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Apoptotic cell death in the central nervous system of *Bufo arenarum* tadpoles induced by cypermethrin

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Abstract

Tadpoles of the toad *Bufo arenarum* treated with cypermethrin (CY) at concentrations above 39 μg CY/L showed dose-dependent apoptotic cell death in immature cells of the central nervous system as demonstrated by morphometric analysis, the TUNEL method, and DNA fragmentation assay. Light-and electron-microscopic studies showed structural alterations in the intermediate and marginal layers of the brain. Immature cerebral tissue showed cellular shrinkage, nuclear fragmentation and increase of intercellular spaces. In this study we demonstrated high toxicity of CY to larval stages of *Bufo arenarum*. Our results show that doses lower than those used in routine insecticide applications can cause massive apoptosis in the immature cells of the central nervous system. These results coincide with our previous studies in *Physalaemus biligonigerus*, confirming the severe toxic effects of CY to the central nervous system of anuran species from Argentina. This may increase the mortality index in wild animals and contribute to the loss of biodiversity in our agroecosystems. We postulate that CY induces apoptosis in central nervous system cells of *Bufo arenarum* tadpoles by specific neurotoxic mechanisms.

Abbreviations: APW, artificial pond water; CY, cypermethrin; DAPI, 4′,6-diamidino-2-phenylindole; EDTA, ethylenediaminetetraacetic acid; GABA, γ-aminobutiric acid; hCG, human chorionic gonadotropin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick-end labeling

Introduction

Cypermethrin (CY; (*R,S*)-α-cyano-3-phenoxybenzyl-(1*R,S*)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate) is a racemic mixture of eight isomers, a highly active synthetic pyrethroid type II. These are among the most active insecticides and at concentrations as low as 0.02–0.05% (5–200 g/L per ha) can be as efficient as organophosphorus compounds (Kaloyanova et al., 1991). The alpha-CN derivatives (type II) include deltamethrin, cypermethrin, cyhalothrin, fenvalerate, fenpropathrin. These contain the cyano group in the *S*-configuration of the 3-phenoxybenzyl alcohol, showing 3-fold to 6-fold higher potency against insects than the non-CN

derivatives (type I) permethrin, alphamethrin and bioallethrin (Kaloyanova et al., 1991). Pyrethroids are used as insecticides in agricultural fields, orchards, and greenhouses, and in stored farm products. They are effective against pests resistant to other insecticides (Kaloyanova et al., 1991). Synthetic pyrethroids have gained popularity for controlling insect pests in agricultural and aquatic systems (Smith and Stratton, 1986) because they combine high insecticidal activity with a moderate to low oral mammalian and avian toxicity and good physicochemical persistence (Elliott, 1976; Miyamoto, 1976). However, arthropods, fish, and frogs are very sensitive to the toxic effects of pyrethroids (Jolly et al., 1978; Holcombe et al., 1982; Cole and Casida, 1983; Salibián, 1992; Little et al., 1993; Izaguirre et al., 2000, 2001). Species specificity is conferred by differences in both detoxification and target site sensitivity (Casida et al., 1983).

Pyrethroids are characterized by strong, broad-spectrum insecticidal activity, based on their neurotoxicity (Cole and Casida, 1983; Kaloyanova et al., 1991; Salibián, 1992; Izaguirre et al., 2000; 2001). They act on the axons of the peripheral and central nervous system and displace the specific binding of kainic acid by interacting with sodium channel gating mechanisms. Synthetic pyrethroids induce delay in closing of sodium channels (Kaloyanova et al., 1991), producing alterations in the ion conductance of nerve cell membranes, which may be one of the mechanisms of pyrethroid toxicity (Salibián and Marazzo, 1995). This in turn results in increased transmembrane sodium influx and inhibition of ion-dependent ATPases in the nervous systems of insects, squids, and toads (Berlin et al., 1984). Changes in the flow of K^+ and Na^+ are known to play a critical role in the activation of neuronal apoptosis. Our previous studies demonstrated that CY caused apoptotic cell death in the telencephalon of anuran larvae (Izaguirre et al., 2000, 2001). These results provide evidence of ion-mediated neurotoxicity mechanisms for CY. Furthermore, calcium-sensitive proteins involved in synaptic transmission appear to be the major active targets of type II pyrethroids (Enan and Matsumara, 1993).

In fish brains, pyrethroids interfere with the function of GABA receptors indirectly through interaction with the voltage-dependent sodium channel and consequently perturbing chloride influx, possibly through a voltage-dependent chloride channel (Eshleman and Murray, 1991). In mammals, pyrethroids cause muscle contraction and positive inotropic effects by increasing transmembrane sodium influx and catecholamine release from sympathetic nerve terminals (Berlin et al., 1984). Moreover, pyrethroids have recently been shown to induce apoptosis in the testicular tissues of rats, by mechanisms mediated via nitric oxide and other reactive oxygen species (El-Gohary et al., 1999).

The purpose of this work was to study the apoptotic effects of CY in *Bufo arenarum* tadpoles, a toad species that is one of the most important representatives of anurans in our region. It is a model system that can easily be fertilized and is easily manipulated in the laboratory, and it is sensitive to CY toxic effects similar to those previously reported in a more vulnerable species (Izaguirre et al., 2000, 2001).

Materials and methods

Animals

The production of oocytes in *Bufo arenarum* adult females was induced using intraperitoneal injection of 2500 IU human chorionic gonadotropin (hCG, Endocorion, Elea, Buenos Aires, Argentina). Larvae were obtained by artificial fertilization, mixing oocytes with testis homogenates, and cultured in Holtfreter's solution (Holtfreter, 1931).

Embryos were maintained in Holtfreter's solution until hatching. The tadpoles were acclimatized in glass tanks (35 cm diameter and 60 cm high) containing artificial pond water (APW, pH

8.1, conductivity 410 μS cm^{-1}, hardness 83 mg/L as $CaCO_3$ at 22 ± 2°C, dissolved oxygen concentration 5.5 mg/L) and exposed to 12 h–12 h light–darkness cycles.

Bioassay

The 96-hour toxicity test was conducted according to USEPA (1975, 1989) standard methods, with prometamorphic tadpoles of stage 28 (Gosner, 1960). The assayed product was a commercial formula containing 25% CY in xylene (Sherpa, Rhône Poulenc Argentina Fomental, Buenos Aires, Argentina). Bioassays were done by triplicate, at population density in the containers (35 cm diameter, 60 cm high) of 10 larvae/L ($n = 3$), at 20 ± 2°C and in 12 h–12 h light–darkness conditions.

Solutions were renewed daily. In the final toxicity survival test, the concentrations used were 39, 156, 625, 2500, and 10, 000 μg CY/L and the mortality was registered at 24, 48, 72, and 96 h. Tadpoles were considered dead when they did not exhibit heartbeat or did not respond to gentle prodding, and dead tadpoles were removed from assay containers. Results were expressed as cumulative mortality (Ferrari et al., 1997).

Controls were conducted in triplicate in APW with or without vehicle (xylene) during the same periods. The 50% lethal concentration (LC_{50}) with confidence limits ($p < 0.05$) was estimated by using a Probit analysis program (Finney, 1971). Data from control and experimental groups were analyzed by one-way analysis of variance in conjunction with LSD test (Sokal and Rohlf, 1979). Neurotoxicity was evaluated as described previously Salibián (1992).

Histological and ultrastructural studies

Control and treated surviving tadpoles from 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 mol/L, phosphate-buffered saline (PBS), pH 7.2 for 4 h at room temperature. Animals treated with 10 000 μg CY/L were not analyzed because of the high mortality exhibited as early as 24 h of treatment.

Brains of fixed animals were dissected, washed in PBS, post-fixed in 1% OsO_4 for 2 h at room temperature, dehydrated in increasing concentrations of acetone, and embedded in araldite resin (Araldite 6005 Resin, Ladd Research Industries, Inc. Burlington, VT, USA). After polymerization, the larval brains were cross-sectioned at 0.5 μm every 50 μm using a Reichert Ultracut–S ultramicrotome. Sections were stained with toluidine blue and photographed using a PM20 camera in an Olympus BX50 microscope. Electron microscopy was done in 70 nm thick sections from preselected areas of the brain in a 201 EM Philips electron microscope.

Tissue processing for morphometry and TUNEL studies

For morphometric studies, groups of control and treated surviving tadpoles were fixed in 4% formaldehyde in 0.1 mol/L PBS at pH 7.2; for TUNEL studies, tadpoles were fixed in Carnoy's solution. The tissues were washed in PBS and dehydrated in increasing-concentration ethanol series, cleared with xylene, and embedded in paraffin (Cicarelli, San Lorenzo, Santa Fe, Argentina). Sagital 5 μm thick sections were obtained with a Reichert Hn 40 microtome and placed on 1% gelatin-coated glass slides.

Morphometry

Brain serial sections of the five surviving tadpoles from the control and 96 h treatments, for each CY concentration, were stained with hematoxylin and eosin. The apoptotic cell counting of the brain was done manually by two different operators using a Leitz compound microscope with a ×400 magnification. For the morphological identification of apoptotic cells versus normal cells, a previously defined criterion (Izaguirre et al., 2000) was followed. Cells were considered apoptotic when they

appeared surrounded by wider intercellular spaces and exhibited pyknotic nuclei and cellular shrinkage.

For each brain, an apoptotic index was calculated as follows:

$$A_i = A_n / B_d$$

where A_i = apoptotic index, and A_n/B_d = apoptotic cell number/brain diameter. When the apoptotic index exceeded 0.60, cerebral apoptosis was considered significant.

Each experimental point was obtained from groups of 5 animals for each CY concentration and the data were expressed as mean ± standard deviation. Variance analysis of the results was performed using the Kruskal–Wallis test (nonparametric ANOVA) using the multiple-comparison Dunn post test. Differences were considered significant at $p < 0.05$.

In addition, morphometric data were transformed ($1/x$) to calculate the 50% effective concentration (EC_{50}) at 96 h treatment. These values were estimated within confidence limits ($p < 0.05$) using a Probit analysis program (Finney, 1971). All the analyses were processed with a statistical computer program (GraphPad Instat Software 3.1 1994, serial number 50533-353).

TUNEL assay

To confirm the presence of apoptotic cells in brain of CY-treated tadpoles, a fluorescein-based TUNEL assay was used (Cell Death Detection, Boehringer Mannheim, Germany). Sagital 0.5 μm thick sections of control *Bufo arenarum* tadpoles and tadpoles exposed to cypermethrin for 96 h were deparaffinized in xylene and rehydrated in an ethanol series and distilled water. Sections were incubated with K proteinase (20 μg/ml in 10 mmol/L Tris-HCl, pH 7.4–8) for 15–30 min at 37 °C, and washed twice in PBS.

Oligonucleosomal DNA fragmentation characteristic of apoptotic cells were visualized by labeling the 3′-OH ends of DNA strands with fluorescein-labeled nucleotides through an enzymatic reaction catalyzed by the terminal deoxynucleotidyl transferase (TdT) enzyme for 60 min at 37 °C in a moisturized, dark chamber. The reaction was carried out following the manufacturer's instructions, using 50 μl volumes per section. Negative controls were done using enzyme-free solution.

Sections treated with DNase I (100 μg/ml in Tris-HCl 50 mmol/L, pH 7.5, $MgCl_2$ 1 mmol/L, 1 mg/ml BSA) for 10 min at room temperature to induce cleavage of DNA strands were used as positive controls. Slides were incubated in a moist, dark chamber for 60 min at 37 °C, washed, and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). The sections were analyzed and photographed using a fluorescence BX50 Olympus microscope.

DNA fragmentation assay

For analysis of DNA fragmentation by agarose gel electrophoresis, total DNA was isolated from brain tissues of three surviving tadpoles from the control and 96 h treatments, for each CY concentration, using a DNA extraction kit (ApopLadder EXTM, TaKaRa, Shiga, Japan). The isolated DNA was suspended in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.5) and quantified by absorbance at 260 nm. The DNA samples, 200 μg/ml per lane, were loaded onto 2.0% agarose gel (NuSieveR 3:1 Agarose, FMC BioProducts, Rockland, ME, USA) containing TBE buffer (89 mmol/L Tris-borate, 2 mmol/L EDTA, pH 8.0) and ethidium bromide and were separated by electrophoresis for 30 min at 100 V. The gels were dried for 3 h at room temperature. DNA bands were visualized with a UPV Transilluminator TFM-20 (Kodak Co.) before being photographed with a Polaroid (Cambridge, MA, USA) camera.

In each agarose gel, images were measured using software developed in Matlab 6.5. The data were plotted as integrated intensity, taking 30 random lines of each band fragment.

Data were expressed as mean ± standard deviation and analyzed with GraphPad Instat 3.06 (GraphPad Software, San Diego, CA, USA). Variance analysis of the results was performed using the Kruskal–Wallis test (nonparametric ANOVA) using the multiple-comparison Dunn post test. Differences were considered significant at $p < 0.05$.

Results

Bioassay

High death rates (∼65–70%) were observed on exposing tadpoles at CY concentrations of 39 and 156 μg CY/L for 96 h (Figure 1). There were no deaths among tadpoles treated with vehicle solution. At 625 and 2500 μg CY/L, the survival rate was approximately 15% at 96 h. At 24 h, practically all the tadpoles treated with 10, 000 μg CY/L died (Figure 1).

LC_{50} values found at different times of exposure to CY are shown in Table 1. The LC_{50} values were 6, 13, and 30 times lower at 48, 72, and 96 h respectively, than at 24 h, indicating a linear increase in toxicity with increased exposure time.

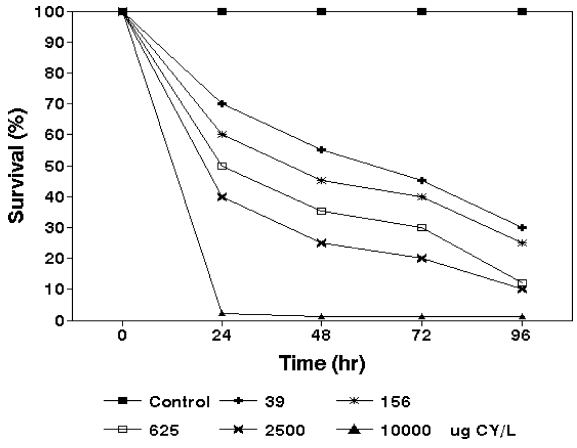

Figure 1. Survival curves for *Bufo arenarum* tadpoles exposed to cypermethrin; $n = 3$ independent experiments, 10 larvae each.

Table 1. Acute toxicity response (LC_{50}) of *Bufo arenarum* tadpoles exposed to cypermethrin, $n = 3$

Time (h)	LC_{50} (μg CY/L)	95% Confidence limits	
		Lower	Upper
24	3274	428	9550
48	488	47	2350
72	249	12	1104
96	110	3	476

Moreover, a dose-dependent hyperactivity syndrome and abnormal behavior of the tadpoles were observed, including lateral curving of the tails, arching of the cephalic–caudal axis, twisting and sinuous writhing of the body, and circular movement activity.

Histological and ultrastructural studies

The marginal and intermediate cell layers surrounding the brain ventricles of *Bufo arenarum* tadpoles treated for 96 h with increasing doses of CY (39, 156, 625, and 2500 μg CY/L) were the most affected areas. The tissues showed a decrease in cell volume and number, and loss of cell adhesion (Figure 2a–d). The number of neurons also decreased beneath the intermediate cell layer. Increased intercellular spaces and shrunken, apoptotic cells containing pyknotic nuclei and condensed cytoplasm were observed (Figure 2b–d). Although this effect was more evident at doses higher than 156 μg CY/L, apoptotic cells were observed at all doses and times analyzed, showing a dose-dependent increase (Figure 2a–d). No significant changes were observed in other tissues, even with the highest doses used (not shown).

At the electron-microscopic level, immature brain cells from animals treated with vehicle control showed no structural abnormalities (Figure 3a) and only rare apoptotic cells in untreated normal tissue (not shown). A dose- and time-dependent increase in apoptotic cells was observed in CY-treated tadpoles. Immature cells showed nuclei with the chromatin condensed into multiple clumps, dense aggregates lining

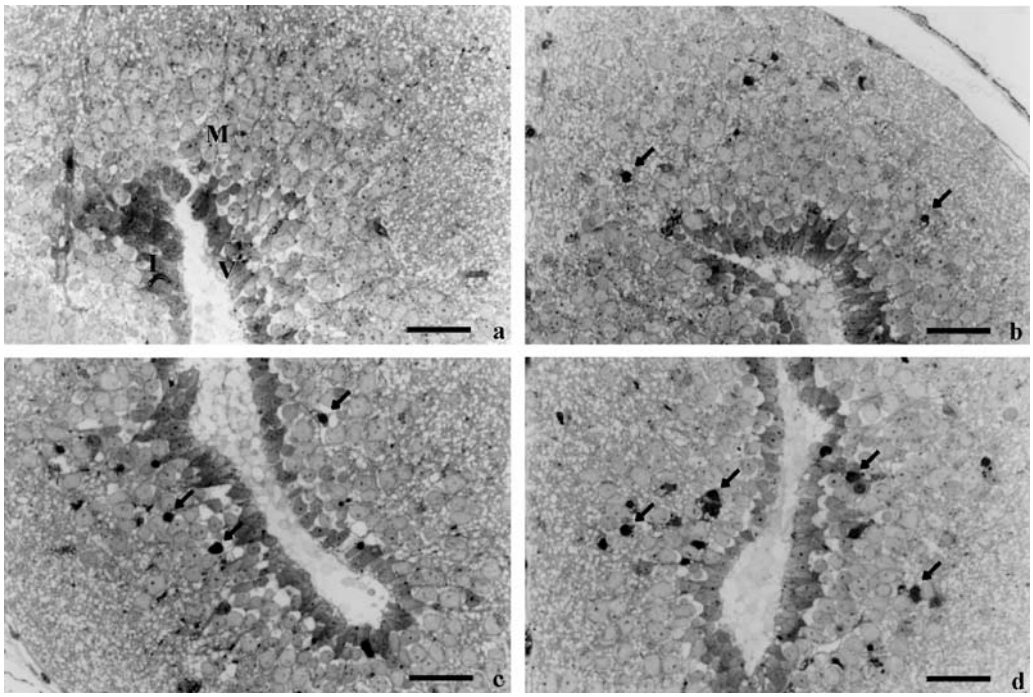

Figure 2. Cross-sections of *Bufo arenarum* tadpole telencephalon. (a) Control (xylene), ×133. Brain tissue shows three normal cellular layers: ventricular, intermediate, and marginal. (b) 39 μg CY/L at 96 h, ×133. Few apoptotic cells are seen and cell shrinkage and intercellular space increase are limited. (c) 625 μg CY/L at 96 h, ×133, showing high number of shrunken cells, increased intercellular spaces, and decreased cell number with consequent decrease in marginal and intermediate layer thickness. (d) 2500 μg CY/L at 96 h, ×133, showing massive apoptosis. Arrows, apoptotic figures; V, ventricular layer, I, intermediate layer; M, marginal layer. Bar = 8.6 μm.

the nuclear membrane (Figure 3b–e), and segmented nuclei (Figure 3c–e). Convoluted plasma membranes forming blebs and membrane clusters were observed, often containing mitochondria and other organelles (apoptotic bodies) (Figure 3c–e). The cytoplasm of apoptotic cells was condensed, but the organelles were mostly intact, except for increased density of the mitochondrial matrix (Figure 3c–e). Numerous apoptotic bodies were seen to be phagocytosed by macrophages or other adjacent viable cells (Figure 3b). The extracellular spaces were widened (Figure 3b–e).

Morphometry

Significant differences were found in the apoptotic index of brains from *Bufo arenarum* tadpoles treated with 625 and 2500 μg CY/L compared to vehicle-treated control groups (Figure 4). The most significant increase in apoptosis was observed at concentration of 625 μg CY/L ($p < 0.01$). Although the levels of apoptosis were not statistically significantly different, numerous apoptotic brain cells were observed at doses of 39 and 156 μg CY/L.

The correlation index (r) was 0.8122, indicating a dose-dependent CY effect (39–2500 μg/L). The EC50 value at 96 h was 4.5 μg CY/L (upper and lower confidence limits of 0.0934 and 18.85 μg CY/L, respectively).

TUNEL

The TUNEL method confirmed the dose-dependent increase in apoptotic cell number (Figure 5a–d). CY-induced initial apoptosis was

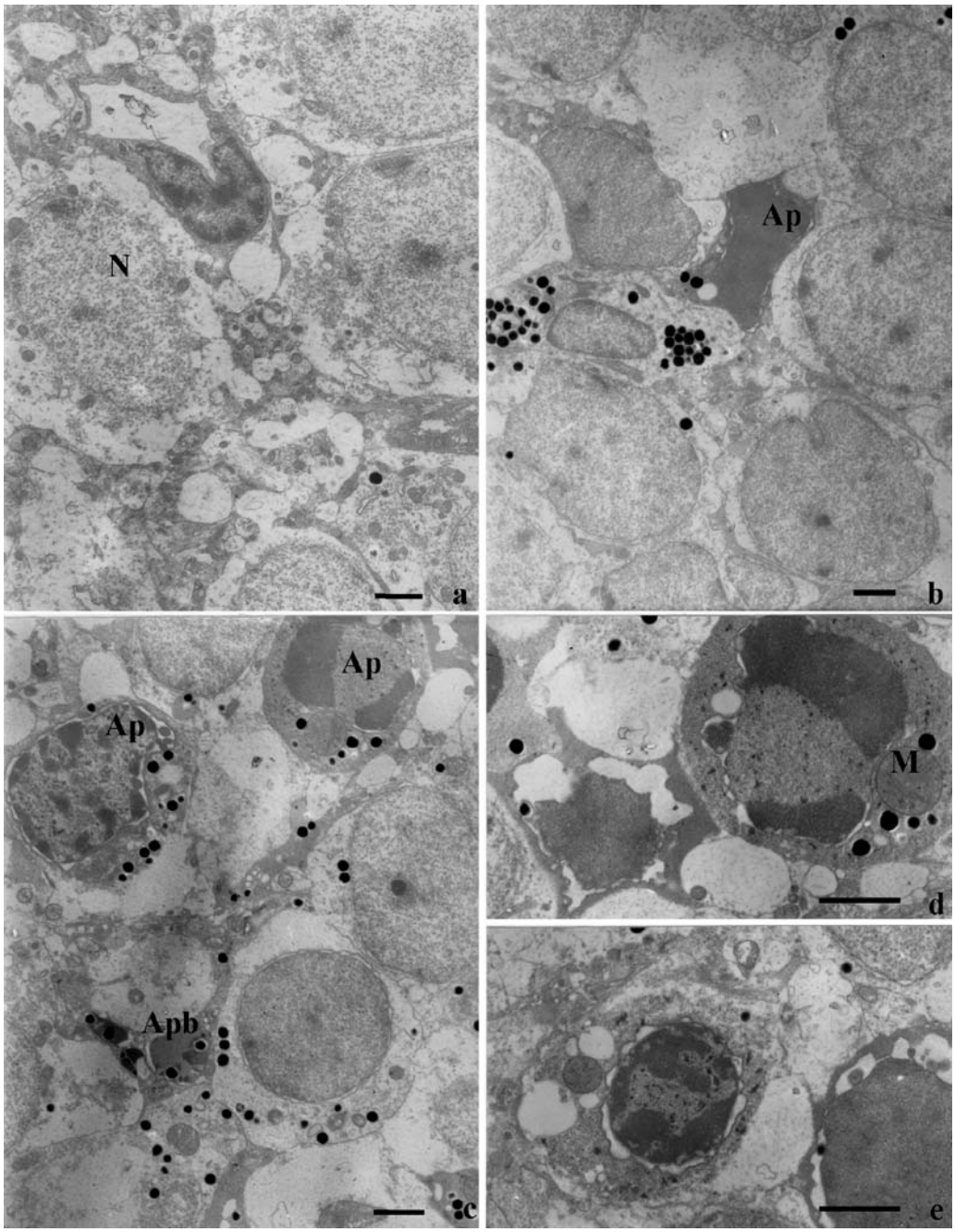

Figure 3. Electron microscopy of *Bufo arenarum* tadpole telencephalon. (a) Control (xylene), ×3200. (b) 39 μg CY/L at 96 h, ×3200. Note the presence of apoptotic cell with highly pyknotic nucleus. (c) 2500 μg CY/L at 96 h, ×3200. The increase in apoptotic bodies and apoptotic cells with segmented or condensed nuclear chromatin and dense cytoplasm is evident. (d) 2500 μg CY/L at 96 h, ×7000. Detail of two apoptotic cells at different stages of cell death. Note that the cytoplasmic organelles remain relatively well preserved. (e) 2500 μg CY/L at 96 h, ×7000. Detail of an apoptotic cell showing chromatin segmentation. Ap, apoptotic cell; N, viable neural cell; Apb, apoptotic bodies; M, mitochondria. Bar = 1 μm.

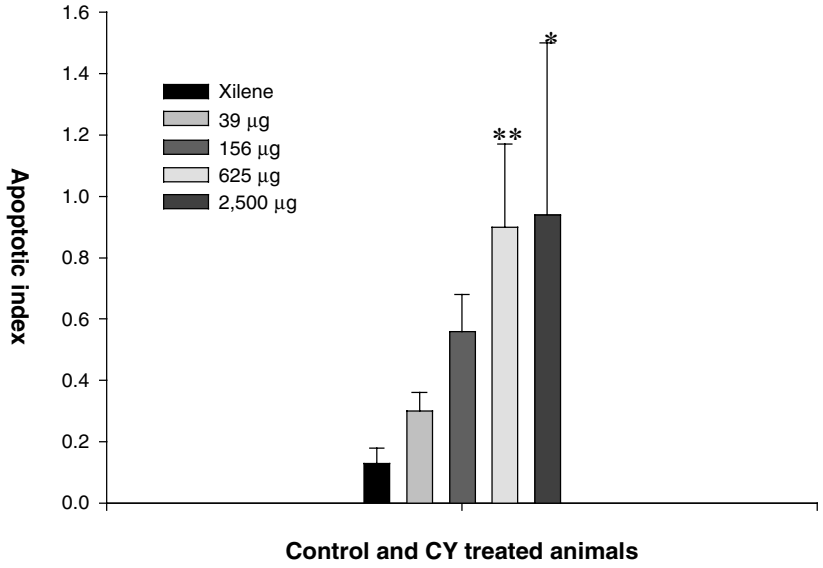

Figure 4. Apoptotic index of *Bufo arenarum* tadpole brain (mean ± SE). Xylene represents control. $^{*}p < 0.05$, $^{**}p < 0.01$.

observed in brain cell nuclei from 39 µg CY/L (Figure 5a). The highest number of apoptotic cells was detected at a dose of 2500 µg CY/L, identified by numerous fluorescent nuclei (Figure 5c). No selectivity was observed in the different cerebral regions studied in relation to the number of apoptotic cells.

DNA fragmentation assay

For the different treatments, lines of DNA fragmentation were evaluated as the integrated intensity of the electrophoretic bands using Matlab-based software designed for that purpose.

The DNA fragmentation assay showed that the DNA breakup pattern increased parallel to the CY concentration. Compared to the control line, statistically significant differences were found with 156, 625 and 2500 µg CY/L (Figures 6 and 7).

Analysis of DNA fragmentation shows no significant differences between 39 µg CY/L and control.

Discussion

Amphibians are particularly vulnerable to pesticides because their life-cycle stages evolve around ponds and streams, and most species remain near these sites even in adulthood. Fresh water can accumulate pesticides and toxic products from cultivated land where pesticides are applied. Moreover, amphibian larval development occurs in spring–summer contemporaneously with increase in pesticide applications (Materna et al., 1995). In Argentina, the application rates of insecticides in cultivated lands are between 1.5 and 120 g CY active ingredient per hectare (CASAFE, 1995).

Variations in the acute toxicity effects depend on the chemical structure and isomerism of the individual compounds and on the vehicle used, as well on as the species, sex, age, and diet of the animal. In such studies, the values reported differed markedly depending on the model used (Kaloyanova et al., 1991). Cypermethrin is toxic to fish (in laboratory tests 96 h LC_{50} values were obtained at doses of 0.4–2.8 µg/L) and aquatic

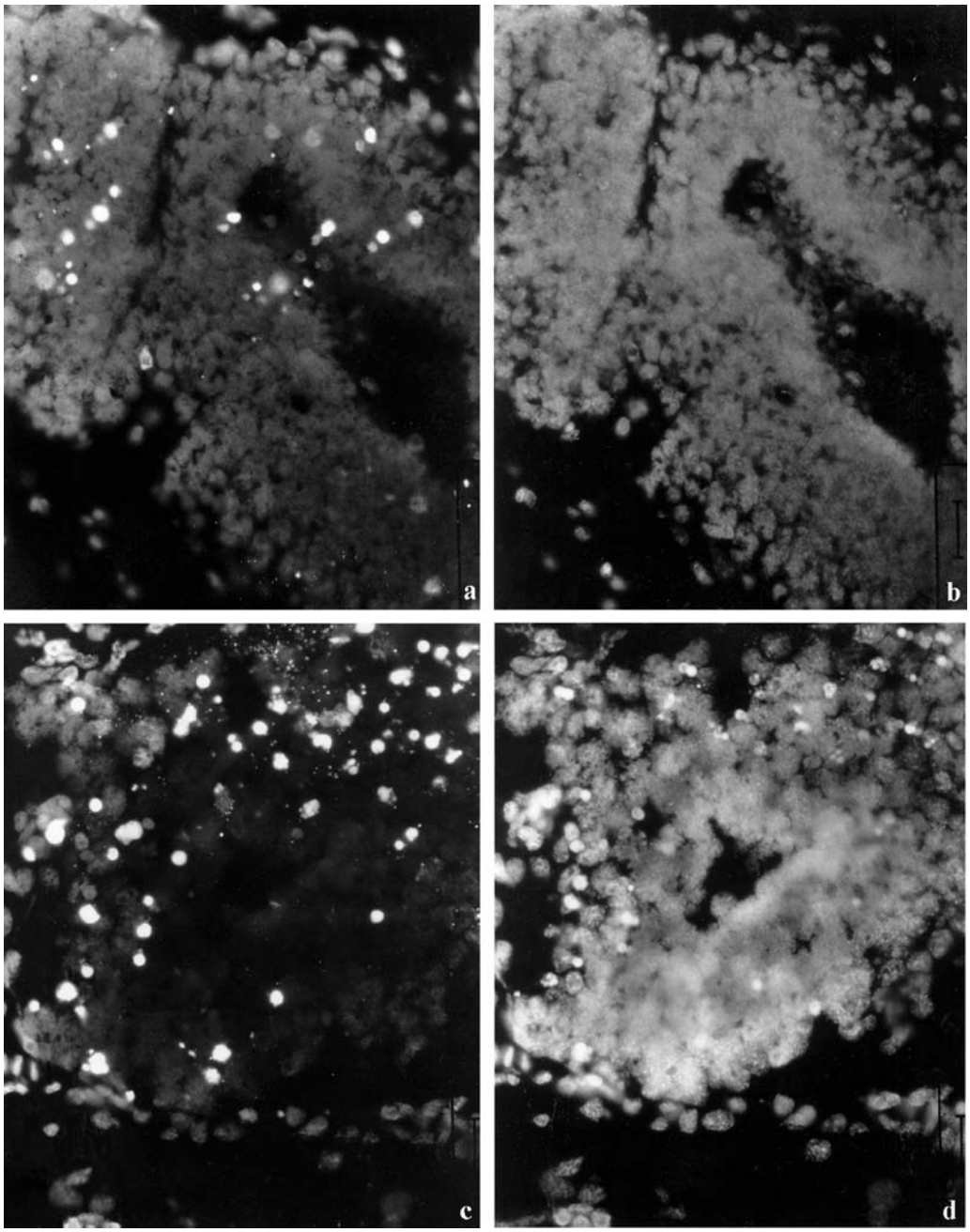

Figure 5. Detection (by TUNEL or DAPI) of fragmented DNA in *Bufo arenarum* tadpole brain. (a) 39 μg CY/L at 96 h (TUNEL). (b) 39 μg CY/L at 96 h (DAPI.) (c) 2500 μg CY/L at 96 h (TUNEL). (d) 2500 μg CY/L at 96 h (DAPI). Bar = 10 μm.

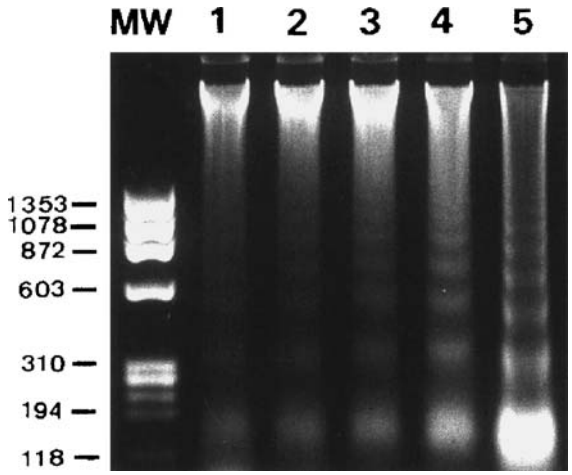

Figure 6. DNA fragmentation pattern in *Bufo arenarum* cerebral cells by electrophoresis. MW, molecular weight ladder; lane 1, control; lane 2, 39 μg CY/L; lane 3, 156 μg CY/L; lane 4, 625 μg CY/L; lane 5, 2500 μg CY/L.

invertebrates (at doses of 0.01 to >5 μg/L) (WHO, 1989) but is not very toxic for birds, and earthworms are not sensitive (WHO, 1989). In studies involving deliberate spraying of experimental ponds under field conditions, concentrations of 2.6 μg CY/L were measured in the water and, although fish were not severely affected at those concentrations, populations of crustaceae, mites, and surface-breathing insects were drastically reduced (WHO, 1989).

Frogs are highly sensitive to most pyrethroids, including CY in subcutaneous and intraperitoneal applications (Cole and Casida, 1983). LC_{50} values for frogs treated subcutaneously are more similar to those for insects treated topically than those for rats or mice receiving oral, intraperitoneal, or intravenous treatments (Casida et al., 1983). Acute toxicity tests showed high sensitivity to CY treatment in *Physalaemus biligonigerus* frog tadpoles (Izaguirre et al., 2000, 2001).

In this work, CY treatments of *Bufo arenarum* tadpoles with doses starting at 39 μg CY/L induced all the neurotoxic phases I to IV described by Salibián (1992), similarly to the effects found in adult frogs (Cole and Casida, 1983). The toxic effects were characterized by clustering, swimming

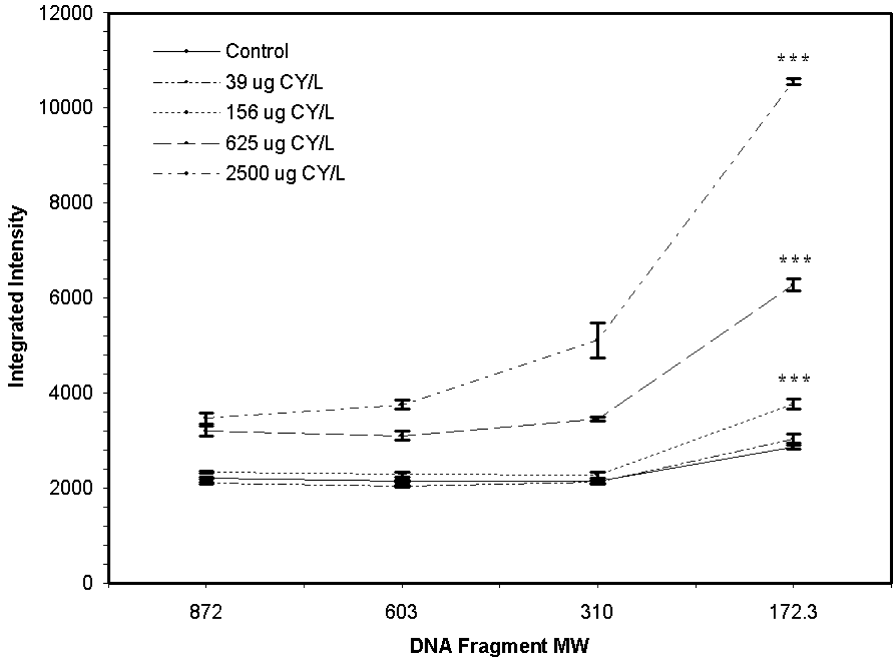

Figure 7. Quantification of DNA fragments, based on integrated intensity of electrophoresis bands. ** $p < 0.01$, *** $p < 0.001$.

at full speed, twisting, lateral curving of the tail, and arching and sinuous writhing along the cephalic–caudal axis. After 96 h of treatment, the 110 μg CY/L LC_{50} values were higher than those found in fish (96 h LC_{50} 0.4–2.8 μg/L) (WHO, 1989). Morphological and morphometric studies, TUNEL analysis, and DNA fragment quantification of treated tadpoles showed pathological alterations in the central nervous system, even at CY concentrations as low as 39 μg CY/L.

Dose-dependent apoptosis was observed in the central nervous system cells starting at 39 μg CY/L with a dramatic increase in apoptosis at a dose of 156 μg CY/L. Most significantly, morphometric analysis showed that CY concentration as low as 4.5 μg CY/L can produce toxic effects, approaching fish toxicity values. The dose-dependent apoptosis produced by CY in the brain of *Bufo arenarum* tadpoles was similar to the effect found in *Physalaemus biligonigerus* tadpoles (Izaguirre et al., 2000, 2001). The 96 h LC_{50} (129 μg CY/L) values for the above-mentioned species were also similar to *Bufo arenarum* 96 h LC_{50} values (110 μg CY/L).

Morphologically, we observed brain alterations in the intermediate and marginal layers. Cerebral immature cells showed cellular shrinkage, nuclear fragmentation, and increase of intercellular spaces, typical of apoptotic cell death (Kerr et al., 1972; Wyllie, 1980; Izaguirre et al., 2000, 2001). The TUNEL method confirmed DNA fragmentation, a typical feature of apoptosis induced by both physiological and pathological mechanisms (Bortner and Cidlowski, 1996). The quantitative method applied to determine the DNA fragment concentration demonstrated that the highest increase in apoptosis is produced from 156 μg CY/L. As was expected, the highest concentration of small DNA fragments (172 bp) occurred at the highest dose of 2500 μg CY/L at 96 h.

A major morphological change during apoptosis is the shrinkage of cells (Kerr et al., 1972; Wyllie, 1980), suggesting a fundamental role of cell volume loss in this form of cell death. However, the mechanism of cell volume loss and its precise role in the activation of apoptosis has not yet been clarified.

Modulation of cell volume appears to regulate cellular metabolism and gene expression, and changes in cell volume can act in signal transducer systems during multiple cellular processes (Haussinger et al., 1994). Several studies have implicated a role for potassium in apoptosis (Barbiero et al., 1995; Beauvais et al., 1995; Benson et al., 1996; Bortner et al., 1997) and have shown that the shrunken apoptotic cells have reduced intracellular levels of K^+ and Na^+ (Bortner et al., 1997). The loss of K^+ and Na^+ during apoptosis appears to occur in most animal species independently of the mode of activation of apoptosis. Although K^+ depletion alone in cells exposed to hypotonic conditions in the absence of an apoptotic stimulus does not trigger apoptosis or alter long-term viability (Bortner and Cidlowski, 1996), a reduced intracellular K^+ concentration enhances the activation of cell death programs (Bortner et al., 1997). *In vitro* studies showed that nucleases that degrade DNA during apoptosis, as well as the activity of caspase-1 (ICE), are effectively inhibited at physiological K^+ concentrations (Walev et al., 1995; Hughes et al., 1997; Montague et al., 1997). Activation of procaspase by dATP and cytochrome *c* is also inhibited by physiological K^+ concentrations (Hughes et al., 1997). These results suggest that the transition of an apoptotic cell from a state of high ionic strength facilitates both the cell volume loss and the activation of enzymes that mediate apoptosis.

In this study we have demonstrated the high toxicity of cypermethrin to *Bufo arenarum* larvae. The present results and previous results (Izaguirre el al., 2000, 2001) show that lower doses than those used in routine applications can cause massive apoptosis in the central nervous system. We postulate that CY-induced apoptotic cell death of cells of the central nervous system, through specific neurotoxic mechanisms, is the major factor for the high mortality of *Bufo arenarum* caused by CY.

Acknowledgements

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