

Alterations in the duodenum myenteric neurons of Wistar rats after ingesting of 2,4 dichlorophenoxyacetic acid

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Abstract

The 2,4 dichlorophenoxyacetic acid (2,4-D) is a systemic herbicide whose effects in animal organic systems have been examined in previous studies, being the neurotoxicity considered the predominant effect. However, the studies that detect the 2,4-D neurotoxicity have merely focused in the central nervous system, and therefore, little is known about the effect of this herbicide in the enteric nervous system. This study aimed to verifying the 2,4-D effects on the myenteric neurons in duodenum of Wistar rats. Ten 60-day-old male Wistar rats (*Rattus norvegicus*) were divided in two groups: control group (C) that did not receive 2,4-D and experimental group (E) that received 5.0 mg of 2,4-D/kg for 15 days. At the end of experimental period, the animal were euthanized, the duodenum was collected and processed for NADPH-diaphorase histochemical analysis in order to expose the nitregeric myenteric neurons (NADPH-dp). In the light microscopy analysis, the whole-mount preparation obtained from duodenum of each animal were image-captured in 120 and 40 fields, for quantitative and morphometric analyses of myenteric neurons, respectively. The neuronal density was not affected when comparing the two groups, but an increase ($p > 0.05$) of 8.5% was observed in the cell body area of neurons in the E group. In conclusion, the ingestion of 2,4-D at a dosage of 5.0 mg/kg body weight for 15 days does not change the neuronal density, but promotes the hypertrophy of NADPH-dp myenteric neurons in duodenum of the rats of this study.

Keywords: herbicide, myenteric plexus, small intestine, nitregeric neuron.

1 Introduction

The 2,4 dichlorophenoxyacetic acid (2,4-D) is a systemic herbicide used in cereal crops, sugarcane, forest control, public and home gardens. During its application, caution is always recommended due to its high volatility, thus it is mentioned to avoid the usage in periods of winds (even weak), since the drift of its vapors can spread out many kilometers away from the point of application (ALMEIDA and RODRIGUES, 1988).

Charles, Bond, Jeffries et al. (1996), Paulino, Guerra and Palermo-Neto (1996), Mattsson, Charles, Yano et al. (1997), Bradbery, Watt, Proudfoot et al. (2000), Ozcan-Oruc, Sevgiler and Uner (2004) studied the administrative effects of different concentration levels of 2,4-D in different organic systems of dogs and rats.

The 2,4-D is rapidly distributed in body tissues being easily absorbed in the gastrointestinal tract, without any bioaccumulation, and, it is excreted in the urine in an unaltered form, or conjugated with amino acids or proteins, with only minimal amounts reaching the body tissues (BRADBERY, WATT, PROUDFOOT et al., 2000). According to Tsman (1991) investigations/studies, the toxic

dose for humans after ingestion is about 3 and 4 g, and the lethal dose is 28 grams, differently, the lethal dose for rats is 375 mg/kg body weight (ALMEIDA and RODRIGUES, 1988); the 2,4-D is considered moderately toxic for birds and non-toxic for fish (THOMPSON, STEPHENSON, SOLOMON et al., 1984).

Acute intoxication by 2,4-D is characterized by complicated conditions in the digestive, cardiac, respiratory, and neuromuscular systems (Charles, Dalgard, Cunny et al. 1996). Hepatic, haematological and renal toxicities were observed in rats administered with 5 mg/kg body weight/day (sub-chronic dose) of 2,4-D added in the food (UNITED..., 1987).

Research studies where the neurotoxicity of 2,4 D is confirmed have only focused on the analyses of this herbicide in the central nervous system, however, the mechanism of action of this herbicide is not yet fully understood (BONGIOVANNI, DE LORENZI, FERRI et al., 2007; BJORLING-POULSEN, ANDERSEN and GRANDJEAN, 2008; KONJUH, GARCIA, LÓPEZ et al., 2008).

The enteric nervous system controls the motility, blood flow, and secretions of the gastrointestinal tract, acting in the mechanisms of digestion and absorption of nutrients; the myenteric plexus is one of its constituents; it is located in the muscular layer of the digestive tract segments (PHILLIPS and POWLEY, 2007).

Symptoms like anorexia, constipation, diarrhea, weight loss, nausea and vomit in diabetes, aging, and malnutrition conditions are attributed to changes in the myenteric plexus (WADE and COWEN, 2004; ZANONI, BUTTOW, BAZOTTE et al., 2003; ARAÚJO, SANT'ANA, MOLINARI et al., 2006; GAGLIARDO, CLEBIS, STABILLE et al., 2008; MARI, CLEBIS, GAGLIARDO et al., 2008; SILVERIO, MARI, CLEBIS et al., 2008).

The nitrergic neurons are, among the total neuronal population of the myenteric plexus, responsible for inhibition of digestive tract muscles (FURNESS, LI, YOUNG et al., 1994). Nitrergic myenteric neurons can be evidenced by histochemical method of the enzyme nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-diaphorase) (SCHERER-SINGLER, VINCENT, KIMURA et al., 1983). These neurons plays a role in the mechanism of defense against free radicals improving it's function of producing nitric oxide, which makes them more resistant to adverse conditions that affect other types of enteric neurons (COWEN, JOHNSON, SOUBEYRE et al., 2000).

Since the 2,4-D acts on the central nervous system, it possibly also acts on the neurons of the enteric nervous system, as among the symptoms of intoxication by 2,4-D are anorexia, gastrointestinal irritation, nauseas, diarrhea and vomit (UNITED..., 1999). For this reason, there is a need of a better understanding of the effects of the 2,4-D in the enteric neurons to prevent the gastrointestinal disorders caused by the herbicide. Thus, the present study aimed to verify the effects of 2,4-D on the NADPH-diaphorase-positive nitrergic myenteric neurons (NADPH-dp neurons) in the duodenum of wistar rats using quantitative and morphometric analyses.

2 Material and methods

This study was conducted according to the ethical principles in animal experimentation (COBEA) and was approved by the Ethics Committee for Animal Research of the Paranaense University (CEEPEA/Unipar).

Ten 60-day-old male Wistar rats (*Rattus norvegicus*) were used in the experiment for this study.

Animals were accommodated in individual polypropylene boxes, under environment conditions with controlled temperature (22 °C) and lighting (12 hours light/dark cycle) for 15 days; water and Nuvital® commercial food was given to the animals *ad libitum*.

The animals were weighted daily and randomly grouped in two groups (n = 5/group) as follow:

- Group E (E): experimental group consisting of animals that received 5.0 mg/kg body weight/day of 2,4-D diluted in one mL of water for 15 days, via gavage; and
- Group C (C): control group consisting of animals that did not receive 2,4-D.

2.1 Animal euthanasia

After the experimental period, and fasting for 12 hours, the animals were euthanized by lethal intravenous dose (tail vein) Thiopental® 40 mg/kg of body weight.

Thereafter, each animal underwent to laparotomy technique and the duodenum (cranial limit – gastroduodenal junction, and caudal limit – duodenojejunal flexure) was collected and processed for NADPH-diaphorase histochemical analysis for NADPH-dp neurons observation.

2.2 Histochemistry for the NADPH-diaphorase (SCHERER-SINGLER, VINCENT, KIMURA et al., 1983).

The duodenum of each animal was rinsed and filled with a phosphate buffer solution (pH 7.4). The ends of the duodenum were tied with suture thread to keep the solution in situ filling.

Then, the samples underwent to two baths in PBS (10 minutes each) followed by permeabilization in PBS with 0.3% Triton X-100 in sodium phosphate buffer (pH 7.3) for 10 minutes. Two additional baths in PBS (10 minutes each) were then performed. The duodenum was incubated in reaction medium containing 50 mg of nitroblue tetrazolium (Sigma®), 100 mg of β-NADPH (Sigma®), and 0.3% of Triton X-100 in Tris-HCl buffer (0.1M; pH 7.6) buffer.

The histochemical reaction was visualized in the stereomicroscope with standard duration of 100 minutes. Afterwards, the ends of the duodenum were released by cutting the suture threads, and each duodenum was rinsed three times in PBS for five minutes, and immersion-fixed in 4% paraformaldehyde for fixation and storage.

2.3 Obtaining whole-mount preparations of duodenum

To obtain the whole-mount preparation, the duodenum was sectioned along the longitudinal axis of the mesenteric insertion and micro-dissected using a stereomicroscope with transillumination. During the micro-dissection, the mucosal and submucosal layers were discarded, and the muscular and serosa layers containing the myenteric plexus were preserved. The whole-mount preparation obtained went through dehydrated series of alcohol solutions of increasing concentrations (90%, 95% and absolute), diaphanized in three consecutive immersions of xylene, and mounted into glass slides using Permount® resin.

The whole-mount preparations were used for quantification and morphometry of the NADPH-dp myenteric neurons.

2.4 Quantification of NADPH-dp neurons

The neuronal quantification per area (cell/mm²), of the whole-mount preparation of the duodenum was observed using a Olympus BX40 light microscope with a 40× objective. The neurons were quantified in 120 microscopic fields per whole-mount preparation, sampling equally all the regions of the duodenal circumference (mesenteric, intermediate, and antimesenteric regions). Half-neurons were considered in alternate fields. The area given by the 40× objective previously measured to determine the average of neuronal density per mm² of duodenum was 0.0746 μm².

2.5 Morphometric analysis of the NADPH-dp neurons

Images of each whole-mount preparation were captured randomly in the field from under 40 microscopic. The images were captured using a light microscope (Motic®, at 400× magnification) coupled to an Axio Cam HRC – Zeiss® digital camera. Cell body area of the NADPH-dp neurons was measured in the captured images using a software Image-Pro Plus 3.0.1 (Media Cybernetics, Silver Spring, Maryland, USA).

2.6 Statistical analysis

The Student's *t*-test was used to compare the data obtained in the quantification and morphometry, between the groups at 5% significance level. The results were expressed in average ± standard deviation.

3 Results

In the light microscopy analysis, the NADPH-dp myenteric neurons of the groups E and C were observed inside the within ganglia (Figure 1) interconnected by bundles of nerve fibers in a spatial distribution fashion.

For quantitative analysis of NADPH-dp myenteric neurons, 120 fields per whole-mount preparation were observed, in the light microscope, and neuronal density per mm² of the mesenteric, intermediate and antimesenteric regions of the duodenal circumference were determined (Table 1).

When comparing the groups C and E, no significant reduction (*P* > 0.05) was observed in the density of NADPH-dp myenteric neurons, in the three studied regions

(mesenteric, intermediate and antimesenteric) under the effect of 2,4-D (Table 1).

The cell body area of the NADPH-dp myenteric neurons from the group E increased significantly (*P* < 0.05) in relation to the group C (Table 1).

4 Discussion

Accordingly to the reported in the literature, the NADPH-dp myenteric neurons was predominantly observed inside the ganglia interconnected by bundles of nerve fibers in the whole-mount preparations of the duodenum (FURNESS, 2006). No changes were verified between the animals of the two groups, indicating that the 2,4-D did not interfere on the spatial organization of the myenteric plexus. The lack of interference of 2,4-D on the organization of myenteric neurons in the duodenum of rats that ingested 2,4-D at doses of 2.5 and 5.0 mg/kg weight, and on nitregic neurons (NADPH-dp) in the jejunum of rats was previously described in Pereira and Stabille (2006) and Correa, Mari, Toledo et al. (2011) studies.

No differences in the density of NADPH-dp myenteric neurons were observed in any duodenum regions analyzed between the groups (*P* > 0.05) administered with 2,4-D. Similar results with NADPH-dp myenteric neurons were described by Correa, Mari, Toledo et al. (2011) in the jejunum of rats that received during the same period time daily doses of 5 mg of 2,4-D/Kg body weight.

The maintenance of neuronal density indicates that there was no loss of NADPH-dp neurons in response to 2,4-D. This result could be attributed to the protective function of the connective tissue cover surrounding the ganglia of the myenteric plexus, which probably would act as a selective

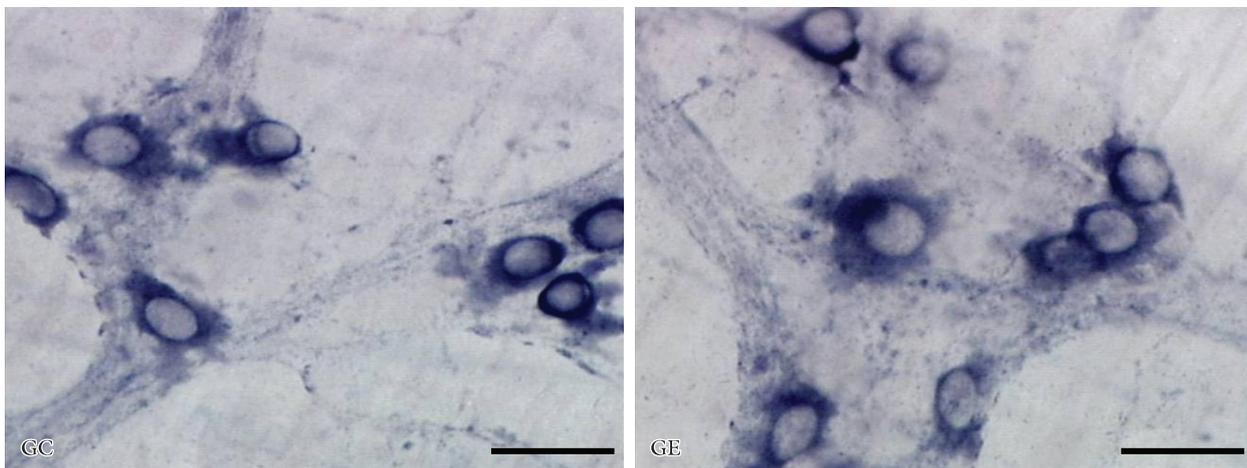


Figure 1. Duodenum whole-mount preparation of the control (C) and experimental (E) animal groups, evidencing NADPH-diaphorase positive neurons.

Table 1. Average and standard deviation of the density of NADPH-dp myenteric neurons in the mesenteric (M), intermediate (I) and antimesenteric (A) regions of the duodenum; and average and standard deviation of the cell body area of the duodenal NADPH-dp myenteric neurons of rats that received (group E) or not received (group C) the 2,4 dichlorophenoxyacetic acid (2,4-D).

Groups	Neuronal density/mm ²			Cell body area (µm ²)
	M	I	A	
E	11,23 ± 2,39 ^a	11,2 ± 2,38 ^a	11,52 ± 1,41 ^a	217,49 ± 98,21 ^b
C	12,37 ± 2,03 ^a	14,15 ± 1,18 ^a	13,5 ± 2,56 ^a	200,56 ± 73,20 ^a

^aMean followed by different letters in the column are different by the test *t* de Student (*p* < 0,05).

barrier (like the blood-brain barrier) preventing the 2,4-D action. In the central nervous system, the 2,4-D is unable to cross the blood-brain barrier (TYNELA, ELO and YLITALO, 1990), although when administered at high concentrations, deleterious effects on the neurons of the central nervous system area have been described, suggesting rupture and damage of the blood-brain barrier (OLIVEIRA and PALERMO-NETO, 1993).

Nevertheless, previous studies with the duodenum of rats that received 2.5 and 5.0 mg of 2,4-D/kg weight showed a reduction of 18.58% in the density of myenteric neurons stained by Giemsa method (PEREIRA and STABILLE, 2006), thus indicating that the cover layer of connective tissue surrounding the ganglia does not prevent the 2,4-D to induce neuronal death. In this view way, the absence of changes in the density of NADPH-dp myenteric neurons is probably due to the greater resistance of this neuronal population to 2,4-D, and not to the protective action of the cover layer of connective tissue ganglia.

The notably neuronal loss reported by Pereira and Stabile (2006) in the duodenum of rats after administration of 2,4-D occurred in neurons evidenced by Giemsa method. This method is used to stain the neuronal myenteric population in general, differently of the histochemical method used in the present study, which is specific to the neuronal subpopulation that expresses nitric oxide (SAFFREY, 2004). The Giemsa method uses the dyes toluidine blue, or methylene blue (BARBOSA, 1978) that have affinity for acid structures present in all neurons, regardless of the neurochemical code, thus precluding to determine which functional type of neuron was lost under the effect of the 2,4-D.

Although neuronal density was not changed, a cell body hypertrophy was observed in the analyzed neurons. The cell body area of the NADPH-dp myenteric neurons in the duodenum of rats was different ($P < 0.05$) between the groups E and C. Neurons of the E group increased cell body area in about 8.5% in relation to the group C. Similar results were described by Correa, Mari, Toledo et al. (2011) with 5% increase in the cell body area of NADPH-dp myenteric neurons in the jejunum of rats that received the same dose of 2,4-D (5 mg/Kg).

Hypertrophy in the cell body area has been associated to the oxidative stress in both NADPH-dp neurons and NOS-immunoreactive neurons (ZANONI, BUTTOW, BAZOTTE et al., 2003; SHOTTON and LINCOLN, 2006; SHOTTOLN, ADAMS and LINCOLN, 2007).

In the case of animals of group E, the production of free radicals by the myenteric neurons can be result of neurotoxicity induced by the ingestion of 2,4-D, since the increase in the production of free radicals is one of the effects produced triggered in the cells by 2,4-D (BJORLING-POULSEN, ANDERSEN and GRANDJEAN, 2008).

A reduction in catalase activity with consequent increase in the production of reactive oxygen species (ROS) were observed with the incubation of the cerebellum with 2,4-D (BONGIOVANNI, DE LORENZI, FERRI et al., 2007). In Ozacan-Oruc, Sevgiler and Uner. (2004) reports an increased activity of antioxidant enzymes in the kidney and brain of fish exposed to 2,4-D was observed. This herbicide induces oxidation in red blood cells, proteins, and increase in production of free radicals (BUKOWSKA, RYCHLIK, KROKOSZ et al., 2008), being also an inhibitor, although moderate, of the oxidative phosphorylation (SCHVARTSMAN, 1991).

Events that may damage or produce functional changes in the neurons, require adaptations in an effort to ensure neuronal viability (EKBLAD and BAUER, 2004). In an attempt to compensate an initial injury or to keep the functional balance under adverse conditions, one way that the neurons respond to the injury is increasing their polysomes, RNA and protein synthesis, leading the cell body to double its size (JESSELL, 1991). Therefore, these adaptive reactions, characteristic to neurons, could justify the hypertrophy observed in the cell body of NADPH-dp neurons in response to the action of the 2,4-D.

5 Conclusion

The ingestion of 5 mg of 2,4-D/Kg body weight for 15 days does not change the spatial organization of myenteric plexus, but promotes cell body hypertrophy of NADPH-dp nitregeric myenteric neurons, maintaining the density of these neurons in the duodenum of Wistar rats unchanged.

Acknowledgements: The authors would like to thank/acknowledge the Paranaense University (Unipar) for financial support of this the project, and the Araucária Foundation, for the scientific initiation scholarship of the first author.

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Received July 17, 2012
Accepted March 1, 2013