin section were cut 5μm-thick slices and stained with hematoxylin and Sirius red (Juncueira et al,

1978) for light microscopic examination. The sections were viewed and photographed using an Olympus microscope with an attached camera.

The diameter of the pneumocytes bodies and their nuclei were measured with an ocular micrometer and the data were statistically analyzed using Student's test.

RESULTS AND DISCUSSION

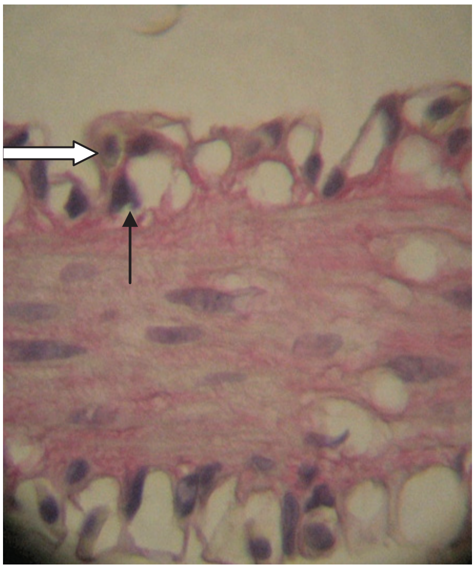

Fig. 1 Lung structure of *Pelophylax ridibundus*. Wight arrow shows erythrocytes near pneumocytes and black arrow shows active pneumocytes. 400×. H-Sirius red staining

The frogs in the first group were evaluated as control animals for the second and the third groups. Lungs are strongly folded by first, second and third order septa, which divide the air space into a network of different sized alveoli (Goniakowska-Witalinska, 1995; Hermida and Fiorito, 1994; Hermida et al, 1998). The respiratory epithelium had pneumocytes, goblet cells and neuroepithelial endocrine cells. The pneumocytes had large and round nuclei and the cytoplasm was stained dark. Goblet cells forming a group among the pneumocytes (fig 1). Erythrocytes in the blood capillaries were observed close to the pneumocytes. The connective tissue possessed smooth muscle cells, fibrocytes, collagen and elastic fibers (fig. 1).

Respiratory organs, lungs suffer structural changes associated with administration of Champion 50WP fungicide.

Copper reaching the lung, acts in animals kept at a temperature of 4-6ºC by thickening and scarring longitudinal connective tissues (fig. 2a). This effect is followed by loss of elasticity in the lung walls, which reduces the amount of air introduced into the lungs, and hence the oxygen existing in the breathing spaces. To accommodate this change, blood capillaries are dilated (fig.2b), and numerous aggregate erythrocytes are to be found around the pneumocytes (fig. 2c). Also, the red cells are dark-colored, evidence of a larger amount of hemoglobin in the cytoplasm, required to take over oxygen which is in a smaller amount. An adaptive response is also the existence of a greater number of goblet cells that secrete protective mucus (fig.2a).

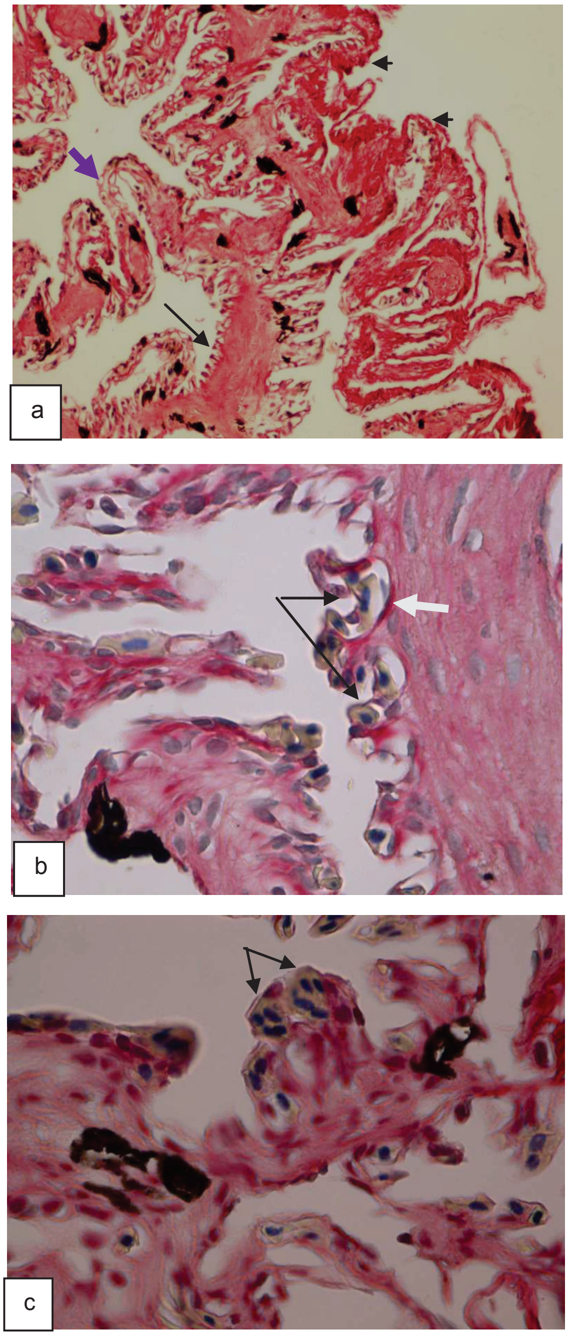

Fig. 2 Lung of *Pelophylax ridibundus* in animals treated with Champion 50WP fungicide and kept at 4-6°C. a- thickened connective tissues lined by pseudostratificated epithelium (black arrow), fibrosis of connective tissues (arrow head) numerous goblet cells (blue arrow). 100×. b- dilated blood capillaries (black arrows); collagen fibers found in pneumocytes (white arrow). c- dark-colored numerous erythrocytes lying around pneumocytes (black arrow). 400×. H-Sirius red staining

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In animals treated with the same concentration of toxic, but kept at a temperature of 22-24ºC, the lung reacts by thickening and scarring the connective tissues (fig. 3a), thus minimizing the amount of oxygen that enters the respiratory spaces. To compensate for the insufficient amount of oxygen, blood vessels dilate (fig. 3b) and the numerous erythrocytes contain a greater amount of hemoglobin in the cytoplasm.

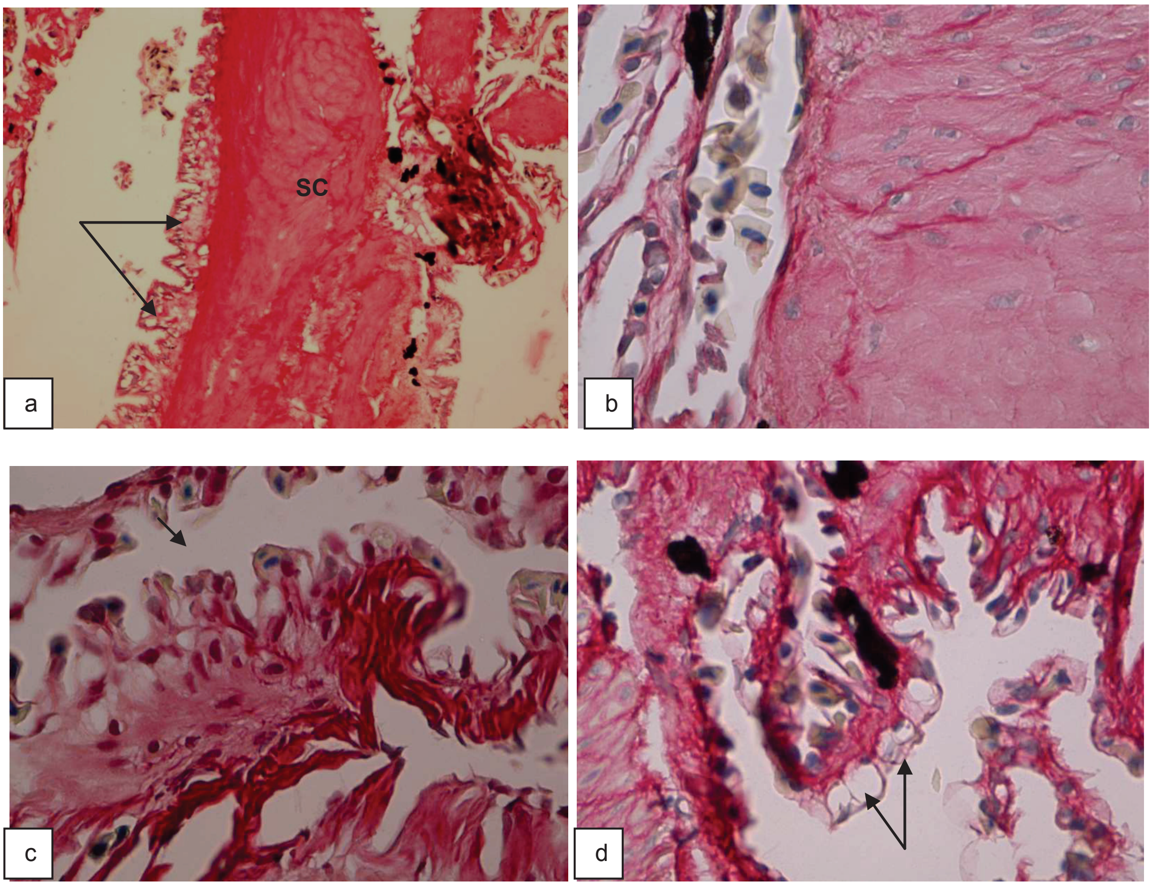

Fig. 3 Lung of *Pelophylax ridibundus* in animals treated with Champion 50WP fungicide and kept at 22-24ºC. a- thick, fibroid connective tissues (CT) lined by hyperplasic epithelium (black arrow). 100×. b- dilated blood capillaries. c- erythrocyte lysates located around inactive pneumocytes (black arrow). d- goblet cells with honeycomb appearance. 400×. H-Sirius red staining.

In parallel, there is a hyperplasia of pseudostratificated epithelium that lines the second order connective tissue (fig. 3a). Pneumocytes are surrounded by numerous inactive hypertrophied goblet cells with honeycomb appearance (fig. 3d). Some pneumocytes are inactive and surrounded by red blood cell lysates (fig. 3c).

The inflammatory reaction to toxic is evidenced by the presence of leukocyte infiltrates.

Histological damage to lung tissues in the present study may be attributed to accumulations of this fungicide, irritation due to elevated mucus secretion, increased ventilation volume and decreased oxygen uptake efficiency. Similar changes were reported by Bradbury et al. (1987), Bradbury and Coats (1989) in gill tissues of fish.

Pneumocytes, respiratory cells, are also affected by the action of copper. They react by changing the ratio of nucleus and cell diameter in both thermic variants (Table 1).

Tabelul 9. Arithmetic means (AM) and standard deviations (SD) of the proportions of pneumocytes and their nuclei

Group	Numbers of cells	AM±SD
C 4-6ºC	200	0.58±0.029
Lot I	200	0.63±0.008
C 22-24ºC	200	0.75±0.022
Lot II	200	0.78±0.011

In both groups studied, there was a slight increase in the value of this parameter, the nucleus diameter being higher than that of control groups, evidence of respiratory cells damage by the toxic action of copper.

Copper affects the respiratory organs of fish. Thus, Osman et al. (2009) describe in the histopathological picture of *Oreochromis niloticus* gill damage, congestion of gill branches, dilated blood vessels, hemorrhage, presence of leukocyte infiltrates, peeling of the gill epithelium. These gill histological alteration has been observed by several authors in fish submitted to copper (Karan et al., 1998; Chen and Lin, 2001; De Boeck et al., 2001). At higher concentrations copper may cause edema, vascular aneurysms, enhanced vasodilatation, hyperplasia of gill epithelium (Figueredo-Fernandes et al., 2007).

In fishes, the gills are the target organ of toxic action due to constant contact between these bodies and the environment. Therefore, structural changes' occurring in the gills is the body's response to the aggression of substances in the environment, being biomarkers of environmental pollution.

Păunescu et al (2010) observe histopathological changes in liver tissues induce by the action of Champion 50WP fungicides in *Rana ridibunda*. They observed 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.

Bufo arenarum embryos treated with copper in toxic concentrations increase their copper contents as was reported by the Herkovits and Perez-Coll (2007). Thus, the amphibian embryo could be a good model to study the feedback mechanism for copper transport at different concentrations (Herkovits and Perez-Coll, 2007).

CONCLUSIONS

All the histopathological observation indicated that exposure to sub-lethal concentrations of Champion 50WP fungicides caused destructive effect in the lung tissues of *Pelophylax ridibundus*. Lung histopathological alterations, such as those observed in this study and findings from previous studies, may result in severe physiological problems, ultimately leading to the death of frog. As a conclusion, the findings of the present histological investigations demonstrate a direct correlation between pesticide exposure and histopathological disorders observed in lung tissues. These histological changes provide a rapid method to detect of irritants in lung.

The study was ecologically relevant, demonstrating the potential routes and toxicological impacts of environmental contaminants to frog.

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