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Organophosphorus insecticides affect normal polyamine metabolism in amphibian embryogenesis

Cecilia I. Lascano [a,b], Ana Ferrari [a,c], Lidia E. Gauna [b], Claudia Cocca [d], Adriana C. Cochón [e], Noemí Verrengia [e], Andrés Venturino [a,b,*]

[a] LIBIQUIMA, Facultad de Ingeniería, IDEPA, Universidad Nacional del Comahue – CONICET, Buenos Aires 1400, 8300 Neuquén, Argentina
[b] Facultad de Ciencias Agrarias, Universidad Nacional del Comahue, Ruta 151 Km 12.5, 8303 Cinco Saltos, Río Negro, Argentina
[c] Facultad de Ciencias Médicas, Universidad Nacional del Comahue, Toschi y Arrayanes, 8324 Cipolletti, Río Negro, Argentina
[d] Laboratorio de Radioisótopos, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 954, Ciudad Autónoma de Buenos Aires, Argentina
[e] Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pab. II. 4to piso, Nuñez, 1428 Ciudad Autónoma de Buenos Aires, Argentina

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ABSTRACT

The objective of the present study was to evaluate the concentration- and time-dependent effects of the organophosphorus insecticides malathion and azinphos-methyl on polyamine metabolism, and relate them to normal and altered embryonic development of the common toad *Rhinella arenarum*. Control embryos showed that the higher polyamines spermidine and spermine acquired importance with respect to the diamine putrescine as embryonic development progressed. The activity of ornithine decarboxylase significantly decreased in complete operculum embryos. Continuous exposure to malathion caused a decrease in polyamine levels during embryonic development. However, there was an increase in putrescine levels in complete operculum embryos exposed to a sublethal concentration of the insecticide. Embryos exposed to malathion displayed a decrease in fresh weight and size, along with an increase in the number of malformed individuals. *R. arenarum* embryos exposed to a lethal concentration of azinphos-methyl showed an increase in putrescine levels and a decrease in spermidine and spermine levels, accompanied by an increase in ornithine decarboxylase activity. In conclusion, as the embryonic development of the toad *R. arenarum* progresses, polyamine metabolism shifts to higher polyamine levels with a more preponderant contribution of spermidine and spermine with respect to putrescine and involves a dramatic change in ornithine decarboxylase activity, one of the key regulatory enzymes of the pathway. Organophosphorus insecticides are capable of altering polyamine metabolism, slowing embryo development in parallel with a reduction in spermidine and spermine levels. An increase in the oxidative degradation of polyamines might be involved in the toxic action of organophosphorus insecticides and might also be related to other effects such as teratogenesis.

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1. Introduction

Polyamines are aliphatic polycations essential for normal cell growth, differentiation and progression of developmental processes [1,2]. Due to their polycationic nature, they are capable of interacting with nucleic acids and proteins. Polyamines stimulate protein synthesis [3], regulate the activity of ion channels [4,5], the activity of G-proteins in signal transduction [6] and the expression of proto-oncogenes [7]. Polyamines also act as scavengers of reactive oxygen species [8]. Consequently, organisms have developed complex regulatory machinery to control intracellular levels of polyamines [9]. The first and key point of control in polyamine metabolism is the activity of ornithine decarboxylase (ODC), which catalyzes the decarboxylation of ornithine into the diamine putrescine (Put). Studies conducted in mice harboring a disrupted ODC gene and in pregnant mice exposed to the suicide ODC inhibitor α-difluoromethylornithine (DFMO), revealed that the enzyme ODC is essential for cell survival during early murine development [10,11].

Many studies, performed with different classes of pesticides, report both delay and arrest of embryonic development in several species [12–14]. In spite of the fact that polyamine metabolism is clearly related to normal and altered development, works connecting the effects of pesticides and polyamines are scarce. The herbicide paraquat has been the main compound employed in this kind of study, as its uptake into the cell is driven by the polyamine transport system. Paraquat's effects on polyamine metabolism

* Corresponding author at: LIBIQUIMA, Facultad de Ingeniería, IDEPA, Universidad Nacional del Comahue – CONICET, Buenos Aires 1400, 8300 Neuquén, Argentina. Fax: +54 299 4490385.
E-mail address: a.venturino@conicet.gov.ar (A. Venturino).

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have been studied on plants, mammals, and cultured cells [15–17]. Cochón et al. [18] performed a study in freshwater invertebrates, using a commercial formulation of paraquat, relating oxidative stress parameters to polyamine levels. Several authors have reported that aquatic vertebrates, such as amphibian embryos and larvae, exposed to organophosphorus (OP) and carbamate pesticides display diverse developmental alterations [19–22]. Some studies have been conducted to address the interaction between the toxicological effects of the anticholinesterasic OP pesticides and polyamines in aquatic organisms. These studies employed exogenously applied Put, spermidine (Spd) and spermine (Spm). Venturino et al. [23,24] determined that exogenously applied polyamines increased malathion (Mtn) toxicity and acetylcholinesterase inhibition, and decreased glutathione levels in *R. arenarum* larvae.

The objective of the present study was to evaluate the concentration- and time-dependent effects of the OP pesticides Mtn and azinphos-methyl (Azm) on polyamine metabolism, and relate them to normal and to altered embryonic development of the common toad *R. arenarum*.

2. Materials and methods

2.1. Chemicals

Azinphos-methyl (99.0% purity) was purchased from Chem Service (West Chester, PA, USA). Malathion (98% purity) was kindly provided by Cyanamid, Argentina, and purified by thin layer chromatography to eliminate possible storage contaminants. Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, pyridoxal 5′-phosphate monohydrate, L-ornithine monohydrochloride and bovine serum albumin were purchased from Sigma Co. (St. Louis, MO, USA). L-[14C]-ornithine was purchased from New England Nuclear. Scintillation liquid Optiphase "Hisafe" 3 was purchased from Perkin–Elmer (Shelton, CT, USA). All the other reagents used were of analytical grade.

2.2. Toad embryo development and insecticide exposure

Three independent experiments were performed for each pesticide, using three different sets of parents. Ovulation was induced by intraperitoneal injection of 2500 international units (IU) of human chorionic gonadotrophin. *R. arenarum* embryos were obtained by *in vitro* fertilization as previously described [25]. Groups of 500 newly fertilized embryos were transferred to glass recipients containing either amphibian Ringer's solution (0.65 g/L NaCl; 0.01 g/L KCl; 0.02 g/L Ca_2Cl) (control group) or pesticide solution, keeping a ratio of 1 embryo/mL solution. Pesticide solutions of 0.5 mg/L, 2 mg/L, and 9 mg/L Azm, and 22 mg/L and 44 mg/L Mtn were prepared by diluting an insecticide standard solution prepared in acetone, with an appropriate amount of amphibian Ringer's solution, keeping acetone to 0.3% in the final solution. The exact concentration of the insecticide in the standard solutions was checked by gas chromatography with a nitrogen-phosphorus detector (GC-NPD). Controls of 0.3% acetone were also performed. The treatments were carried out in duplicate. The solutions were renewed every 48 h until embryos reached the stage of complete operculum (CO) [26]. The embryos were maintained at 18–20 °C in a 12 h light–12 h dark photoperiod without feeding. Viability of individuals and malformations were monitored with a stereoscopic microscope [27]. Embryonic development was assessed in samples of non-treated embryos and embryonic stages were determined according to [26]. During the continuous exposure, samples were taken at different embryonic stages to evaluate polyamine levels and ODC activity.

2.3. Ornithine decarboxylase activity

Fifty tail bud-(TB), twenty-five open mouth-(OM), and twenty-five CO embryos were sampled for determination of ODC activity and homogenized in 1 mL of 50 mM Hepes buffer pH 7.4 containing 0.1 mM EDTA, 0.04% Triton X-100, 1 mM dithiothreitol, 0.5 mM pyridoxal-5′-phosphate and protease inhibitors (0.05 mM phenylmethanesulfonyl fluoride and 0.001 μg/μL leupeptin, aprotinin and NaF). Homogenized samples were centrifuged at 20,000*g* for 40 min at 4 °C and the supernatant was collected for determination of ODC enzymatic activity. ODC activity was assayed by measuring the release of $^{14}CO_2$ from L-[14C]-ornithine according to [28], with slight modifications. The standard reaction mixture consisted of homogenization buffer plus L-ornithine and L-[14C]-ornithine (1 mM, 0.1–0.2 μCi) in a final volume of 50 μL. Enzyme reaction was initiated by the addition of 15 μL of samples. Blank solvent and sample controls were run in parallel, replacing sample with homogenization buffer and difluoromethylornithine (DFMO)-inactivated samples, respectively. The reaction was performed for 1 h at 30 °C in agitation and $^{14}CO_2$ was trapped on a 2 cm × 2 cm piece of filter paper soaked with 2 N KOH. The reaction was stopped by the addition of 50 μL of 0.25 N $HClO_4$ and maintained under the same conditions for 1 h. Filter papers were then transferred to scintillation vials and 0.5 mL 1% Triton X-100 were added along with 5 mL of scintillation liquid Optiphase "Hisafe" 3. Radioactive CO_2 was then measured in a liquid scintillation counter (Wallac Winspectral 1414).

2.4. Polyamine content determination

Fifty embryos from early developmental stages were homogenized in 1 mL of 143 mM potassium phosphate buffer pH 7.5 with 6.3 mM EDTA and 0.2 N $HClO_4$. Twenty-five embryos from OM stage on were also processed in this way. Samples were incubated on ice for 1 h and centrifuged 10 min at 3000*g*. The supernatant was derivatized with 20 mg/mL dansyl chloride [29] and polyamine content was assessed by HPLC reverse-phase separation and fluorometric quantitation [30]. 1,7-Diamine heptane was used as an internal standard both for samples and calibration standards.

2.5. Evaluation of morphological parameters

Embryo morphology was evaluated with the aid of a stereoscopic microscope, and the type and number of malformations was registered. The malformations were typified according to [31]. Mortality was determined by the absence of blood circulation in the gills and caudal fin, or the lack of a heart beat. Dead embryos were removed and were not included in any morphological or biochemical analysis. Control embryos at the end of development displayed <5% mortality under laboratory conditions.

Embryo length was measured with a submillimetric scale under a stereoscopic microscope. Fresh weight was determined in groups of 20–40 embryos using an analytical balance, after a careful elimination of residual water with filter paper.

2.6. Protein determination

Protein content was determined according to [32] using bovine serum albumin as the standard.

2.7. Data analysis

Three independent experiments were performed for each pesticide and, within each assay, the different treatments were tested in duplicate. For the statistical analysis, data from the three experiments were pooled ($n = 6$ for each embryo stage and treatment)

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