HISTOPATHOLOGICAL CHANGES IN MARSH FROG (*Pelophylax ridibundus*) LUNG TISSUE INDUCED BY THE ACTION OF ROUNDUP® HERBICIDE

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Abstract. The histopathology of Roundup® on the lung tissues in marsh frog (*Pelophylax ridibundus*) was investigated. The animals were exposed to sub-lethal concentrations ($0.138x10^{-3}$/g of body weight) of Roundup® administrated by intraperitoneal shots (one shot every two days in a scheme of 3 weeks) at two thermic levels (4-6ºC and 22-24ºC). Light microscopy of lung revealed hyperplasia of the respiratory epithelium, an increase in the number of goblet cells, increasing the nucleus diameter of pneumocytes, interstitial edema, presence of melanin deposit. Highly degenerative changes in lung tissue such as interstitial edema, presence of melanin deposit, an increase in the number of hyperplasiated and active goblet cells, were evident in animals that was treated with toxic and kept at 22-24ºC.

Keywords: frog, hyperplasia, respiratory epithelium, interstitial edema, pneumocytes, Roundup®.

Rezumat. Modificări histopatologice în ţesutul pulmonar la broasca-de-lac (*Pelophylax ridibundus*) induse de acţiunea erbicidului Roundup®. În prezentul studiu au fost investigate efectele histopatologice induse de acţiunea erbicidului Roundup® asupra ţesutului pulmonar la broasca *Pelophylax ridibundus*. Animalele au fost expuse acţiunii unor concentraţii subletale de Roundup® ($0.138x10^{-3}$/g greutate corporală) administrate prin injecţii intraperitoneale (o injecţie la 2 zile timp de 3 săptămâni) la 2 nivele termice (4-6ºC respectiv 22-24ºC). Microscopia optică a indicat prezenţa hiperplaziei epiteliului respirator, creşterea numărului de celule mucoase, creşterea diametrului nucleului pneumocitelor, edeme interstiţiale, prezenţa depozitelor de melanină. Cele mai severe modificări (edeme interstiaţiale, prezenţa depozitelor de melanină, creşterea numărului de celule hiperplaziate) au fost înregistrate în cazul animalelor tratate şi ţinute la o temperatură de 22-24ºC.

Cuvinte cheie: broască, hiperplazie, epiteliu respirator, edeme interstiţiale, pneumocite, Roundup®.

INTRODUCTION

The application of environmental toxicology studies on non-mammalian vertebrates is rapidly expanding (RELYEA & JONES, 2009).

Aquatic ecosystems can be contaminated with agrochemical products by leaching, run-off or direct or indirect spraying, this latter by action of the wind (WHO, 1994). In this regard, considerable research efforts have focused on the global decline of amphibian populations across the globe.

One of the widely used agrochemical products is glyphosate the herbicide (GP) - N (phosphonomethyl) glycine - known commercially as Roundup® (RAMÍREZ-DUARTE *et al.,* 2008). The Roundup® contains, in addition to GP, a cationic surfactant denominated polyoxyethylamine (POEA) that confers toxicological properties different from those of GP (FOLMAR *et al*., 1979).

Previous studies demonstrated that the toxicity of Roundup® in fish and mammalian is moderate. In the case of amphibians, because interest in the group is relatively recent, there are few amphibian data upon which to base these assessments.

In fish exposed to commercial formulations of GP, several authors have reported the development of necrotic and proliferative lesions, aneurysms and leukocyte infiltration in the gills (NEŠKOVIC *et al*., 1996; JIRAUNGKOORSKUL *et al*., 2002, 2003), as well as degenerative changes, lipidic vacuolization and hyaline droplets in hepatocytes (SZAREK *et al*., 2000; JIRAUNGKOORSKUL *et al*., 2003)

The aim of this study was to evaluate sublethal effects of Roundup® herbicide exposure on marsh frog lung tissues at two thermic levels (4-6ºC, respectively 22-24ºC).

MATERIAL AND METHODS

The animals used in this study were adult of *Pelophylax ridibundus*, 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 aquaterrariums 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 and the animals were fed "ad libitum".

The study was performed with the approval of the local Committee of Bioethics according to the Romanian law 205/2004 art.7, 18, 22 and regulation number 143/400/2002 for care and use of animals for research purposes.

After adaptation in the lab, the frogs (twenty healthy adult frogs male and female) 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 every day, (1) one lot containing animals which were subjected to treatment with Roundup® in a dose of $0.138x10^{-3}$/g of body weight and kept at 4-6ºC, (2) a second lot containing animals which were subjected to treatment with Roundup® in a dose of

$0.138x10^{-3}$/g of body weight and kept at 22-24ºC 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 toxic was not lethal as none of the subjects died through the experiment.

We began sacrificing the animals at the end of 3rd week of treatment; the frogs in each lot were sacrificed after chloroform anaesthesia and lung pieces were taken to assess histological changes via light microscope examination. Tissues samples were fixed in 8% neutral formalin for poikilotherms for 24h. Samples were then processed using a graded ethanol series and embedded in paraffin. Paraffin section were cut 5µm-thick slices using a rotary microtome (Slee Maintz Cut 5062) and stained with: haematoxylin (H) as a general screening method and Sirius red (JUNCUEIRA *et al*., 1979) for collagen stain (fibrosis). The sections were viewed and photographed using an Olympus microscope with an attached camera.

RESULTS AND DISCUSSIONS

The herbicide Roundup® in the lung works in animals kept at a temperature of 4-6˚C, by thickening the second-order connective septa (Fig. 1a). They are composed of smooth muscle fibers, along with collagen fibers and are covered by a pseudo-layered epithelium with a tendency of hyperplasia (Fig. 1a). In this case, hyperplasia of the epithelium is a defence mechanism of the deep structures under the action of irritating stimuli.

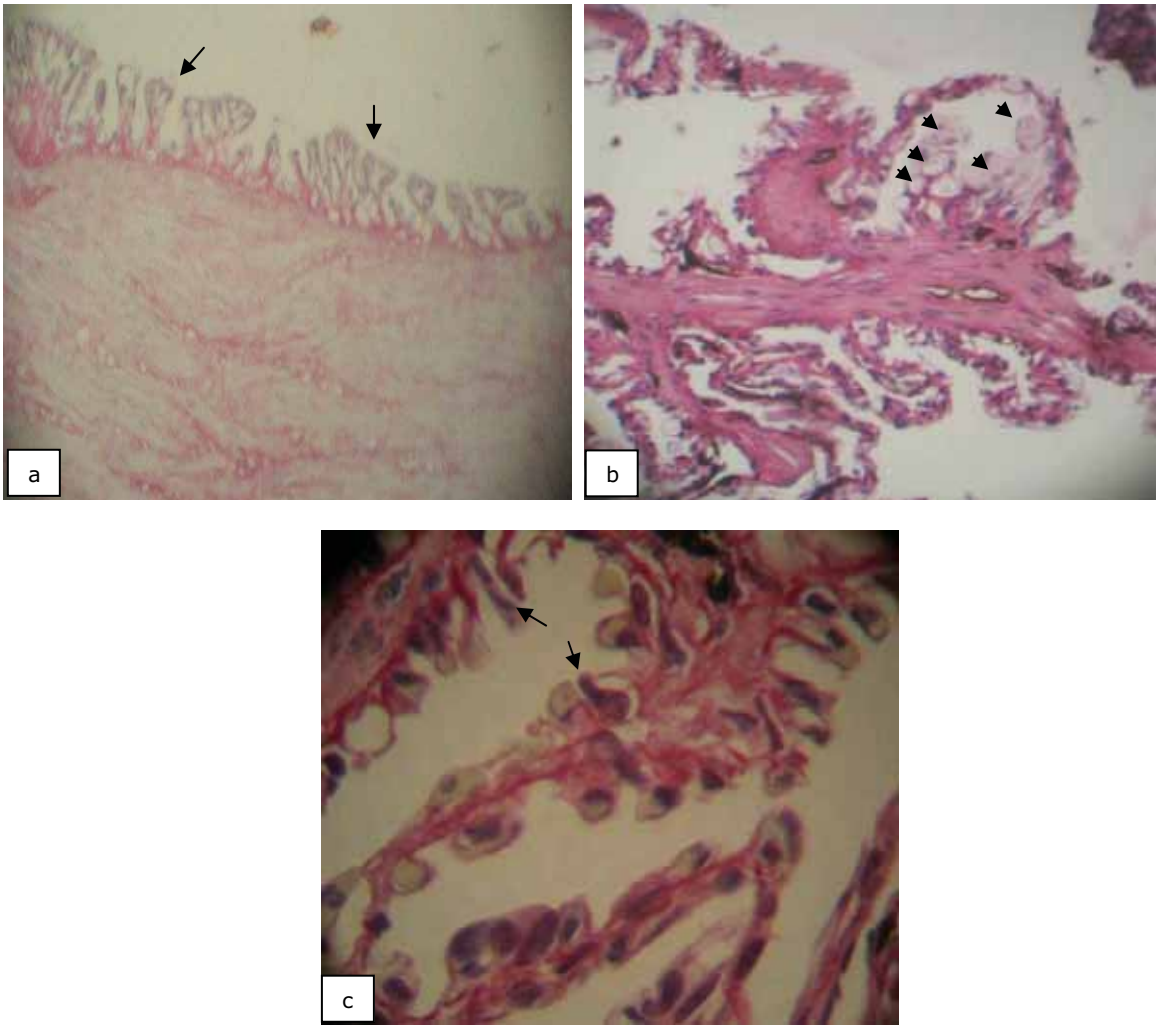

Figure 1. Lung of *Pelophylax ridibundus* species treated with Roundup® herbicide and kept at a temperature of 4-6˚C. a - thickened longitudinal septa composed of smooth muscle fibers and collagen fibers; hyperplasia of the epithelium (arrow). b - hyperplasiated goblet cells (arrow head). 100×. c - pneumocytes with elongated nuclei (arrow). 400×. H-Sirius red staining. / Figura 1. Plămân la exemplarele de *Pelophylax ridibundus* tratate cu erbicidul Roundup® şi ţinute la o temperatură de 4-6˚C. a - septe longitudinale îngroşate formate din fibre musculare netede şi fibre de colagen; hiperplazia epiteliului (săgeata). b - celule caliciforme hiperplaziate (cap de săgeată). 100×. c - pneumocite cu nuclei alungiţi (săgeata). 400×. Coloraţie H-Sirius red (original).

In the epithelium, there is an increase in the number of goblet cells, which are also hyperplasiated (Figs. 1b, c), having a honeycomb appearance. The goblet cells react to the toxic action through the synthesis of large quantities of mucus. This hypersecretion favours diluting or neutralizing of toxic substances reaching this level (FERGUSON *et al*.,

1992), but reduces the respiratory function of the epithelium, by increasing the distance between the air and the respiratory cells (BOLS *et al.*, 2001).

Pneumocytes react by increasing the nucleus diameter in relation to the cell diameter (Fig. 1c). They are enriched with intensely coloured erythrocytes, proved by the presence in their cytoplasm of a large quantity of haemoglobin.

Also, the lung parenchyma shows the presence of interstitial edema, characterized by the presence of dilated blood vessels prone to bleeding, as a result of the irritating action of the glyphosate and surfactant in Roundup® commercial product.

Histopathological effects on lung, following the administration of Roundup® are similar in the animals treated and kept at a temperature of 22-24˚C.

Therefore, one can notice a significant thickening of the second-order longitudinal septa and the same hyperplasia of the epithelium covering the septa (Fig. 2a). In the lung parenchyma there appear dilated blood vessels with bleeding areas (Fig. 2b), and deposits of melanin involved in the detoxification processes. The respiratory epithelium responds to the large number of hyperplasiated, active goblet cells, which synthetize a large quantity of mucus (Fig. 2c). Hypersecretion of these cells, which reduces the diffusion of xenobiotic substances, is a response to the electrolyte imbalance caused by the action of glyphosate and / or surfactant in the commercial product, on the permeability of the cell membrane (LIN & RANDALL, 1995).

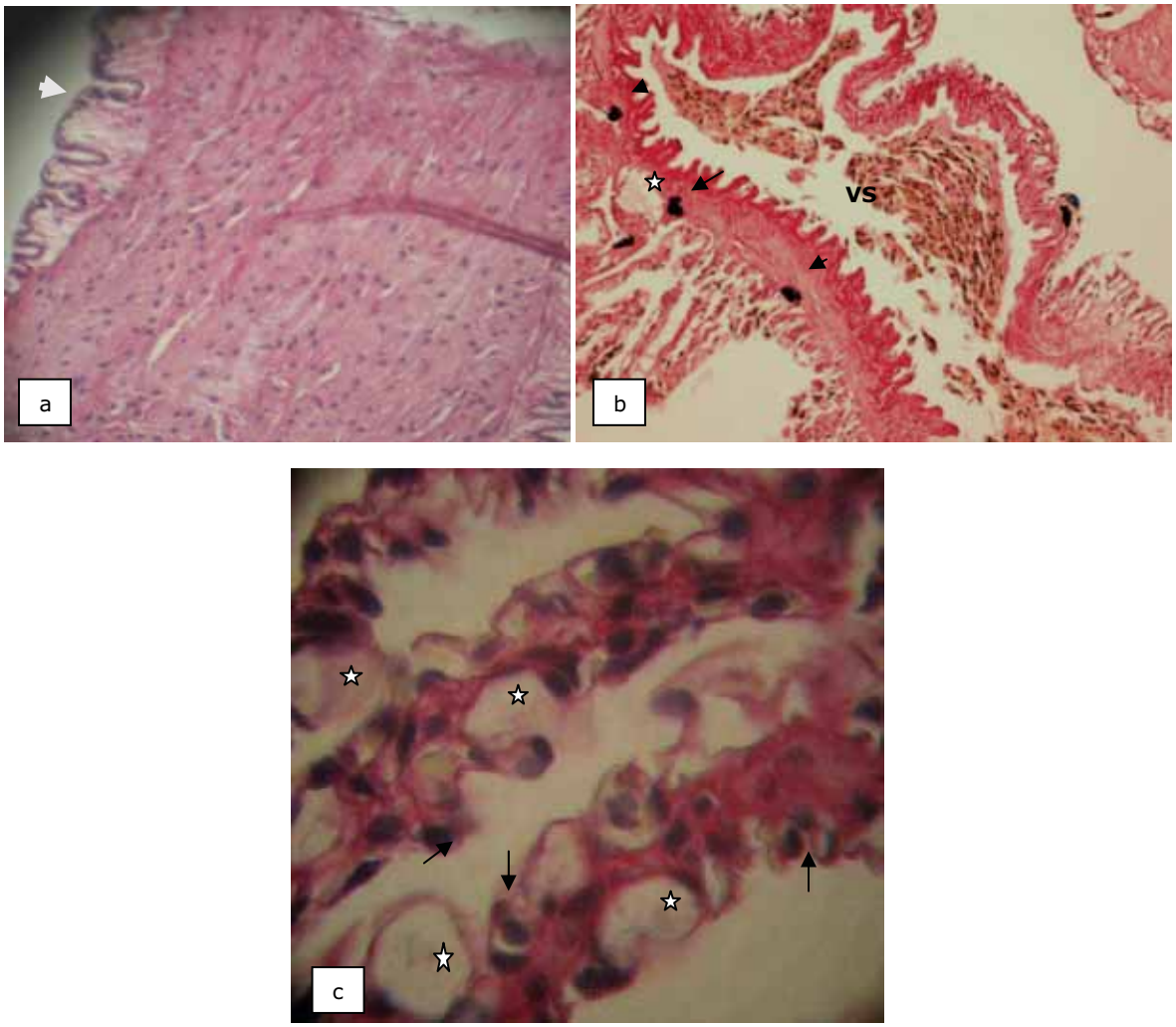

Figure 2. Lung of *Pelophylax ridibundus* species treated with Roundup® herbicide and kept at a temperature of 22-24˚C. a - thickened longitudinal septa composed of smooth muscle fibers and collagen fibers; hyperplasia of the epithelium (arrow). b - congested blood vessel (VS); bleeding area (asterisk); deposits of melanin (arrow). 100×. c - hyperplasiated goblet cells (asterisks); pneumocytes with elongated nuclei (arrow). 400×. H-Sirius red staining. / Figura 2. Plămân la exemplarele de *Rana ridibunda* tratate cu erbicidul Roundup® şi ţinute la o temperatură de 22-24˚C. a - septe longitudinale îngroşate formate din fibre musculare netede şi fibre de colagen; hiperplazia epiteliului (săgeata). b - vas de sânge congestionat (VS); zona hemoragică (steluţă); depozite de melanină (săgeata). 100×. c - celule caliciforme hiperplaziate (steluţe); pneumocite cu nuclei alungiţi (săgeata). 400×. Coloraţie H-Sirius red (original).

There is an increase of the nucleus surface in the pneumocytes. Around the pneumocytes there are erythrocytes containing more haemoglobin in the cytoplasm.

In order to explain the toxic effect of Roundup® herbicide on the pneumocytes, it was calculated the ratio between the nucleus and the cell diamter, the results being shown in table 1, observing an increase in the value of this ratio in both heat levels studied, due to the increase of the nucleus diameter.

Table 1. The proportions of pneumocytes and their nuclei.
Tabel 1. Raportul dintre diametrul nucleului şi diametrul celulei.

Lots	Number of cells	Arithmetic mean ± Standard deviations
C 4-6ºC	200	0.58±0.029
Lot I	200	0.63±0.020
C 22-24ºC	200	0.75±0.022
Lot II	200	0.81±0.016

All these changes should reduce the diffusion of toxic substances through the lungs. Similar changes associated with administration of Roundup® herbicide were recorded in the gills of *Clarias gariepinus* species by OLURIN *et al*. (2006), the authors noticing hyperplasia of the epithelium and the goblet cells, fusion of gills lamellae, the presence of bleeding areas in the parenchyma.

In both adults and juveniles of *Oreochromis niloticus* species there were identified histopathological changes in the gills, induced by the action of Roundup® herbicide (JIRAUNGKOORSKULA *et al.*, 2002; AYOOLA, 2008), which causes reduced oxygen consumption and ultimately, death of the bodies by suffocation.

Gills lesions characterized by hyperplasia of the epithelium, interstitial edema, congestion of blood vessels, were identified in *Piaractus branchypomus* species, under the treatment with Roundup® herbicide (WILSON *et al.*, 2008).

CONCLUSIONS

These observations lead us to conclude that Roundup® in a dose of $0.138x10^{-3}$/g of body weight determinates morphologic modifications in the lung tissues of *Pelophylax ridibundus* in both thermic variants (at 4-6º and at 22-24ºC). Lung tissue of the frogs showed marked pathological changes such as: hyperplasia of the respiratory epithelium, an increase in the number of goblet cells, which are also hyperplasiated. Also, pneumocytes react by increasing the nucleus diameter in relation to the cell diameter. The lung parenchyma shows the presence of interstitial edema, characterized by the presence of dilated blood vessels and deposits of melanin. Highly degenerative changes in lung tissue were evident in animals that were treated with toxic and kept at 22-24ºC.

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