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landeira@puc-rio.br
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Behavioral and histological effects of rotenone in fish (Guppy, *Poecilia reticulata*)

André Walsh-Monteiro[1], Wylfredo Pragana[2], Edmar Tavares Costa[2], and Amauri Gouveia Jr[2]

1- Instituto Federal do Pará, Tucuruí, PA, Brazil

2- Universidade Federal do Pará, Belém, PA, Brazil

Abstract

Rotenone is a toxic compound that causes neuronal death in dopaminergic areas and is commonly used as a pesticide in agriculture. The aim of the present study was to investigate the effects of acute rotenone poisoning on the telencephali ventralis pars ventralis (Vv; i.e., an area of dopaminergic innervation) in adult *Poecilia reticulata* fish and its behavioral effects in the open field test. Forty adult guppy fish (King Cobra) were divided into five groups: 0, 5, 10, and 12.5 µg/L rotenone exposure for 24 h. The fish were then tested in the open field for 10 min and then sacrificed. The encephalon was removed, and Nissl staining was performed. The cell counts were performed in a 200 μm^2 area of Vv tissue. Rotenone increased locomotor activity in the open field, enhanced exploratory activity in the center of the open field, and reduced the number of cells in the Vv. **Keywords**: behavior, rotenone, toxicology, animal model.

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Introduction

Rotenone is a secondary metabolite product (Edwards & Wratte, 1981) with pesticide and ictiotoxic properties. It is found in plants and commonly used in artisanal fishing in South America and Southeastern Asia (Chacon, 1973). It is a lipophilic compound that easily crosses biological barriers because of its hydrophobicity, thus having high absorption and uptake in the brain. Despite being widely distributed in the brain, rotenone can cause selective neurodegeneration in specific regions (Talpade, Greene, Higgins, & Greenamyre, 2000) and has been directly associated with the onset of Parkinson's disease (PD; Tanner et al., 2011). Heikkila, Nicklas, Vyas, and Duvoisin (1985) injected rotenone in rat brains, which caused the death of dopaminergic neurons. Later studies showed that rotenone can be used as a model for studying PD because its effects mimic the features of the disease (Schmidt & Alam, 2006), including the selective degeneration of nigrostriatal dopaminergic neurons and movement disorders (Sherer, Kim, Betarbet, & Greenamyre, 2003).

Rotenone promotes the systemic inhibition of mitochondrial complex I, in which the production of reactive oxygen species (ROS) blocks the electron transport chain and causes neurodegeneration (Miyoshi, 1998; Betarbet, Sherer, Mackenzie, Garcia-Osuna, Panov, & Greenamyre, 2000). Meng et al. (2011) studied the association between pesticides, such as rotenone, and gene products related to PD. They found that prolonged exposure to environmental toxins tends to increase oxidative stress in exposed subjects.

Although rotenone is used as a pesticide, its level of exposure is small relative to other pesticides, making epidemiological and toxicological studies of rotenone quite scarce. One of the few epidemiological reports on rotenone in humans was published by Hancock et al. (2008). Toxicological studies on the lethal and sublethal effects of this neurotoxin in fish are even rarer (Cheng & Farrell, 2007). In one of these studies, Ling showed that fish are sensitive to acute doses of rotenone, indicating that 1 ppm or lower concentrations are sufficient to cause death within a few hours. The LC_{50} in 24 h for these animals was determined to be between 5 and 100 µg/L (Ling, 2003). Considering ecological aspects, toxicological studies with fish may help clarify the role of toxins in the environment, in which animals come into contact with xenobiotic elements that are dissolved and incorporated into food chains (Ahmad, 1995).

The effect of neurotoxins can be studied through behavioral tests, such as the open field test (Hall, 1934),

André Walsh-Monteiro, Laboratório de Biociências e Comportamento, Instituto Federal do Pará, Campus Tucuruí. Wylfredo Pragana and Edmar Tavares Costa, Laboratório de Doenças Neurodegenerativas, Instituto de Ciências Biológicas, Universidade Federal do Pará. Amauri Gouveia Jr., Laboratório de Neurociências e Comportamento, Núcleo de Teoria e Pesquisa do Comportamento, Universidade Federal do Pará. Correspondence regarding this article should be directed to: André Walsh-Monteiro, Laboratório de Biociências e Comportamento, Instituto Federal do Pará, Campus Tucuruí, Rua Salto Santiago s/nº, Vila Permanente, Tucuruí, PA, 68464-000, Brazil. Phone: +55 (94) 8114-0236. Fax: +55 (94) 3778-4684. Email: andre.walsh@ifpa.edu.br

which assesses emotional behavior by measuring the time spent at the edge and center of the circular apparatus and locomotor activity (Prut & Belzung, 2003) in fish when they explore new bright environments. Another way to study the effects of neurotoxins is to histologically analyze tissue to identify the brain areas that are affected by exposure to neurotoxins.

Rotenone is associated with PD, and we neurohistologically analyzed the telencephali ventralis pars ventralis (Vv) area. Several studies have indicated this area is rich in dopamine and homologous to the substantia nigra in mammals (Wullmann & Vernier, 2009). This area in mammals undergoes substantial changes when exposed to drugs that are commonly used in experimental models of PD (e.g., 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, paraquat, and rotenone; Gorell, Johnson, Rybicki, Peterson, & Richardson, 1998). Furthermore, comparative analyses of vertebrates have strongly suggested that the Vv is a septal formation area.

In the present study, *Poecilia reticulata*, a benthopelagic nonmigratory Amazonian fish that is commonly known as Guppy or Lebiste, with a King Cobra lineage (Whitney & Hahnel, 1980), was used. This teleost fish is widely used in toxicological studies because it is quite sturdy and easy to handle. In the open field test, this fish presents a stereotyped behavioral pattern, in which exposure to the new environment is followed by higher activity in the peripheral zone than in the central zone. This pattern is only modified through habituation (Warren & Callaghan, 1976).

The purpose of the present study was to describe the effects of acute rotenone exposure at different concentrations on *Poecilia reticulata* using behavioral and neurohistological analyses.

Materials and methods

All of the experimental procedures were consistent with the standards of the International Guiding Principles for Biomedical Research Involving Animals (National Research Council, Institute for Laboratory Animal Research, 2004) and received approval from the Ethics Committee for Animal Use of the Federal University of Pará.

Subjects

In the current investigation, the experimental subjects were adult male fish of the *Poecilia reticulata* species, King Cobra lineage, weighing between 0.5 and 0.6 g, that were purchased from an Aquanorte store (Belém, Pará, Brazil). The animals were housed in 20 L aquaria (pH 7.0-7.6, 25.90ºC and 27.00°C at room temperature), with constant aeration and filtration with an hydraulic pump (HF-0400, Atman, Brazil). The photoperiod was 12 h light/12 h dark. The water was supplied by the public network, and the limnological parameters were adjusted according to the limits recommended by the American Public Health Association (2012). The animals were fed three times per day with specific food for the species (Guppy Mep 200, Alcon, Brazil). Feeding was interrupted only during the 24-h period of exposure to rotenone.

A 1 week acclimation period was used to observe and control health conditions, recover from transportation stress, and adapt to the new environment, as recommended by Murty (1988).

Test apparatus

During the 24 h of intoxication, the animals were individually placed in aquaria with a volume of 1 L. The aquaria were coated with black vinyl to protect the rotenone solution from photobleaching.

For the open field test, a circular tank was used, with matte walls and background (37.70 cm^2) and a 2 L capacity. The background had two perpendicular lines that intersected at the center of the apparatus where they met a circle (7.54 cm^2). This structure resulted in the formation of four peripheral quadrants and one central circle. Thus, the animal's displacement in two directions (center-periphery and periphery-periphery) could be measured.

Intoxication

Four groups were formed ($n = 10$). Three groups were exposed for 24 h to different sublethal concentrations of rotenone (5, 10, and 12.5 µg/L) diluted in water. The fourth group was not exposed to rotenone (negative control). Rotenone has limited solubility (0.2 mg/L at 20°C), and successive dilution was performed to obtain the final concentrations. The concentrations were determined to be within the minimum sensitivity range for the family Poecilidae, which is 17 µg/L (Ling, 2003). All of the groups, including the negative control, were exposed to their respective solutions for 24 h.

Open field test

The open field test was conducted in a controlled environment with low noise and fluorescent lamps positioned above the test tank to avoid shadow formation from the animal and walls of the apparatus.

One fish was placed in the center of the apparatus at the beginning of each session, and the test duration was 10 min. The experiment was recorded on a webcam (LifeCam, Microsoft, USA) coupled to a notebook computer (Vostro 3560, Dell, USA). Each animal was tested 10 times.

Craniotomy and brain section

After 24 h exposure to rotenone, the animals were anesthetized with 5% tricaine methamesulfonate and then subjected to craniotomy. After extraction, the brains were fixed in 4% paraformaldehyde and diluted in a solution of 0.1% phosphate-buffered saline (PBS) and 2.5% glutaraldehyde for 24 h at room temperature. The brains were then transferred to a cryoprotectant solution that consisted of 30% sucrose in 0.1% PBS for 48 h at -4°C.

Coronal sections of the brains, as thin as 40 μm, were cut with a cryostat (CM 1520, Leica, Germany). The histological sections were then mounted on gelatinized slides, and Nissl staining was performed.

Nissl staining

For the neurohistological study, the tissue was stained with Cresyl violet. The staining protocol was adapted from Klüver & Barrera (1953). The sections were mounted on gelatin-coated slides, dehydrated in ethanol, delipidated in xylene, rehydrated in ethanol, and immersed in 0.25% Cresyl violet acetate for 2 min. The sections were then dehydrated again, immersed in xylene, and covered with DPX mounting medium (BDH, Poole, United Kingdom).

Histological study

A Lucida optical microscope equipped with an image capture system (Labophot-2, Nikon, Japan) was used at 400× magnification. Cell counting was performed in a previously defined area of approximately 200 μm^2. The limits of this area were defined by considering the natural border circumscribed by the group of cells (cluster) and neuroanatomical features, such as bundles of nerves that cross the area. Cell counting was always performed with the fifth section of the telencephalon. Five sections were manually counted for each animal.

Statistical analysis

The results were analyzed using BioEstat 5.0 software for Windows (Ayres, Ayres, Ayres, & Santos, 2007). The arithmetic mean and standard deviation were first displayed. The nonparametric Kruskal-Wallis test (H test) was then performed, with a significance level of $p < .05$.

Results

In the open field test, locomotor activity was measured in the four peripheral quadrants and central circle. Table 1 shows the values obtained in the analysis of overall crossings (total) and statistical comparisons between crossings in the center-periphery (central) and periphery-periphery (periphery).

Table 2 shows the *p* values of the comparisons between groups that were exposed to rotenone and the control group. Peripheral, central, and total locomotor activity was analyzed. The comparison between the 5 μg/L and 10 μg/L groups indicated no differences in any of the variables, suggesting that the subjects were equally affected by these doses.

Figure 1 presents central, peripheral, and total locomotor activity in the open field after 24 h exposure to rotenone. All the groups preferred the periphery of the open field over the center.

The number of crossings in the peripheral region of the apparatus was not significantly different between the 5 μg/L and 10 μg/L groups and the control group. The number of crossings in the 5 μg/L and 10 μg/L groups was higher than in the control group. No significant difference was found between the 5 μg/L and 10 μg/L groups. Significantly differences were found in all of the other comparisons. The 5 μg/L and 10 μg/L groups had a very similar average number of crossings. Both concentrations apparently had a similar anxiogenic

Table 1. Locomotor activity (number of crossings) in the open field.

Group	Central	Periphery	Total	*p*
Control	1.80 ± 1.16	11.44 ± .66	13.24 ± 1.15	.0005**
5 μg/L rotenone	2.89 ± .64	15.77 ± 2.48	18.66 ± 2.47	< .0001**
10 μg/L rotenone	3.26 ± .80	15.33 ± 2.45	18.59 ± 2.47	< .0001**
12.5 μg/L rotenone	4.63 ± 1.99	10.77 ± 2.15	15.40 ± 1.97	.0857

Table 2. Comparison of number of crossings in the open field between groups.

Group	Central H = 19.20 *p* < .0007	Periphery H = 36.47 *p* < .0001	Total H = 38.90 *p* < .0001
Control × 5 μg/L	.0542	.0197*	.0009**
Control × 10 μg/L	.0102*	.0384*	.0008**
Control × 12.5 μg/L	.0002**	.5497	.0960
5 μg/L × 10 μg/L	.5194	.7943	.9572
5 μg/L × 12.5 μg/L	.0790	.0034**	.0992
10 μg/L × 12.5 μg/L	.2661	.0076**	.0886

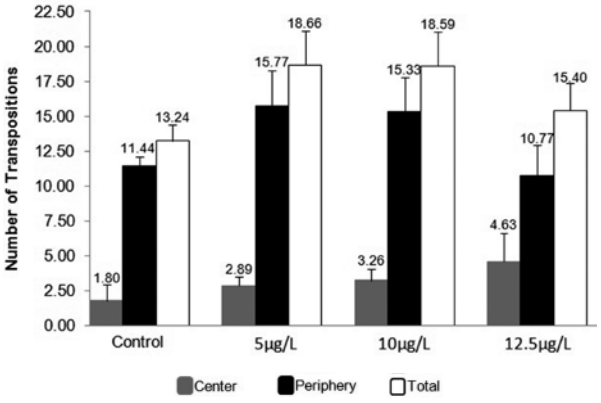

Figure 1. Locomotor activity, presented as the number of crossings, between groups.

Table 3. Comparison of total number of neurons in Vv between groups.

Group	Neurons H = 32.24 *p* < .0001
Control × 5 µg/L	.0362*
Control × 10 µg/L	.0018**
Control × 12.5 µg/L	.0001**
5 µg/L × 10 µg/L	.3017
5 µg/L × 12.5 µg/L	.0005**
10 µg/L × 12.5 µg/L	.0147*

effect. This anxiogenic effect was less noticeable in the 12.5 µg/L group.

With regard to central ambulation, crossings were less frequent than in the peripheral area. The 10 µg/L and 12.5 µg/L rotenone groups exhibited a significantly greater number of crossings compared with the control group. Locomotor activity in the 10 µg/L and 12.5 µg/L groups was equivalent to the control group.

Total ambulation in the 5 µg/L and 10 µg/L groups was significantly higher than in the control group, demonstrating the anxiogenic effect of these concentrations. Average ambulation was significantly higher In the groups exposed to rotenone.

The quantitative analysis of cells in the Vv showed that the number of neurons was inversely proportional to the concentration of rotenone (Figure 2). A significant reduction of the number of cells was observed in the 5 µg/L (390.40 ± 2.59), 10 µg/L (387.60 ± 3.34), and 12.5 µg/L (359.30 ± 5.29) groups compared with the control group (394.80 ± 1.55; Table 3). The 12.5 µg/L group had significantly elevated neuronal loss compared with the other rotenone groups.

Discussion

Rotenone is deleterious to mitochondria. It binds to complex I proteins of the electron transport chain, blocks adenosine triphosphate production, and promotes the formation of free radicals, which activate mechanisms of oxidative stress (Drechsel & Patel, 2008), resulting in neurodegeneration. Although this effect occurs uniformly throughout the brain, only dopaminergic nigrostriatal neurons appear to suffer degeneration (Betarbet et al., 2000). In the present study, the neurodegenerative effects of rotenone on the Vv were identified, and the cell count in this area was inversely proportional to the pesticide concentration with acute exposure.

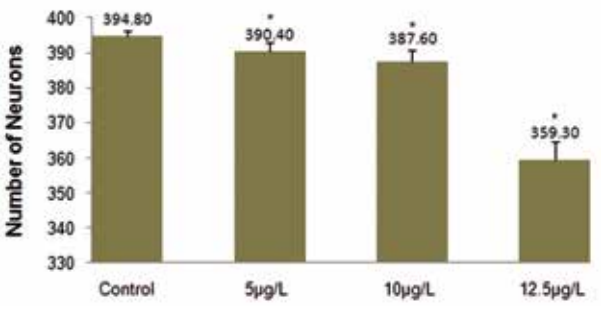

Figure 2. Total number of neurons in Vv.

Even the lowest concentration of rotenone (5 µg/L) caused significant neuronal loss in the Vv compared with the control group (p = .0362). The 12.5 µg/L concentration caused an approximately 9% loss of neurons in the Vv compared with the control group (control: 394.80 ± 1.55; 12.5 µg/L: 359.30 ± 5.29; p < .0001). The 12.5 µg/L group also exhibited higher locomotor activity in the central area of the apparatus in the open field test. Therefore, the Vv in fish may have the same intrinsic sensitivity to defects in mitochondrial complex I as the neurons in the substantia nigra. In the present study, higher rotenone concentrations increased locomotor activity, and rotenone also exerted an anxiolytic effect, reflected by behavioral disinhibition and an increase in central ambulation.

A significant increase in locomotor activity (total ambulation) was found in the 5 µg/L (p = .0009) and 10 µg/L (p = .0008) rotenone groups compared with the control group. The lesions observed in the Vv are consistent with the hypothesis that rotenone directly affects locomotion. Thus, although the low (5 µg/L) and moderate (10 µg/L) doses of rotenone enhanced total ambulation, preference for the periphery was preserved (p < .0001) in both groups compared with controls, even with equivalent percentages of peripheral ambulation (84.51%, 82.46%, and 86.40%, respectively). These results indicate an anxiogenic effect of these concentrations, similar to the findings reported by López-Patiño, Yu, Cabral, and Zhdanova (2008), who studied the effects of cocaine in *Danio rerio*, and Hallgren, Volkova, Reyhanian, Olse, and Hällström (2011), who studied the effects of 17α-ethynylestradiol in *P. reticulata*.

Locomotor alterations were also found in the 12.5 µg/L rotenone group, in which central ambulation was significantly higher than in controls (p = .0002).

Although preference for the peripheral areas of the apparatus occurred at the detriment to the central area, which was observed in the control group and 5 and 10 µg/L groups, the difference in locomotor activity was not significant, and the percentage of peripheral crossings decreased by 69.93%. Thus, the rate of locomotor activity in the central area of the apparatus was two-times higher in the 12.5 µg/L group compared with the lower concentrations, suggesting an antagonistic effect. The 12.5 µg/L concentration exerted a bimodal pharmacological effect, in which peripheral activity was reduced (related to fear) and exploratory behavior was increased (crossings toward the center of the apparatus).

The anxiolytic effect induced by 12.5 µg/L rotenone, reflected by a significant increase in central ambulation compared with the control group (p = .0002), appeared to be associated with extensive damage in the Vv caused by this concentration. Neurodegeneration was significant, even when compared with the 10 µg/L concentration (p = .0147). These results appear to indicate higher motivation to explore at the detriment of avoidance behavior (McNaughton & Corr, 2004).

Conclusions

In the present study, the adverse effects of rotenone were observed at the lowest concentration (5 µg/L), causing significant neuronal loss in the Vv and resulting in progressive motor hyperstimulation (Viggiano, 2008) up to a concentration of 12.5 µg/L. This may have caused a progressive collapse of the inhibitory system, which is equivalent to septal regions, resulting in an increase in locomotor activity and behavioral disinhibition. However, more behavioral, biochemical, and cellular studies are necessary to confirm the possibility of using rotenone as a model of PD in fish similarly to rats.

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