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RESEARCH ARTICLE

The effect of paraoxon on spermatogenesis in *Dugesia gonocephala* from the Chilean Altiplano: proliferation and apoptosis

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Abstract
Introduction and aims The Chilean Altiplano ecosystem is conserved free from contaminants and pollutants because of the absence of major local human activities such as agriculture or other industries. We studied the effects of paraoxon on proliferation and apoptosis of testicular cells during active spermatogenesis in *Dugesia gonocephala* collected from a pristine river (Guacollo) in the Altiplano region nearby Visviri town, Chile.
Materials and methods Adult planarians were incubated in varying concentrations of paraoxon (0.8, 0.4, 0.04, 0.004, and 0.0004 mM) for 4 h. After 3 h of incubation, bromodeoxyuridine (BrdU) was added. Effects on cell proliferation (BrdU) and apoptosis (Apaf-1) were determined by immunohistochemistry.
Results Paraoxon concentrations of 0.4 and 0.8 mM caused 100% mortality in the respective treatment groups. The lowest tested concentration (0.0004 mM) caused a significant increase on cell proliferation in the seminiferous tubules, as well as an increase in the number of apoptotic cells. All other tested concentrations significantly inhibited cell proliferation and induced apoptosis.
Conclusions Paraoxon inhibits DNA synthesis and induces apoptosis during spermatogenesis in adult planarians from a high-altitude, pollution-free environment. This could suggest its use as a biosensor or biomarker for contamination with agro pesticides.

Keywords High altitude · Planaria · Spermatogenesis · *Dugesia* · Paraoxon · Reproduction

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1 Introduction

Freshwater planarians are an attractive model organism for toxicology research. They are excellent experimental tools, as they allow research to be conducted in vivo and enable the assessment of the mechanisms, function, and regulation of cell turnover in adult seminiferous tubules (Villar et al. 1994; Horvat et al. 2005).

Dugesia gonocephala (Duges 1830) (Turbellaria, Suborder Tricladida, Infraorder Paluldicola, Family Dugesiidae) is a common and widespread freshwater triclad species in the Old World (cited by Malhao et al. 2007). In these hermaphroditic animals, during the adult life stage, the male reproductive system shows a complete spermatogenesis with stability of somatic cell populations. Planarians have been used as indicators of water quality and toxic effects of various substances (Nano et al. 2002; Lau et al. 2007). One critical parameter appears to be the interaction between stem cells and their local tissular microenvironments. Both heterologous cells and the extracellular matrix can provide clues as to what regulates stem cell behavior (Molofsky et al. 2004).

Through apoptosis, certain differentiated cells are regularly eliminated and replaced by the progeny of adult stem cells (Hwang et al. 2004). This often involves an exogenous stimulus that causes an acute damage to tissues and provokes a strong inflammatory response (Pfister et al. 2007; Pellettieri and Sanchez 2007). Incorporation studies with bromodeoxyuridine (BrdU) show that 99% of the neoblasts are labeled with BrdU after 3 days of continuous BrdU treatment (Lau et al. 2007).The availability of BrdU-labeling for planarian stem cells, and thousands of planarian cDNA sequences are soon to be released into public databases (Robb and Sanchez 2002; Moraczewski et al. 2008).

Also, proliferative processes can be altered by the presence of toxic agents, which are capable of inhibiting the synthesis of DNA and proteins, as reported for organophosphate insecticides (Rodriguez et al. 2001; Espinoza-Navarro and Bustos-Obregon 2005; Rodriguez et al. 2006).

Parathion is transformed by oxidative reactions through the P_{450} enzyme complex to paraoxon (its active metabolite) (Kim et al. 2000). Paraoxon is a parasympathomimetic agent and is one of the most potent acetylcholinesterase-inhibiting insecticides available. However, it carries the risk of being poisonous to humans and other animals (ATSDR 2001; Favari et al. 2002).

Both chemicals have been shown to induce apoptosis in cell cultures of neuroblasts (Carlson et al. 2000).

Adapted *D. gonocephala* lives in the Chilean Andes, an area free of anthropogenic pollutants, constituting an excellent biological model for studying the effects of toxic agents on the regulation of cell populations via proliferation and apoptosis (as has been demonstrated with other species of invertebrate animals) (Espinoza-Navarro and Bustos-Obregon 2004).The objective of this research is to analyze the spermatogonial cellularity in adult *D. gonocephala* (with complete spermatogenesis) under the effects of different concentrations of paraoxon in vitro.

2 Materials and methods

The ecosystem in the Chilean Altiplano is unique and conserved with few and small human settlements. It is located in the Andes, coincident with most of the country limits and consists of straight-line segments plain between high mountain peaks. From the Argentina tripoint of Cerro Zapaleri, it extends northward through more than five degrees of latitude to the Peru tripoint at 17° 29′ 55.0″S. latitude and 69° 28′ 28.8″W. longitude. They are defined as areas free of human contamination. These regions are defined as areas of low or no human impact including contamination. This region is also the origin of many rivers that are fed by glaciers and, thus, are characterized by low temperature regimes throughout the year. Due to the pristine nature of this environment, local aquatic life form are representing ideal sentinel organisms for the detection of effects of chemicals commonly used in agriculture such as paraoxon because no pre-exposure occurred.

2.1 Test organism

High-altitude planarians *D. gonocephala* were collected in the Guacollo River, located in the town of Visviri (Arica, Chile) during the month of January (summer). The samples from this high-altitude zone are of low human contamination due to natural barriers of temperature (high temperatures during the day and below 0°C at night) and low atmospheric pressure. The collection of animals was made early in the morning, stored in transparent glass bottles with water from the same river. They were transported to the Central Laboratory of the University of Tarapaca, Arica, Chile (58 m a.s.l.) and were kept in the same river water in an aquarium. Animals were fed ad libitum with daily replacement of chicken liver and/or cooked chicken egg yolk and always kept under laboratory ambient conditions (8–10°C and at 58 m a.s.l.). After adaptation, the animals were distributed into experimental groups.

2.2 Experimental design

Animals were placed in aquariums with river water prior to preparing test dilutions (Guacollo river) for an acclimation period of 15 days. The *D. gonocephala* specimens were distributed into groups of 25 individuals, and each group was incubated for 4 h in a total volume of 5 mL with different concentrations of paraoxon (Sigma Co., D9286, USA) and control (0.8, 0.4 mM, 0.04, 0.004, and 0.0004 mM). First, a solution of higher concentration (0.8 and 0.4 mM) was prepared, and then from the solution, 0.4 log dilutions were made up 0.0004 mM for a period of 4 h. At the third hour of treatment, 50 μL of BrdU (thymidine-base analog, Zimed R Cat. 93-3943-3944, USA) was

added. Paraoxon was diluted in 20 mL of filtered Guacollo river water. Incubations were replicated three times, and each one was shaken for 30 min. These concentrations and dilutions were selected on the basis of concentration ranges of contamination that have been reported in tap water for human consumption (Amaya-Chavez et al. 2009).

2.3 Histology and immunohistochemistry

After a total of 4 h of incubation, animals were fixed in alcoholic Bouin's solution and embedded in paraffin (Paraplast plus McCormick Ref. 502004, USA) with a melting point of 56°C to 58°C. Longitudinal and transversal cross sections (5 μm) were mounted on silanized slides. Then, selective immunohistochemical assays for the analysis of the cellularity of the male gonad of planarians were performed (BrdU for cell proliferation and Apaf-1 for apoptosis; Vertor Co. Cat. RB-9263-P-R7, USA), both according to the manufacturer's instructions for the Staining BrdU and Apaf-1 kit based on the streptavidine–biotin and DAB system (diaminobenzidine, Bio SB HRP/DAB 0003, USA). For the implementation of immunohistochemical protocols, deparaffinized samples were subjected to antigen retrieval with citrate (Sigma-Aldrich S4641, USA) buffer at pH 6.0.

For both antibodies, quantification of positive and negative cells in the seminiferous tubules of the gonad was performed. Evaluations were done using different objectives (×10 and ×20) of the optical microscope (Nikon E400 microscope, Nikon, Melville, NY, USA).

The immunohistochemical reaction (S-phase cell cycle in DNA synthesis, or apoptosis via mitochondrial route) was considered positive when cells exhibited a brown color (DAB) with the hematoxylin contrast (basofilia). We quantified the total of positive cells per seminiferous tubule.

2.4 Statistical analysis

Results were expressed as percentage of positive and negative cells in the total seminiferous tubules as mean± SD; with the normal distribution dates (Fisher test) later, they were analyzed by comparing the differences in means within and among groups using statistical t test and variance multiple (ANDEVA) double-entry and t test ($p \leq 0.05$), with Origin 6.0 software. The images were recorded on a Nikon E400 microscope with a digital camera Coolpix 4500.

3 Results

There were statistically significant and dose-dependent effects on mortality. At the two greatest paraoxon concentrations, 100% of test animals were dead after 4 h. None of the other concentration tested were lethal, and animals development apparently normal behavior (displaced and distribution).

Exposure to all sublethal concentrations of paraoxon resulted in significant alterations of cell proliferation and apoptosis markers. Cells that underwent active cell proliferation or apoptosis are indicated by positive (brown) staining after incubation with BrdU and Apaf-1 antibodies. Exposure to the lowest exposure concentration of 0.0004 mM of paraoxon resulted in a significant increase in both cell proliferation and apoptosis as indicated by the increased presence of brown stained cells (Table 1; Fig. 1e, f control groups for BrdU and Apaf-1 without PO).

The exposure to low (0.04 mM) and very low (0.004 mM) paraoxon concentrations had a strong inhibition effect on the promotion of cell proliferation. This was evaluated through the incorporation of BrdU during entrance of the cells of the seminiferous tubules into cell cycle (Fig. 1c, e). The averages of positive cells in these groups were statistically different with respect to the control group ($p \leq 0.05$). However, exposure to 0.0004 mM paraoxon caused significant increases in cell proliferation compared with the control group ($p \leq 0.05$), where an augment close to 50% was observed (Table 1).

In the quantification of the effects of different concentrations of paraoxon on the proportion of apoptotic cells in the seminiferous tubules of the planarians (Table 2; Fig. 1d, f), it was possible to demonstrate that all concentrations tested generated large numbers of apoptotic cells in the seminiferous tubules that were statistically different from the control group ($p \leq 0.05$).

4 Discussion

In males, the presence of seminiferous tubules can be observed in the stroma with spermatogenesis at different stages of development (Fig. 1a, b). The present work demonstrated the usefulness of BrdU and Apaf-1 in the histochemical detection of cell proliferation and apoptosis in the testis of *D. gonocephala*. Furthermore, we demonstrated

Table 1 BrdU(+) cells distribution in the presence of different concentrations of paraoxon (mM) in seminiferous tubules of planarians

	Control	0.04 mM	0.004 mM	0.0004 mM
BrdU+(%)	51.0±3.7	16.0±2.8*	41.0±5.1*	78.0±6.4*
IF		−0.7	−0.2	+ 0.5

IF inhibition fraction=(experimental−control)/control BrdU

*$p \leq 0.05$

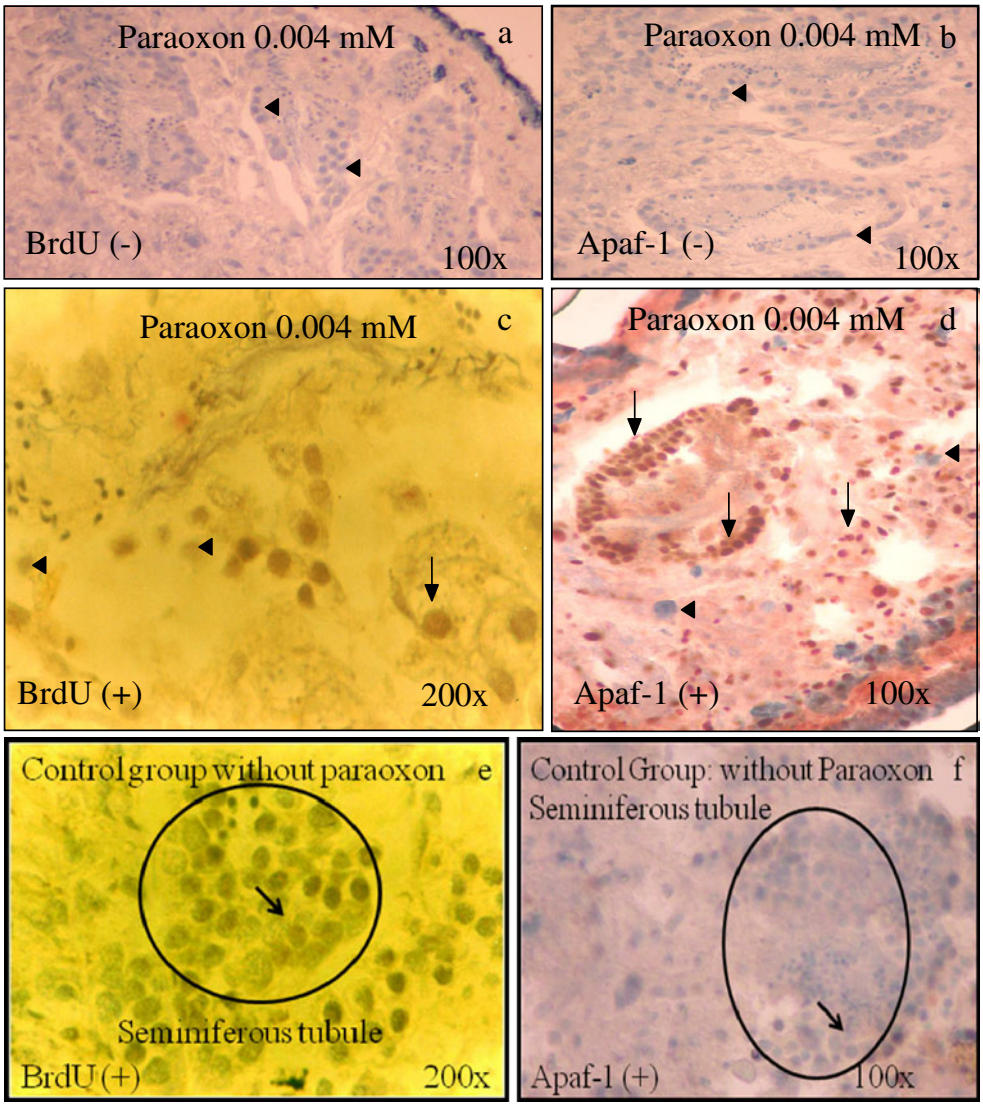

Fig. 1 **a**–**f** Seminiferous tubules of *D. gonocephala* incubated with paraoxon and immunohistochemistry to show cell proliferation (BrdU+, **c**) and apoptosis (Apaf-1+, **d**); **a** and **b** are the respective controls. *Arrows* indicate cells with positive immunohistochemical reaction, and *arrowheads* indicate negative cells. Control animals without PO incubation are in **e** and **f**

the utility of these histochemical markers to detect the effects of paraoxon on the proliferation of neoblasts and germ cells in the seminiferous tubules, suggesting planarians as a proper animal model to act as a biomarker or sensor of toxicity (Fig. 1 and Table 1).

Planarians that were collected from an area that is free of agricultural pesticides (Table 1) showed a great number of basal cells in proliferation inside the seminiferous tubules, indicating that planarians have the ability to incorporate exogenous DNA precursors, specifically BrdU, which allows germ cells to mark the tubules, similar to that described by Newmark and Sanchez (2000).

At concentrations greater than or equal to 0.004 mM, exposure to paraoxon resulted in a suppression of positive BrdU staining in epithelial tissue as revealed by primarily staining with hematoxylin (negative reaction), suggesting alterations in the cell cycle that are indicative of an inhibition of proliferative activity. This indicates inhibition of DNA synthesis, which would alter the mitotic replacement processes of damaged cells (Best and Morita 1982), in turn altering enzymatic pathways. At least in humans, the organophosphorates inhibit the "granzyme" enzymatic complex (Li et al. 2002), a process which would be triggered by the inhibitory action of acetylcholinesterase and subsequent accumulation of acetylcholine, with an acceleration of neural energy metabolism, which ultimately leads to the death of the individual, as reported by Surendra

Table 2 Apaf-1(+) cell distribution in the presence of different concentrations of paraoxon (mM) in seminiferous tubules of planarians

	Control	0.04 mM	0.004 mM	0.0004 mM
Apaf-1+ (%)	1.3±0.7	68±8.1*	51±5.3*	43±3.7*

*$p \leq 0.05$

and Surendra (1999) for the silkworm. It also leads to the expression of tumors in other species (Cabello et al. 2001; Espinoza-Navarro and Bustos-Obregon 2005).

This study demonstrate that the process of labeling DNA in cell proliferation in *D. gonocephala* (Fig. 1c) is an accurate and useful procedure for the study of molecular processes of cell proliferation. However, very little is known about the metabolic mechanisms that control proliferation and differentiation of the stem cell population in planarians (Baguna 1981; Baguna and Romero 1981; Salo and Baguna 1984; Salo and Baguna 1985).

Additionally, it has been shown that paraoxon affects the synthesis of proteins, RNA, and DNA in mice (Rodriguez and Bustos-Obregon 2000; Rodriguez et al. 2001, 2005), and is responsible for the causes of the inhibitory effects on cell proliferation during spermatogenesis.

With a paraoxon concentration of 0.004 mM, the values of cell proliferation begin to normalize, and further in a paraoxon concentration of 0.0004 mM, a stimulating effect on cell proliferation is observed. This would indicate that *D. gonocephala* possesses defense mechanisms as compensation in response to the increased apoptosis rate that was observed at the same concentration. A similar hypothesis was proposed by Salo and Baguna (2002), who suggested that the neuropeptide substance P would act as a powerful mitogen, reinforcing the increased cell proliferation, and mitosis would be stimulated. Analysis of the results clearly indicates the deleterious effect of paraoxon, demonstrating disruption of the cell cycle and cell differentiation, specifically affecting the epithelium of the seminiferous tubules of planarians, and, thus, having the potential of altering reproductive functions in this species.

Sanchez and Newmark (1998) and Sanchez (2000) determined that the planarian is an excellent model for biological studies of cell proliferation because of its relative simplicity, developmental plasticity, and evolutionary position. Furthermore, this concept of a model or biological sensor could also include apoptosis as a variable.

With regard to the effects of paraoxon on induction of programmed cell death, it was possible to show that this process is highly sensitive to the presence of the toxic agent studied. It was possible to demonstrate that the apoptotic processes in planarians, as in other animal species of different phyla, occur throughout the cytoplasmatic factor Apaf-1 and mitochondrial cytochrome *c*, which is probably associated with the system of the caspase cascade. Therefore, it is an interesting variable in its usefulness in the proposed animal model of planarians as sensors and biomarkers levels of environmental pollution.

The reason why apoptosis arises in the highly active region is still unclear, but it is a general fact that apoptosis regulates the number of proliferating cells during organization and normal development (Sommer and Rao 2002).

Similar to what has been described by Hwang et al. (2004), our study suggests that apoptosis is indeed extensively involved in the regeneration of planarian cells after exposure to a toxic substance. It also suggests that apoptosis plays a similar role to the one seen in the normal development of most metazoans: controlling the cell proliferation, structure patterning, and reconstruction of old tissue parts.

5 Conclusion

Paraoxon inhibits DNA synthesis and induces apoptosis in spermatogenesis in adult planarians obtained from a high-altitude and non- or low-impacted, pollution-free iatrogenic habitat and could suggest their use as a biosensor and biomarker of contamination with these agro pesticides.

The results presented allow us to conclude that paraoxon alters the germinative epithelium in the seminiferous tubules of planarians originating from a high-altitude environment (free of human pollutants).

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