Bull. Environ. Contam. Toxicol. (2000) 65:501–507

DOI: 10.1007/s001280000152

Bulletin of Environmental Contamination and Toxicology

Cypermethrin-Induced Apoptosis in the Telencephalon of *Physalaemus biligonigerus* Tadpoles (Anura: Leptodactylidae)

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Received: 12 February 2000/Accepted: 20 July 2000

Worldwide attention has been focussed recently on amphibians because of concerns that populations are declining globally (Wake 1991; Hedges 1993). Pesticide pollution is probably one of the most important factors causing amphibian decline in agricultured regions (Berrill et al., 1994).

Cypermethrin (CY) (RS)-alpha-cyano-3-phenoxybenzyl (1RS)-cis-, trans-3- (2,2, -dichlorovinyl)-2,2-dimethylcyclopropane carboxylate) is a highly active synthetic pyrethroid type II. Synthetic pyrethroids have increased in popularity for control of insect pest in agricultural and aquatic systems (Smith and Stratton 1986). They are characterized by strong, broad-spectrum insecticidal activity, based on their neurotoxicity (Salibián 1992). Salibián and Marazzo (1995) noted that the biocidal action of most pyrethroids is likely produced by alterations in the ion conductance of nerve cell membranes. Berlin et al. (1984) showed that pyrethroids cause increase transmembrane sodium influx and inhibition of ion-dependent ATPases in nervous tissues of insects, squids and toads. Moreover, pyrethroids have been recently postulated to induce apoptosis in the testicular tissues of the rat, by mechanisms mediated via nitric oxide and other reactive oxygen species (El-Gohary et al. 1999).

The purpose of this work was to examine under laboratory conditions the sub-lethal and acute doses of CY in *Physalaemus biligonigerus* tadpoles, through their survival and the effects of CY on brain morphology.

MATERIALS AND METHODS

Foam nests of *Physalaemus biligonigerus* were collected from temporary ponds in the Paraná River floodplain (31°43′ S; 60°34′ W), Paraná, Argentina. The tadpoles were acclimatized in glass tanks with artificial pond water (APW): pH 8.1, conductivity: 410 μmhos/cm^{-1}, dissolved oxygen concentration: 5.5 mg/L, hardness: 83 mg/L as CO_3Ca at 22°C ± 2°C for 7 days, and 12 h-12 h light-dark cycles

The 96-h acute toxicity and sub-lethal tests were conducted according to

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USEPA (1975; 1989) standard methods, with prometamorphic larvae (from stages 26 up to 36) (Gosner 1960). The glass tanks (35 cm diameter and 60 cm high) with 4 L of (APW) and 10 tadpoles (average weight: 0.06 ± 0.01 gr.) per tank were used in the experiments. The assayed product was SHERPA® a commercial formulation containing 25 % of CY in xylene. In the final acute toxicity survival test, the concentrations used were 375; 750; 1,500; 3,000 and 6,000 µg CY/L. The sub-lethal test was realized in the same way of the acute toxicity test, but the concentrations used were 12.9, 21.6, 36, 60 and 100 µg CY/L.. Controls were conducted in APW with or without vehicle (xylene) during the same periods. Both control and test solutions were made by triplicate. Solutions were renewed daily. Mortality was recorded every 24 h. The LC-50 with confidence limits ($p<0.05$) were estimated by using a Probit Analysis Program based on Finney (1971). Data from control and experimental groups were analyzed by one-way analysis of variance in conjunction with LSD test.

Control and treated survival tadpoles, emerging of the acute and sub-lethal tests at 24, 48, 72 and 96 h, were fixed in a solution of 3% glutaraldehyde, 3% formaldehyde, saturated solution of 1% picric acid in 0.1 M phosphate buffered saline (PBS), pH 7.4 for 4 h at room temperature. Animals treated with 6,000 µg CY / L were not analyzed because of the high mortality exhibited at 24 h.

Following fixation, brain, skin and eyes were carefully dissected microscopically, and immersed in the same fixative for 1 h at 4°C. The tissues were extensively washed in PBS and rinsed in 1% OsO_4 for 2 h, washed in PBS and dehydrated in increasing concentrations of acetone and embedded in araldite resin (Ladd®). After polymerization, the different tissues were cut at 0.5 µm with a Reichert Ultracut-S and stained with toluidine blue. Thin sections of 80 nm for electron microscopy analysis were done from preselected areas of each tissue under study.

RESULTS AND DISCUSSION

The average rate of pyrethroids application to crops is 10-200 g of active ingredient (CY/ha) WHO, 1992. In Argentina the levels of application oscillate between 1.5 g and 120 g of active ingredient (CY/ha) (CASAFE, 1995). CY is one of the most light-stable pyrethroid (Ruzo et al., 1977) and its degradation rates were similar in both laboratory and field surveys (Roberts and Standen 1981).

Pyrethroids are relatively nontoxic to birds and mammals but they are extremely toxic to aquatic organisms, including fish and invertebrates (Jolly et al. 1978, Holcombe et al. 1982, Little et al. 1993). In laboratory tests 96-h LC 50 was within the range of 0.4 - 2.8 µg CY / L for fish and 0.01 - 5 µg CY / L for aquatic invertebrates (WHO 1989).

In the present work, we determined that the 96h-LC50 for *Physalaemus biligonigerus* tadpoles induced by CY/L was 129 µg CY/L, a concentration higher

than the values reported for fish (96h-LC50: 0.4-2.8 μg CY/L) (WHO 1989) (Table 1). However, comparing the confidence limits of our acute test and the morphological analysis of the sub-lethal test it could be inferred that, even lower concentrations than 96h-LC50 might cause neuroalterations. Our estimated value range, was similar to Alpha-Cypermethrin (0.7 and 350 μg/L depending upon formulation; WHO 1992). Our study indicates that native populations of *Physalaemus biligonigerus* tadpoles can be affected by the application of CY, even at a concentration lower than LC-50 obtained in the laboratory (Fig. 1).

In experiments using CY as a field spray, at an initial concentration of 24,000 μg/L showed a decline of approximately 50 μg/L after the first week of treatment (Crossland 1982). Estimating a linear degradation, the CY concentration in stagnated waters would be 10,000 μg/L after 96 h of treatment. Those values are much higher than the dose used by us in determining the 96 h LC-50 for *Physalaemus biligonigerus* tadpoles.

Table 1. Acute toxicity response (LC 50) of *Physalaemus biligonigerus* tadpoles exposed by Cypermethrin, $n = 30$.

Time (h)	LC50 (μg CY/L)	Confidence Limits Lower	Upper
24	1012	393	1787
48	592	133	1065
72	253	97	629
96	129	29	414

The neurotoxic effects of CY can be divided in four phases (see: Salibián 1992). In our experiments we determined that the sub-lethal doses induced signs equivalent to toxic phases I and II, whereas acute 96 h tests induced the four toxicity phases in the larvae.

The most noticeable morphological process observed at concentrations higher than 100μg CY /L was the presence of massive cell death resembling apoptosis (programmed cell death) in neural cells. CY-induced apoptosis of neural cells was dose- and exposure time-dependent. Brain ventricular, marginal and intermediate cell layers were differentially affected showing loss of cell volume and cell junctions. By light microscopy, apoptotic bodies were evident as round or oval dense masses of condensed nuclei and cytoplasm, which were invariably smaller than the cell of origin (Figs 2b, c, d). There was a very prominent intercellular increase in space, even in brains of animals treated with 100 μg CY / L for 48 hs. However, we could not distinguish any apoptotic figures (Fig 2b).

At one electron microscopic level, immature brain cells of control animals, treated with xylene alone, had typical characteristics of normal tissue (Fig. 3a). Only few physiological apoptotic figures were seen in these control animals (Fig.3a).

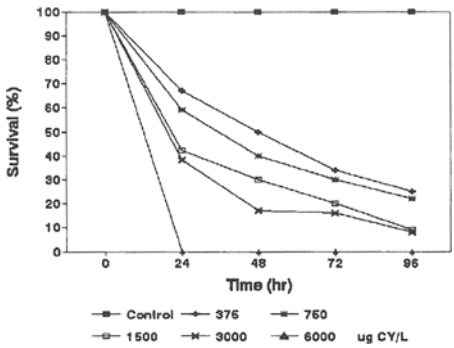

Figure 1. Survival curves for *Physalaemus biligonigerus* tadpoles.

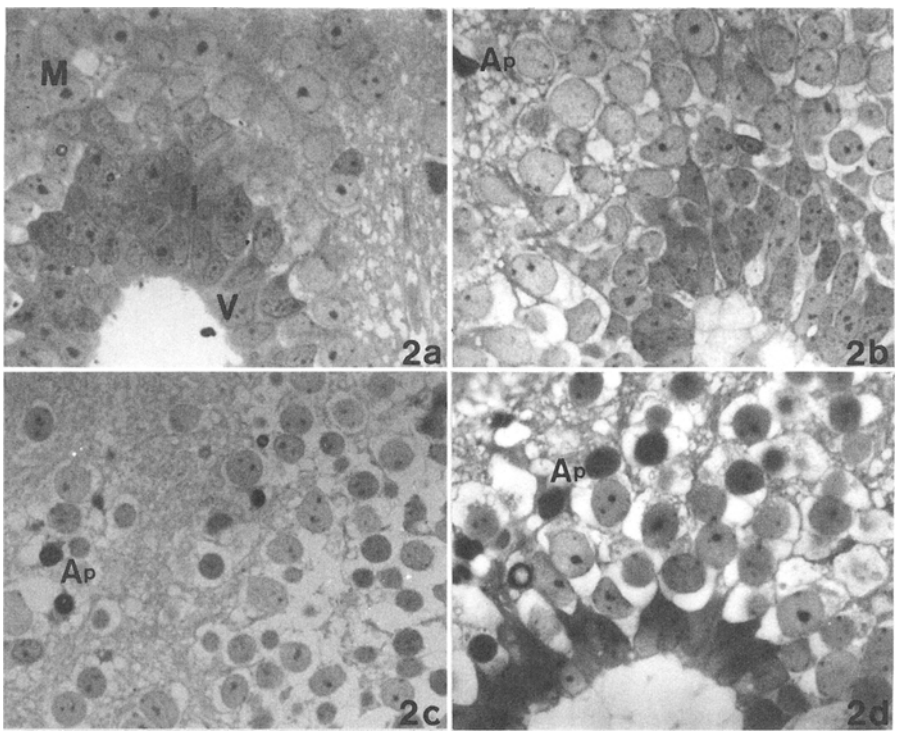

Figure 2. Cross sections of *Physalaemus biligonigerus* tadpole telencephalon. (a) Control section. Observe the three normal cellular layers: ventricular, intermediate, and marginal; (b) 100 μg CY / L - 48 h. Note the incipient cell shrinkage and loss of cell junctions; (c) 100 μg CY / L - 96 h. Long exposure-induced cell shrinkage and increased intercellular space. At this time point, numerous apoptotic figures can be seen and the tissue organization of the layer is lost. (d) 1,500 μg CY / L - 48 h. Short exposure time and higher dose of CY cause similar neuroalterations as shown in c. V: ventricular layer, I: intermediate layer and M: marginal layer, Ap: apoptotic figures. (X 330).

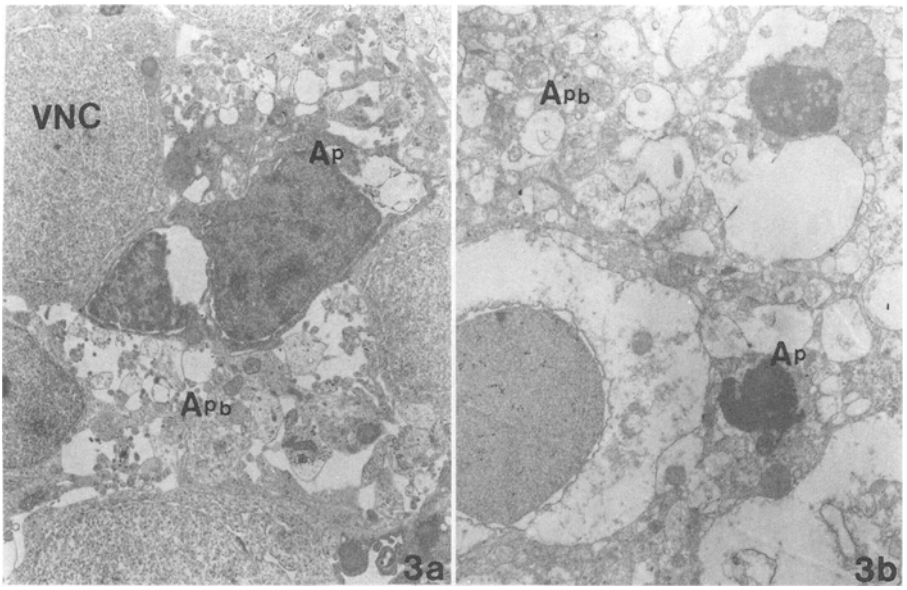

Figure 3. Electron microscopy of *Physalaemus biligonigerus* tadpole telencephalon. (a) Detail of an apoptotic cell in the telencephalon of a control animal (X 7,000) (b) Detail of telencephalon after 100 μg CY / L - 96 h treatment showing two apoptotic cells surrounded by enlarged intercellular spaces (X 7,000). Ap: apoptotic figure; VNC: viable neural cell; Apb: apoptotic bodies

In all treated tadpoles, we verified a massive wave of apoptosis, characterized by convoluted surfaces and membrane blebbings. While the cytoplasmic organelles were largely intact, the endoplasmic reticulum was dilated and a series of crater-like cavities were fused with the cell surface (Fig. 3b).

The morphology of mitochondria and ribosomes was normal in contrast to cells dying by necrosis. The chromatin formed dense crescent-shaped aggregates lining the nuclear membrane, and nuclear pores were absent from nuclear membranes, resulting in segmented nuclei. The convoluted plasma membranes formed clusters of membrane bound segments, termed "apoptotic bodies", which often contained morphologically normal mitochondria and other cellular organelles (Fig. 3b). There were also numerous apoptotic bodies phagocytized by macrophages or other adjacent normal viable cells (not shown).

We did not found any significant morphological alteration in other common target tissues. Therefore, we concluded that the toxic effect of CY appears specific to the developing nervous tissue. Our results agree with previous reports suggesting a correlation between use of CY and the mortality of tadpoles (WHO, 1989).
In the present report we did not determine the pathophysiological and molecular mechanisms of CY apoptotic toxicity. However, there is evidence indicating that

pyrethroids toxic activity is mediated mainly by alteration of the membrane Na^+ transport. We suggest that the effect of CY on ion transport may be a mechanism by which CY induces massive apoptosis of neurons in the central nervous system of tadpoles.

From an ecological point of view, although fish are more sensitive to pyrethroid toxicity than amphibians, the high frequency of temporary pond tadpole habitats in Northeast Argentina makes the amphibians more vulnerable to CY than lotic fish. We propose that *Physalaemus biligonigerus* tadpoles can be used as a sensitive indicator organism for aquatic bioassays of pyrethroids.

Acknowledgments. These studies were supported by a SCYTFRH-UNER (PID: 6053-1) grant.

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