HISTOLOGICAL CHANGES INDUCED BY THE ACTION OF THE INSECTICIDE RELDAN 40EC IN *RANA RIDIBUNDA*

MODIFICĂRI HISTOLOGICE INDUSE DE ACȚIUNEA INSECTICIDULUI RELDAN 40EC LA *RANA RIDIBUNDA*

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Abstract. *The main purpose of this work is to study the histological changes induced by the action of the insecticide Reldan 40EC (chlorpyrifos-methil) on Rana ridibunda. Reldan 40EC is one of the most widely used organophosphorus insecticides in agriculture with its attendant adverse health outcomes. The animals used in the experiment were divided in four experimental lots: two lots of control individuals (first lot was kept at 4-6ºC and the second lot at 22-24ºC) and two experimental lots in which the animals were treated with 0.01 ml/g body weight Reldan 40EC and kept at 4-6ºC, respectively at 22-24ºC. The toxic was administrated with intraperitoneal shots (one shot every two days, in a scheme of three weeks). At the end of the experiment we observe histological changes in skin.*

Key words: stratified epithelium, mucous gland, chlorpyrifos-methil, *Rana ridibunda*

Rezumat. *Scopul acestei lucrări este acela de a urmări modificările histologice induse de acțiunea insecticidului Reldan 40EC la broasca de lac (Rana ridibunda). Reldan 40EC este unul dintre cele mai utilizate insecticide organofosforice în agricultură cu efecte negative asupra sănătății omului şi animalelor. Animalele utilizate au fost împărțite în 4 loturi: 2 loturi martor (unul în care animalele au fost ținute la o temperatură de 4-6ºC şi al 2-lea în care animalele au fost ținute la o temperatură de 22-24ºC) şi 2 loturi de experiență în care animalele au fost tratate cu 0.01ml/g greutate corporală Reldan şi ținute la 4-6ºC, respective 22-24ºC. Toxicul a fost administrat prin injecții intraperitoneale (câte o injecție la 2 zile timp de 3 săptămâni). La sfârşitul tratamentului s-au constatat modificări histologice la nivelul tegumentului.*

Cuvinte cheie: epiteliu statificat, glande mucoase, clorpirifos-metil, *Rana ridibunda*

INTRODUCTION

Dramatic declines in amphibian populations have been described all over the world since the 1980s. 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 (Relya, 2006).

The evidence that the sensitivity to environmental threats is higher in amphibians than in mammals has been generally linked to the observation that amphibians are characterized by a rather permeable skin (Boone and Bridges, 2003, Rowe et al., 2003). Some researchers have focused attention on the study of the antioxidant defense mechanisms of mammal skin after exposure to oxidative stressors, including chemical pollutants and ionizing, ultraviolet, and UV irradiation (Kohen, 1999).

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 (Duellman and Trueb 1986). Amphibian skin is highly permeable because it is physiologically involved in gas, water, and electrolyte exchange with the environment (Quaranta et al., 2009).

Several studies have suggested that in frogs, epidermal principal cells are mainly involved in active transport, whereas MRCs (flask-shaped mitochondria-rich cells) are specialized in proton and bicarbonate secretion (Lindeman and Voûte l976; Fox, 1986; Ehrenfeld and Klein 1997). Numerous investigations on frog skin have focused mainly on morphologic and ion-transport changes under both, natural and experimental conditions (Barni et al. 1987; De Piceis Polver et al. 1988; Vanatta and Frazier 1989; Malvin and Hlastala 1989) and in relation to environmental contamination as well (Ferrari and Salibian 1999; Suwalsky et al. 2000, 2001, 2004). Johnson et al. (2000) have undertaken studies to date on the detoxifying and antioxidant responses of the amphibian epidermis to counteract effects of contaminants.

The aim of this study was to assess the several of histological alterations observed in skin frogs who were intoxicated with Reldan 40EC in a dose of 0.01 ml/g body weight.

MATERIAL AND METHOD

In all the variants, frogs (*Rana ridibunda*) of both sexes captured from the bordering lakes of Pitesti (Romania) were used. Animals were kept unfed in freshwater aquaria for 5 days and 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^{o}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 Reldan 40EC in a dose of 0.01 ml/g of body weight and kept at 4-6ºC, (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 22-24ºC. 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 by decapitation, under chloroform anesthesia, and fragment of ventral skin 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 H&E, Sirius red for collagen (Juncueira et al. 1979).

The toxic substance used was the insecticide commercialized under the generic name „Reldan 40EC" which has as an active substance chloropyrifos-methyl. Reldan 40EC is an organophosphorous pesticide that is currently registered, or has tolerances pending, for crops and livestock, ornamental plants, turf, household pests, and mosquito control. The most obvious threat to the aquatic environment is its use as a mosquito larvicide's; fish and aquatic invertebrates can also be affected through runoff due to certain terrestrial uses (Cebrián, 1992).

RESULTS AND DISCUSSIONS

The stratified epidermis of adult anurans consists of several layers: germinative (or basal), spinous (or spinosum), granulous (or granulosum), and hornified (or corneum), all consisting of principal cells or keratinocytes showing a peculiar morphologic aspect depending on the layers (Fox 1986; Lindeman and Voûte 1976).

Compared with amniotes, anuran epidermis shows some structural differences; in particular, it exhibits a relatively thin hornified surface composed of one or two layers of flattened cells that retain their nuclei (Alibardi, 2003). Based on these structural traits, frogs can use the epidermis for respiratory gas (mainly carbon dioxide) and ion exchange. In fact, being exposed to both, air and pond water, the skin of the frog represents one of the principal organs for body-fluid homeostasis (Fenoglio et al., 2006).

At light microscopy, frog epidermis that were treated with Reldan 40EC in a dose of 0.01ml/g body weight and kept at 4-6ºC was found to consist of four layers of cells with a superficial flat layer of hornified cells showing picnotic nuclei (fig.1).

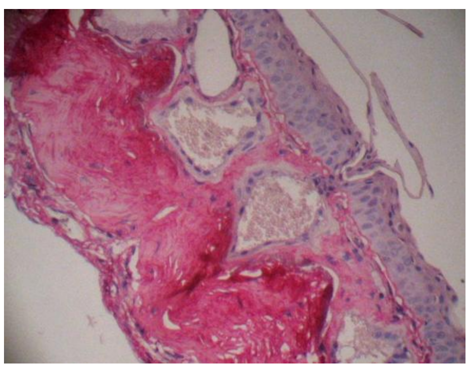

Fig.1. Skin of Rana ridibunda treated with Reldan 40EC in a dose of 0.01ml/g body weight and kept at 4-6ºC. HE and Sirius red. 10X

Epidermis displayed a tendency to become thicker. In addition, the presence of some pale keratinocytes was noticed in the subcorneal layer. Cells in the deeper layers (basal to granulous) displayed more abundant cytoplasm and spheroid or oval nuclei. A thin interstice was present between the external horny layer and the epidermal layer just below (fig.2).

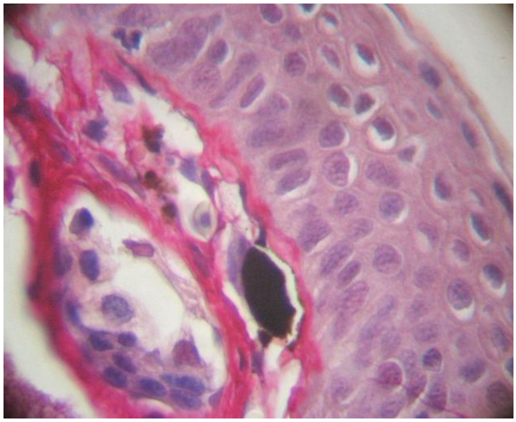

Fig.2. Skin of Rana ridibunda treated with Reldan 40EC in a dose of 0.01ml/g body weight and kept at 4-6ºC. HE and Sirius red. 40X

The skin presented a high number of hypertrophied acinose glands. In parallel, we registered an intense secretion of the mucous (fig. 3).

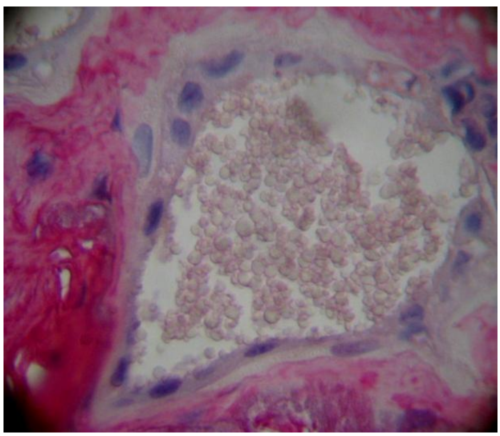

Fig.3. Secretor cells in mucous glands. HE and Sirius red. 40X

In the skin sections of frogs that were treated with Reldan 40EC in a dose of 0.01ml/g body weight and kept at 22-24ºC was found to consist more layers of cells and less keratinized superficial cells in the epidermis (fig.4, 5). We concluded that the highly stratified epidermis was directly linked to the insecticide action (Păunescu et al., 2008, 2009). The skin presents a more pronounced hypertrophy of the dorsal and ventral mucous glands.

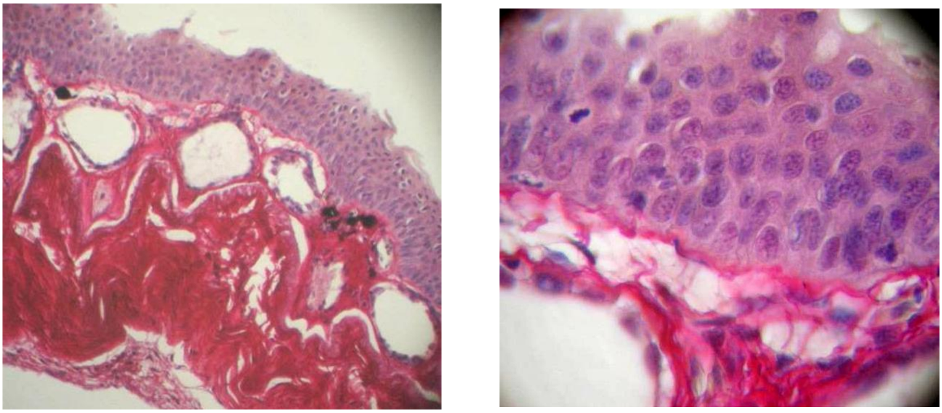

Fig.4. Skin of Rana ridibunda treated with Reldan 40EC in a dose of 0.01mlțg body weight and kept at 22-24ºC. HE and Sirius red. 10X, 40X

CONCLUSIONS

Generally, in the skin sections of frogs, no degenerative alterations were detected but remarkable morphologic modifications were detected. However, the epidermis become more thickness in animal treated with Reldan 40EC in a dose of 0.01ml/g body weight and kept at 4-6ºC. At 22-24ºC, Reldan 40EC in a dose of 0.01ml/g body weight determines a highly stratified epidermis with less keratinized superficial cells. In both variants skin presented a high number of hypertrophied mucous glands and an intense secretion of mucous.

REFERENCES

1. **Alibardi L.,** 2003 - *Adaptation to the land: The skin of reptiles in comparison to that of the amphibians and endotherm amniotes*. J Exp Zool 15:l2–41.
2. **Barni S., Bernocchi G., Gerzeli G.,** 1987 - *Nuclear changes and morphology of the epidermis in the hibernating frog.* Tissue Cell 19(6):817–825.
3. **Boone MD, Bridges CM,** 2003 - *Effects of carbaryl on green frog (Rana clamitans) tadpoles: timing of exposure versus multiple exposures*. Environ Toxicol Chem 22: 2695–2702.
4. **Cebrián C., Andreu-Moliner E. S., Fernández-Casalderrey A., Ferrando M. D.**, 1992 - *Acute Toxicity and Oxygen Consumption in the Gills of Procambarus clarkii in relation to Chlorpyrifos exposure*. Bull. Environ. Contain. Toxicol. 49:145-149.
5. **De Piceis Polver P., Fenoglio C., Rapuzzi G., Gerzeli G., Barni S.,** 1988 - *Potassium-dependent p-nitrophenyl phosphatase and adenylate cyclase activity in Rana esculenta skin during natural hibernation and active life: A cytochemical study*. Arch Biol 99:183–195.
6. **Duellman W., Trueb L.,** 1986 - *Biology of the amphibians*. New York, NY, McGraw-Hill.
7. **Ehrenfeld J., Klein U.,** 1997 - *The key role of the H^+ V-ATPase in acidbase balance and Na^+ transport process in frog skin*. J Exp Biol 200:247–256.
8. **Fenoglio C., Grosso A., Boncompagni E., Milanesi G., Gandini C., Barni S.,** 2006 - *Morphofunctional Evidence of Changes in Principal and Mitochondria-Rich Cells in the Epidermis of the Frog Rana kl. esculenta Living in a Polluted Habitat*. Arch. Environ. Contam. Toxicol. 51: 690–702.

9. Ferrari L., Salibian A., 1999 - *Effect of cadmium on the epidermic structure of Bufo arenarum tadpoles: Influence of the chemical composition of the incubation media.* Arch Physiol Biochem 107:91–96.

10. Fox H, 1986 - *The skin of amphibia: Epidermis*. In: Bereiter-Hahn J, Matoltsy AG, Richards KS (eds).The integument. Volume 2. Vertebrates. Springer, New York, pp 472–498.

11. Johnson M.S., Vodela J.K., Reddy G., Holladay S.D., 2000 - *Fate and the biochemical effects of 2,4,6-trinitrotoluene exposure to tiger salamanders (Ambystoma tigrinum).* Ecotoxicol Environ Safe 46:186–191.

12. Juncueira L.C.U., Bignolas G., Brentani R.R., 1979 - *Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue section*. Histochem J. 11:447–455.

13. Kohen R., 1999 - *Skin antioxidants: Their role in aging and in oxidative stress—New approaches for their evaluation.* Biomed Pharmacother 53:181–192.

14. Lindeman B., Voûte C., 1976 - *Structure and function of the epidermis.* In: Llinas RR, Precht H (eds). Frog neurobiology. Springer Verlag, New York, pp 169–210.

15. Malvin G.M., Hlastala M.P., 1989 - *Effects of environmental O_2 on blood flow and diffusing capacity in amphibian skin*. Respir Physiol 76:229–241.

16. Păunescu Alina, Ponepal Cristina Maria, Drăghici O., Marinescu Al. G., 2008 - *Histologic modification induced by the action of the insecticide Samurai on the skin and liver of Rana ridibunda*. Lucrări ştiinţifice, seria B-LI-2008: 666-670.

17. Păunescu Alina, Ponepal Cristina Maria, Drăghici O., Marinescu Al. G., 2009 - *The influence of Carbetox insecticide upon histological and physiological indices in Rana ridibunda*. Bulletin USAMV Agriculture, 66(2): 173-178.

18. Quaranta A., Bellantuono V., Cassano G., Lippe C., 2009 - *Why Amphibians Are More Sensitive than Mammals to Xenobiotics.* PLoS ONE 4(11).

19. Relyea R. A., 2006 - *The effects of pesticides, pH, and predatory stress on amphibians under mesocosm conditions*. Journal Ecotoxicology 15:503-511.

20. Rowe C.L., Hopkins W.A., Bridges C.M., 2003 - *Physiological ecology of amphibians in relation to susceptibility to natural and anthropogenic factors.* In Linder G, Krest S, Sparling D, eds. Amphibian Decline: An Integrated Analysis of Multiple Stressor Effects. Pensacola: SETAC press. pp 9–57.

21. Suwalsky M., Norris B., Cardenas H., 2000 - *Mercuric chloride ($HgCl_2$) and methyl mercury (CH_3HgCl) block sodium transport in the isolated skin of the toad Pleurodema thaul.* Bull Environ Contam Toxicol 65:794–802.

22. Suwalsky M., Ungerer B., Villena F., Norris B., Cardenas H., Zatta P., 2001 - *Effects of AlCl3 on toad skin, human erythrocytes, and model cell membranes.* Brain Res Bull 55:205–210.

23. Suwalsky M., Schneider C., Norris B., Cardenas H., 2004 - *Effects of Pb^{2+} ions on Na^+ transport in the isolated skin of the toad Pleurodema thaul.* Biometals 17:655–668.

24. Vanatta J.C., Frazier W., 1989 - *Histological changes in the skin of Rana pipiens produced by metabolic alkalosis*. Tissue Cell 21:219–227.