Original article

Effects of the ingestion of 2,4 Dichlorophenoxyacetic acid on jejunal myenteric neurons in rats

Correa, OP.[1], Mari, RB.[2], Toledo, EL.[1], Guimarães, JP.[2], Pereira, JNB[1], Germano, RM.[1], Faria, HG.[4], Souza, RR.[3] and Stabille, SR.[1]*

[1]Science Animal Research Laboratory, Department of Biological Sciences, Universidade Paranaense – UNIPAR, Paranavaí Campus, Av. Huberto Bruning, 360, Jardim Santos Dumont, CEP 87706-490, Paranavaí, PR, Brazil
[2]Aquatic Mammals Foundation, Aracajú, SE, Brazil
[3]Biodinamic and Morphology Laboratory, São Judas Tadeu University – USJT, São Paulo, SP, Brazil
[4]Central Vivarium, Universidade Estadual de Maringá – UEM, Maringá, PR, Brazil
*E-mail: renatamari@unipar.br

Abstract

2,4 dichlorophenoxyacetic acid (2,4-D) is a systemic herbicide. The effects of different levels of 2,4-D on some animal organ systems have been examined, but little is known about its role in the enteric nervous system. The purpose of this study was to verify the effects of 2,4-D administration on the density and morphometry of jejunal myenteric neurons in rats. Ten male rats were assigned to control (C) and experimental (E) groups. For 15 days, group E received, via gavage, 5 mg of 2,4-D.kg^{-1} body weight. On the 16th day, the animals were sacrificed by a lethal dose of thiopental, and the jejunum was removed by laparotomy and used to obtain whole mount preparations for Giemsa staining and NADPH-diaphorase (NADPHd+) histochemistry to identify neurons. The density and cell body area of the myenteric neurons was measured. In the total neuronal population, the neuronal density/mm^2 of the jejunum in groups E and C was equivalent, and the cell body area of the rats in group E was lower ($p < 0.05$) than that of those in group C. For NADPHd+ neurons, the neuronal density did not differ between the groups, although the cell body area was larger ($p < 0.05$) in group E. It was concluded that even though 2,4-D does not alter the neuronal density in the rat jejunum, it induces cell body atrophy in the general population of neurons and hypertrophy of the NADPHd+ nitric oxide producing neurons without promoting cell death.

Keywords: 2,4-D, intestine, enteric nervous system, herbicide, nitric oxide.

1 Introduction

The principal source of phenols in the environment is the degradation of herbicides, such as 2,4 dichlorophenoxyacetic acid (2,4-D). Phenoxyherbicides were synthesised in 1941, but the molecular mechanism of its toxic action in animals is still being investigated (BUKOWSKA, RYCHLIK, KROKOSZ et al., 2008).

The exposure of humans and other animals to 2,4-D or its residues, occurs through air, water, soil or contaminated food during the production and application of the herbicide (AYDIN, ÖZDEMIR and UZUNÖREN, 2005).

2,4-D is the clorofenoxiherbicide that is most commonly used for pest control in crops, and is therefore widely studied (BJORLING-POULSEN, ANDERSEN and GRANDJEAN, 2008). 2,4-D interferes with plant growth and is used as a systemic herbicide to control seasonal and non-seasonal, broad leaf weeds (DI PAOLO, EVANGELISTA DE DUFFARD and DUFFARD, 2001).

Although they are chemically stable, all forms of 2,4-D (salt and esters) are rapidly converted into free acids (AYDIN, ÖZDEMIR and UZUNÖREN, 2005). When administered orally, 2,4-D is easily absorbed in the intestine, metabolised in the liver (MEHMOOD, WILLIAMSON and KELLY, 1996) and excreted in the urine, in the unaltered form, in forms conjugated with amino acids or proteins or as the metabolite 2,4-Dichlorophenol, with only minimal amounts reaching body tissues (BRADBERY, WATT, PROUDFOOT et al., 2000). However, the tendency for accumulation, principally in the kidneys, liver, adipose tissue and central nervous system, has been reported (AYDIN, ÖZDEMIR and UZUNÖREN, 2005).

The effects of different concentration levels of 2,4-D administered in single or continuous doses upon some animal organ systems, principally in dogs and rats, has been analysed. Findings indicate that the LD_{50} rats is 639 and 764 mg.kg^{-1}/day for males and females, respectively (MUNRO, CARLO, ORR et al., 1992) or between 375 and 666 mg.kg^{-1}/day (STEVENS and SUMMER, 1991).

In rats, the no-observed-adverse-effect level (NOAEL) was established as 1 mg.kg^{-1}/day for renal, hepatic and haematological effects (GARABRANT and PHILBERT, 2002). However, it has also been reported that doses of 70 mg.kg^{-1} body weight administered intraperitoneally in rats is eliminated on the same day, with no hepatotoxicity (HANSEN, QUAIFE and HABERMANN, 1971).

Acute intoxication is characterised by digestive complications and by neuromuscular manifestations, breathing difficulties and bradycardia, among other effects (CHARLES, BOND, JEFFRIES et al., 1996a; CHARLES, DALGARD, CUNNY et al., 1996b).

The toxicity of 2,4-D may be mediated by mechanisms that involve lipid peroxidation (DUCHNOWICZ and KOTER, 2003), interference with cellular metabolic pathways involving acetyl coenzyme-A (BRADBERY, WATT, PROUDFOOT et al., 2000), interference in neuronal energy metabolism, with impairment of molecular signalling, regulation of biosynthetic and catabolic reactions, and transport of metabolites and ions (PALMEIRA, MORENO, VASCO et al. 1997) and inhibition of polymerisation of the microtubular protein and disruption of the Golgi apparatus with impaired biosynthesis of gangliosides (ROSSO, CÁCERES, EVANGELISTA DE DUFFARD et al., 2000). However, the specific mechanism of 2,4-D neurotoxicity is still unclear (BORTOLOZZI, EVANGELISTA DE DUFFARD, DUFFARD et al., 2004; BJORLING-POULSEN, ANDERSEN and GRANDJEAN, 2008; KONJUH, GARCIA, LÓPEZ et al., 2008).

The 2,4-D neurotoxicity is caused, in part, by the formation of free radicals with decreased reduced glutathione levels and impaired action of antioxidant enzymes such as superoxide dismutase and catalase (BUKOWSKA, 2003; BONGIOVANNI, FERRI, KONJUH et al., 2007).

In the mitochondria of liver and red blood cells, 2,4-D promoted oxidative stress, as demonstrated by the depletion of antioxidants and increased markers of oxidation (OAKES and POLLAK, 1999; BUKOWSKA, 2003; DUCHNOWICZ and KOTER, 2003). However, Dinamarca, Hidalgo and Cavieres (2007) report that, at doses between 0.1 mg to 1 $mg.kg^{-1}$/day 2,4-D does not seem to induce any oxidative response, whereas at 100 mg, the non-enzymatic antioxidant activity is decreased.

Most research studies verifying the neurotoxicity of 2,4-D have been focused on the central nervous system (UNITED..., 1987; ROSSO, CÁCERES, EVANGELISTA DE DUFFARD et al. 2000; BORTOLOZZI, DUFFARD and EVANGELISTA DE DUFFARD, 2002, 2004), such that its effects on the enteric nervous system are still unknown.

Since 2,4-D acts on the central nervous system, it is also expected to act on the neurons of the enteric nervous system, particularly given that among the manifestations of poisoning by 2,4-D are anorexia, gastrointestinal irritation, nausea, vomiting and diarrhoea (UNITED..., 1987).

The enteric nervous system (ENS), with both a myenteric and submucosal plexus, acts on the mechanisms of digestion and absorption of nutrients by controlling movement, blood flow and secretions from the gastrointestinal tract (PHILLIPS and POWLEY, 2007). Through connections with other components of the autonomic nervous system, the ENS allows the digestive tract function to be integrated harmoniously with other functions of the body (COSTA, SIMON and BROOKS, 1994).

In the various segments of the digestive tract, the myenteric plexus, located between the circular and longitudinal layers of muscular tissue, is related to the control of motility for the transport of luminal contents (PHILLIPS and POWLEY, 2007).

Alterations in the myenteric plexus neurons are responsible for the manifestations of anorexia, constipation, diarrhoea, weight loss, vomiting, nausea and other symptoms present in conditions such as diabetes, aging and malnutrition (ZANONI, BUTTOW, BAZOTTE et al., 2003; WADE and COWEN, 2004; FREGONESI, MOLINARI, ALVES et al., 2005; ALVES, ALVES, FREGONESI et al., 2006; 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 ENS displays adaptive responses to different types of stimuli and conditions by changing its functional structure or its chemical phenotype in order to maintain homeostasis of intestinal functions (GIARONI, DE PONTI, COSENTINO et al., 1999; LOMAX, FERNANDEZ and SHARKEY, 2005)

The myenteric plexus neurons express different types of neurotransmitters, with the same neuron releasing more than one type of neurotransmitter (FURNESS, 2006). Techniques using methylene blue dye, such as Giemsa staining (BARBOSA, 1978) can be used to identify the general population of these neurons. Giemsa staining has a high level of affinity for acidic cellular structures and can be an indicator of cellular activity.

The population of myenteric neurons includes inhibitory neurons that express nitric oxide (NO) and vasoactive intestinal peptide (VIP) (FURNESS, 2006).

NO is produced through the action of nitric oxide synthase (NOS) on L-arginine, via a NADPH, $Ca2^{+}$ and calmodulin-dependent mechanism (WADE, 2002). Neurons that express NO can be detected by techniques that use antibodies for NOS or by nicotinamide adenine dinucleotide phosphatase diaphorase (NADPHd) histochemistry technique, since NOS co-exists with the NADPHd (FURNESS, LI, YOUNG, 1994; SAFFREY, 2004).

Neurons that express NO seem to be more resistant to adverse conditions that affect other enteric neurons, which is consistent with the protective function of NO as an activator of the defence mechanism against free radicals (COWEN, JOHNSON, SOUBEYRE et al., 2000)

Therefore, by applying Giemsa staining and the NADPHd enzyme histochemistry technique to whole mount preparations in order to detect neurons, the objective of the present study was to verify the effects of 2,4-D on some morphologic and quantitative aspects of myenteric neurons in the rat jejunum.

2 Materials and methods

In all stages, this study was conducted in accordance with the procedures established by the Ethics Committee for Animal Research (protocol n. 12754/2008 - CEPEEA/Unipar).

Ten 60-day-old male Wistar rats (*Rattus norvegicus*), from the Central Vivarium of the Universidade Estadual de Maringá, were used for this study.

Animals were housed in individual plastic boxes for 15 days, in a room with controlled artificial light on a 12 hours light/dark cycle and temperature set at 22 °C.

The animals were weighed daily and were randomly assigned to two groups (n = 5/group) as described below:

- Group E: experimental group consisting of animals that received 5.0 $mg.kg^{-1}$ body weight/day of 2,4-D in the form of sodium salt (Sigma, Steinheim, Germany) diluted in 1 mL of distilled water via gavage for 15 days;

- Group C: control group of animals that received 1 mL of distilled water without 2,4-D, also via gavage and for the same period.

All animals were provided with commercial rations (Nuvital®) and water without restriction. The dose of 2,4-D was adjusted daily according to body weight.

2.1 Animal euthanasia

At the end of the experimental period and after a 12 hours fast, the animals were sacrificed with a lethal dose of sodium thiopental (Abbout®) (40 $mg.kg^{-1}$ weight).

Thereafter, each animal was laparotomised by ventral midline incision and the jejunum was removed, from the duodenal-jejunal flexure (proximal cranial limit) to the ileocecal fold (distal caudal limit), its length was measured and its outline was traced onto white paper. These tracings were scanned to determine the area of the jejunum (mm^2) using an image analyser.

The jejunum of each animal was sectioned to obtain two segments. The proximal segment was used for procedures pertaining to the Giemsa staining technique, and the distal segment was used for the NADPHd histochemistry to reveal the myenteric plexus neurons. The neurons were observed in whole mount preparations of the jejunum, as reported below.

2.2 Giemsa technique (BARBOSA, 1978)

The jejunum was washed with 0.9% NaCl and filled with a fixative solution of formalin-acetic acid. The extremities of the jejunum were tied with suture thread to maintain this solution inside the organ. The jejunum was then immersed in the same fixative solution for 24 hours, after which the knots were untied and the jejunum was opened by a longitudinal section performed along its mesenteric border.

The jejunum was microdissected under stereomicroscopy (Olympus®, JAP) with trans-illumination, to remove and dispose of the mucosa and submucosa. The serum and muscular layers containing the myenteric plexus were preserved and together formed the whole mount preparation.

Each whole mount preparation was immersed for at least 12 hours in Giemsa dye solution prepared with methylene blue in 0.1 N Sorensen phosphate buffer (pH 6.9). Subsequently it was dehydrated in an ascending concentration series of ethanol (90, 95% and absolute), cleared by three successive immersions in xylol (Synth®, São Paulo, Brasil) and then mounted between glass slide and cover slip with Permount medium (Fisher Chemical®, USA).

2.3 NADPH-diaphorase histochemical technique (SCHERER-SINGLER, VINCENT, KIMURA et al., 1983)

The jejunum was washed and filled with phosphate buffer (PB pH 7.4) and, to prevent leakage, the extremities of the jejunum were tied with suture thread. The segments were fixed in 4% paraformaldehyde (Merk, Darmstad, Germany), prepared in 0.1 M phosphate-buffered saline (PBS, pH 7.4), for 30 minutes. They were then immersed in 0.3% Triton-X 100 (Sigma, St. Louis, USA), prepared in PBS (pH 7.4), for 10 minutes and then rinsed with PBS 10 times (10 minutes each).

Subsequently, the jejunum was incubated in a reaction medium containing 50 mg Nitro Blue Tetrazolium (NBT, Sigma Chemical Company, St Louis, Missouri, EUA), 100 mg β-NADPH (Sigma, Steinheim, Germany) and 0.3% Triton X-100 dissolved in Tris-HCl buffer (0.1M, pH 7.6).

The progression of the histochemical reaction was controlled visually with stereomicroscopic(Olympus®, JAP) aid and lasted 100 minutes. The suture threads on the jejunum were then cut, and the organ was rinsed three times in PBS, for five minutes in each wash, and then immersed in a 4% paraformaldehyde solution for fixation and storage.

To obtain the whole mount preparation, the same microdissection procedures were used as described for the Giemsa technique. The whole mount preparation was dehydrated in an ascending concentration series of ethanol (90, 95% and absolute), cleared by three successive immersions in xylol and then mounted between a glass slide and cover slip with Permount medium.

2.4 Quantitative analysis of myenteric neurons

For neuronal quantification, each whole mount preparation of the jejunum submitted to either of the staining techniques (Giemsa and NADPHd) was visualised under an Olympus BX40 light microscope with a 40× objective. The image observed on the microscope was captured by a high-resolution AxioCam digital camera (Zeiss, Jena, Germany), digitised into a microcomputer by the Axio Vision 4.1® program and recorded on a compact disk (CD).

Neurons were quantified in images obtained from 120 microcospic fields by whole mount preparation, equally sampling all the regions of the jejunal circumference. The image-analysis software used for neuronal quantification in the images was Image-Pro Plus 3.0.1.(Media Cybernetics, Silver Spring, Maryland, USA).

The area captured by the 40× objective was measured, resulting in 120 fields representing a total jejunal area of 11.52 mm^2. The results were expressed in terms of neuronal density per mm^2 of jejunum.

2.5 Morphometric analysis of the myenteric neurons

The measurement of the cell body area of the myenteric neurons, as evidenced by the Giemsa and NADPHd techniques, was obtained from images of neurons taken by digital camera at the neuronal quantification stage, using the Image-Pro Plus 3.0.1 image-analysis program.

The cell body area (mm^2) was measured for all neurons counted previously in the whole mount preparation of each animal's jejunum.

The neurons were grouped into classification intervals of 50 μm^2 according to the cell body dimensions, for comparison between groups.

2.6 Statistical analysis

The normality of the data was tested using the Shapiro-Wilk test. Data with a normal distribution were expressed as mean ± standard deviation. Data with a free distribution were expressed using median and the 25^{th} and 75^{th} percentiles (P25; P75). The data from the control and experimental groups were compared using Student's t test (data with normal distribution) or the Mann-Whitney test (data with free distribution). The significance level used for all tests was $p < 0.05$.

3 Results

The animals' body weights did not differ ($p > 0.05$) between groups C and E at the beginning or at the end of the experiment, nor did the jejunum area (Table 1).

Under the light microscope, in the whole mount preparation of the jejunum, the myenteric neurons were observed within ganglia interconnected by bundles of nerve fibers (Figure 1) without any difference in the distribution between groups C and E.

The neuronal density per mm² of the jejunum did not differ ($p > 0.05$) between groups C and E for neurons evidenced by the Giemsa technique or for NADPHd positive neurons (NADPHd+) (Table 2).

For neurons revealed by the Giemsa technique (general population), the cell body area ranged from 51.43 to 647.89 μm² in group C and from 54.89 to 511.76 μm² in group E. The neurons presented a smaller cell body area ($p < 0.05$) in group E when compared with group C (Table 2).

Neurons measuring less than 200 μm² were the most common in group E, whereas neurons measuring 200 μm² or more were the most common in group C (Table 2).

Among the NADPHd+ neurons, the cell body area ranged from 50.99 to 508.85 μm² in group C and from 51.15 to 627.52 μm² in group E. Neurons in group E had larger cell body dimensions ($p < 0.05$) than those in group C (Figure 2), with the frequency of neurons with dimensions above 200 μm² greater for group E than for group C (Figure 3).

4 Discussion

Despite a wide variety of research reporting on the effects of 2,4-D in the central nervous system, the mechanism for the neurotoxicity of 2,4-D has not yet been fully clarified (BORTOLOZZI, EVANGELISTA DE DUFFARD,

Table 1. Mean and standard deviation of initial and final body weight and of the area of the jejunum from animals in the control (C) and experimental (E) groups.

Groups	Initial weight (g)	Final weight (g)	Area of jejunum (mm²)
C (n = 5)	266.80 ± 9.2	299.20 ± 9.3	2907.6 ± 172.7
E (n = 5)	254.40 ± 28.3	292.00 ± 4.0	3081.0 ± 124.6

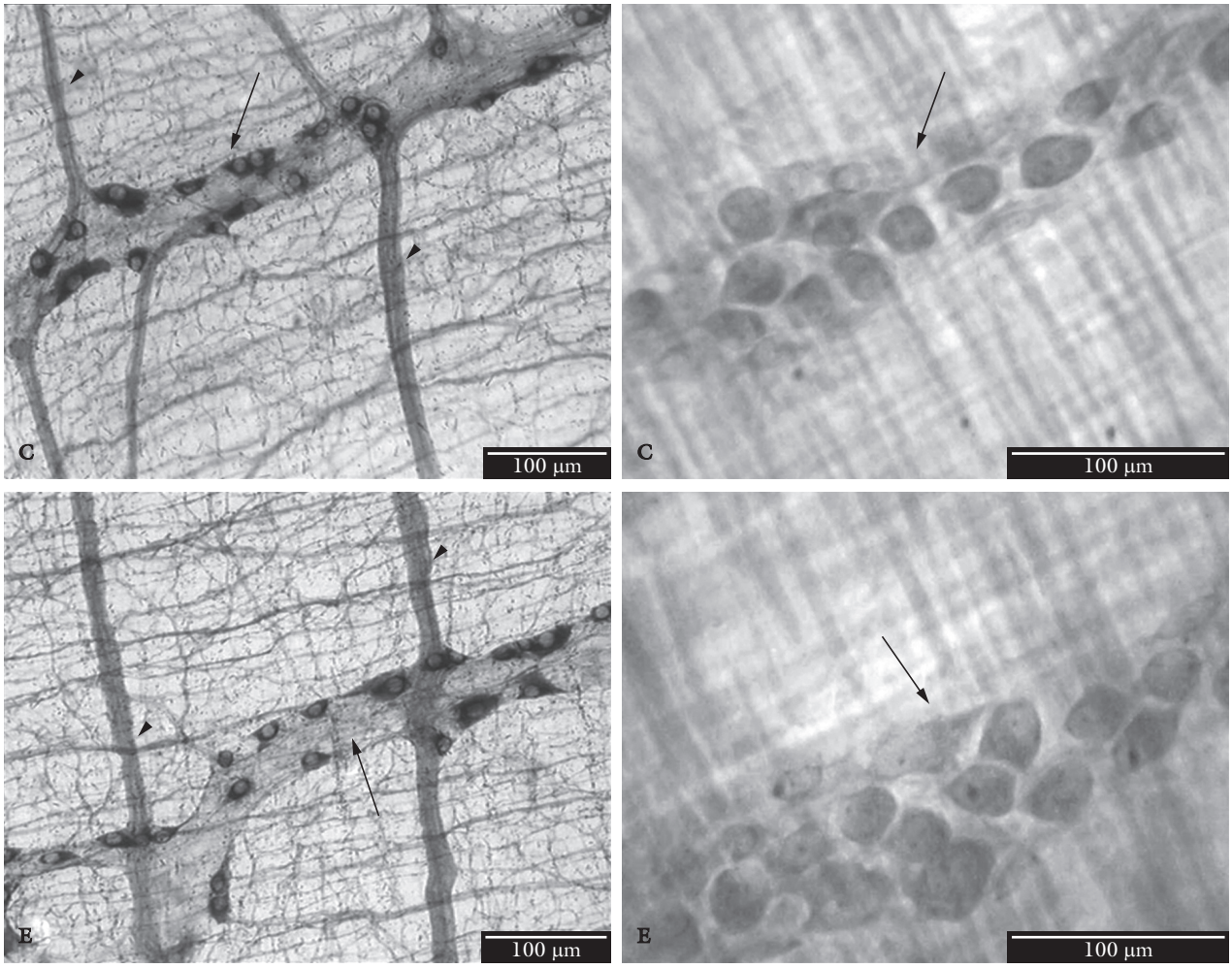

Figure 1. Photomicrograph of the jejunal whole mount preparation of rats from the control (C) and experimental (E) groups showing, on the left, ganglia (arrow) containing NADPH-d+ myenteric neurons interconnected by bundles of nerve fibers (arrowhead) and, on the right, a ganglion containing myenteric neurons stained with Giemsa (arrow). (bar = 100 μm).

Table 2. Mean and standard deviation of neuronal density (neurons/mm² of jejunum and neurons/11.52 mm² of jejunum) and median and the percentiles (P25; P75) of the cell body area of myenteric neurons from the jejunum of animals in the control (C) and experimental (E) groups.

Groups	Neuronal density (11.52 mm² of jejunum)	Neuronal density (mm² of jejunum)	Cell body area (μm²)
		NADPHd+ neurons	
C (n = 5)	589.70 ± 106.7	51.19 ± 9.26	173.9 (141.6; 217.9)*
E (n = 5)	627.84 ± 49.3	54.50 ± 4.27	183.2 (147.3; 230.7)*
		Giemsa neurons	
C (n = 5)	1511.42 ± 182.3	131.2 ± 15.83	224.8 (172.4; 278.7)*
E (n = 5)	1659.02 ± 48.4	160.4 ± 5.30	204.4 (151.3; 261.4)*

Mean values marked with asterisk (*) in the same column show significant difference between groups (p > 0.05).

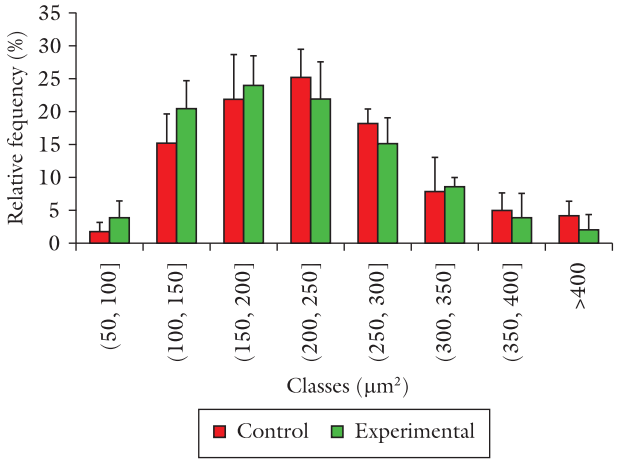

Figure 2. Histogram of the frequency distribution (%) of jejunal myenteric neurons revealed by Giemsa staining of samples from control and experimental animals as a function of the dimensions of the cell body area, grouped into class intervals of 50 μm².

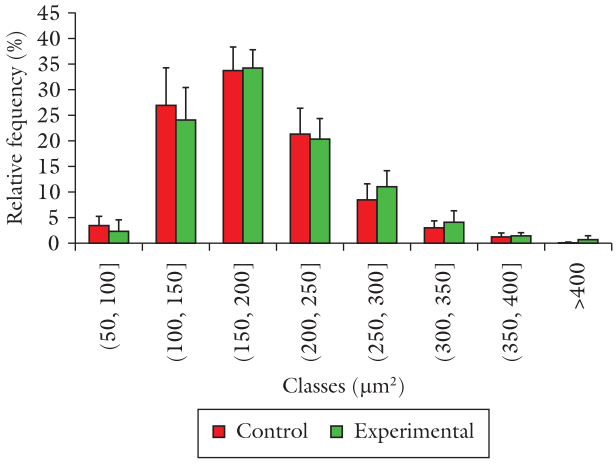

Figure 3. Histogram of the frequency distribution (%) of NADPH-diaphorase positive jejunal myenteric neurons from the control and experimental animals as a function of the dimensions of the cell body area, grouped into class intervals of 50 μm².

DUFFARD et al., 2004; BJORLING-POULSEN, ANDERSEN and GRANDJEAN, 2008; KONJUH, GARCIA, LÓPEZ et al 2008) and little is known about its effects on the myenteric plexus. Therefore, in this initial exploratory study, the possible effects of 2,4-D was evaluated in the general population of myenteric neurons and in the sub-population of NADPHd+ neurons, using quantitative and morphometric analyses of whole mount preparations of the jejunum.

Considering the lack of information on the effects of 2,4-D on the myenteric plexus, a daily dose of 5 mg 2,4-D.kg^{-1} body weight was selected for this study, which is five times higher than the NOAEL (1 mg.kg^{-1}/day) established for renal, hepatic and haematological effects (GARABRANT and PHILBERT, 2002).

The body weights of the animals that did (group E) or did not (group C) receive 2,4-D remained equivalent at the beginning and the end of the experiment, which lasted 15 days, with no significant differences (p > 0.05) between groups C and E. This indicates that at the dose used (5 mg.kg^{-1}/day), 2,4-D did not affect the animal's body weight (Table 1). Body weight changes were also not observed in rats with ingestion of 15 to 70 mg.kg^{-1}/day (STÜRTZ, BONGIOVANNI, RASSETTO et al., 2006; KONJUH, GARCIA, LÓPEZ et al., 2008). However, when the 2,4-D is administered at higher doses (150 and 300 mg.kg^{-1}/day) there are reports of weight loss in Wistar rats (CHARLES, BOND, JEFFRIES et al., 1996a; CHARLES, DALGARD, CUNNY et al., 1996b), indicating that the effects of 2,4-D on the weight of the animals are dose dependent.

To avoid quantitative and interpretive errors, Gabella (1989) emphasises the need to measure the size of the intestinal segment analysed, since an increase in the size of the intestinal tract can lead to greater dispersion of neurons and apparent decrease in neuronal density. 2,4-D did not alter the length nor the area of the jejunum, since there was no difference (p > 0.05) between groups C and E, thus allowing the direct comparison of results between groups without the need to use a correction factor, as recommended by Gabella (1989), Johnson, Schemann, Santer et al. (1998) and Phillips and Powley (2001).

In jejunal whole mount preparations, myenteric neurons were observed within ganglia interconnected by bundles of nerve fibres, with no difference between the animals in groups C and E, also indicating that the 2,4-D did not affect the spatial organisation of the myenteric plexus (Figure 1).

Among the neurons revealed by the Giemsa technique (general population of neurons) the neuronal density/mm² of the jejunum in group E (160.4 ± 5.30) did not differ (p > 0.05) from that in group C (131.2 ± 15.83). The same occurred with the density of NADPHd+ neurons, which remained balanced between the treated group E

(54.50 ± 4.27) and group C (51.19 ± 9.26) without a statistically significant difference ($p > 0.05$), hence confirming that the 2,4-D did not cause neuronal death in the myenteric plexus.

The total neuronal density in the general population and sub-populations of myenteric neurons has been evaluated under different conditions such as ageing (GAGLIARDO, CLEBIS, STABILLE et al., 2008; MARI, CLEBIS, GAGLIARDO et al, 2008), malnutrition and protein restriction (SANT'ANA, MOLINARI and MIRANDA-NETO, 2001; MOLINARI, FERNANDES, OLIVEIRA et al., 2002; SCHOFFEN, SOARES, FREITAS et al., 2005; ARAÚJO, SANT'ANA, MOLINARI et al., 2006), diabetes (ZANONI, BUTTOW, BAZOTTE et al., 2003; FREGONESI, MOLINARI, ALVES et al., 2005; SHOTTON, ADAMS and LINCOLN, 2007; SILVERIO, MARI, CLEBIS et al., 2008), infections (UGAUARA, SANT'ANA, ALMEIDA et al., 2008; SUGAUARA, SANT'ANA, SILVA et al., 2009), Parkinson's disease (ANDERSON, NOORIAN, TAYLOR et al., 2007), Crohn's disease (BOYER, SIDPRA, JEVON et al., 2007) and enteritis (LOMAX, LINDEN, MAWE et al., 2006). These analyses have shown retention, decrease or increase in the general population or sub-populations of neurons in different intestinal segments, depending on the type and severity of the condition imposed. These facts suggest that the length of the experiment (15 days) and the dosage intake of 2,4-D (5 $mg.kg^{-1}$/day) did not induce a high enough degree of severity in our experiment to change the neuronal density by cellular death.

The maintenance of the general population and NADPHd+ subpopulation, as was observed between groups E and C, is not, however, indicative of the absence of neuronal alterations. Many times, the effects of adverse situations are manifested by hypertrophy or atrophy of the neuronal cell body, as a compensatory or protective adaptive response resulting from neuronal plasticity in order to maintain the neuronal viability and functional homeostasis of the gastrointestinal tract.

The increased expression of neurotransmitters, followed by neuronal damage, indicates that specific neurotransmitters take part in a protective response aimed at the recovery of affected neurons (KRISTENSSON, THEMNER-PERSSON and EKBLAD, 2007). Thus, the neuronal cell body area was measured to verify the possible effects of 2,4-D on this parameter.

The cell body area of myenteric neurons varied and differed between groups C and E, indicating that 2,4-D had effects on the jejunal myenteric population. However, these effects are expressed differently in the general population of neurons (Giemsa stain) and in NADPHd+ neurons.

In the general population of neurons, there was a significant ~9 % decrease ($p > 0.05$) in cell body area in the animals receiving 2,4-D (Group E) when compared with those that did not (group C). However, NADPHd+ neurons showed a significant 5% increase ($p < 0.05$) in cell body area in group E in relation to group C.

Neuronal cell body area atrophy in the general population was confirmed when neurons were seen in class intervals of 50 μm^2 according to cell body dimensions. Neurons with dimensions less than 200 μm^2 were more frequent in group E compared with group C, in which neurons measuring 200 μm^2 or more were the most frequent.

Atrophy in the cell body area of the general population of neurons may have been triggered by the 2,4-D. Changes in the environment in response to offensive events, or functional changes, require adaptability of adult neurons to maintain their primary functions (EKBLAD and BAUER, 2004).

The interference in the energy metabolism of neurons impairing, among others, signalling processes, regulation of biosynthetic and catabolic reactions, and transport of metabolites and ions, among others, is suggested among the mechanisms of toxicity for the 2,4-D (PALMEIRA, MORENO, VASCO et al. 1997; BRADBERY, WATT, PROUDFOOT et al., 2000; DI PAOLO, EVANGELISTA DE DUFFARD and DUFFARD, 2001; DUCHNOWICZ and KOTER, 2003). This could also justify a decrease in cell body area of the myenteric neurons observed in group E since neuronal atrophy is a basic and adaptive cellular response of the neuron in response to situations of aggression (BOGLIOLO, 1981). The neuron decreases its metabolism in response to deleterious conditions that could lead to cell death, which is reflected in its decreased size and ensures its functional viability.

On the other hand, in group E, the increase in the cell body area of the NADPHd+ neurons was homogeneous for all classes of neurons. Aside from the class of neurons measuring less than 100 μm^2, the other classes had increased frequency of neurons when compared to group C. The hypertrophy of the cell body area has been attributed to oxidative stress (ZANONI, BUTTOW, BAZOTTE et al., 2003; FREGONESI, MOLINARI, ALVES et al., 2005; SHOTTON and LINCOLN, 2006; SHOTTON, ADAMS and LINCOLN, 2007) in both NADPHd+ and NOS immunoreactive neurons.

The neurotoxicity induced by 2,4-D may be partly due to the production of free radicals (BJORLING-POULSEN, ANDERSEN and GRANDJEAN, 2008). The cerebellum's incubation with 2,4-D decreased the levels of catalase activity, increased the generation of reactive oxygen species (ROS) and the activity of selenium-glutathione peroxidase (BONGIOVANNI, FERRI, KONJUH et al., 2007). Moreover, in the kidney and brain of some fish species exposed to 2,4-D, Ozcan-Oruc, Sevgiler and Uner (2004) reported increased activity of antioxidant enzymes. 2,4-D induces oxidation in red blood cells, protein oxidation and increased production of free radicals (BUKOWSKA, 2003; BUKOWSKA, MICHALOWICZ, KROKOSZ et al., 2007; BUKOWSKA, RYCHLIK, KROKOSZ et al., 2008).

In oxidative stress, there is increased use of NADPH by aldose reductase and glutathione reductase (CAMERON, COTTER and MAXFIELD, 1993). Thus, the availability of this cofactor for the metabolic activity of NOS is expected to be decreased, and NADPHd+ neurons could attempt to circumvent the reduction of NADPH by increasing metabolic pathways responsible for NO synthesis, causing the cell to increase its volume (ZANONI, BUTTOW, BAZOTTE et al., 2003; FREGONESI, MOLINARI, ALVES et al., 2005). Therefore, under the effect of 2,4-D, the jejunal NADPHd+ myenteric neurons would also have increased cell body area, showing hypertrophy of the cell body as a result of neuronal plasticity.

In nature, NO plays a dichotomous role, exerting both beneficial and detrimental effects on cells (DANIELS, CAVILL, MURRAY et al., 2005; WALLACE and MILLER, 2000). Thus, the increase in the size of NADPHd+ neurons with maintenance of neuronal density that is observed under 2,4-D could be a protective response due to oxidative stress, consistent with the protective function of NO. Cowen, Johnson, Soubeyre et al. (2000) suggest that neurons that utilise constitutive NOS (cNOS) should increase their defence mechanisms against free radicals.

A response induced by ingestion of 2,4-D on the mucosa of the jejunum should also be considered. It is known that the enteric reflex circuits may be activated by various stimuli, including the chemical composition of luminal contents, based on non-neuronal components that trigger inflammatory responses including the release of substances that, directly or indirectly, interact with the enteric neural network (LOMAX, LINDEN, MAWE et al., 2006).

Although not showing clear signs of inflammation in jejunal segments of the mucosa, near the end of the experiment we found visible changes in the faeces eliminated by the animals that received 2,4-D (group E). These changes relate to the texture of faeces which become more viscous compared to the faeces of the animals in group C, suggesting that 2,4-D is an irritant agent in the intestinal mucosa. Mild enteritis in rats was triggered by exposure of the intestinal mucosa to repeated low doses of xenobiotic food contaminants (ANTON, THEODOROU, ROY et al., 2002). Therefore, the intestinal mucosa exposed to 2,4-D should be further investigated to evaluate the presence of inflammatory responses.

The doses and treatment time used in research of chronic and sub-chronic acute neurotoxicity of 2,4-D (STEISS, BRAUND and CLARK, 1987; OLIVEIRA and PALERMO NETO, 1993; DUFFARD et al., 1996; BORTOLOZZI, DUFFARD and EVANGELISTA DE DUFFARD, 2002) are, in most cases, greater than those used in this study, which could explain the maintenance of myenteric neuronal density of group E compared to group C. However, since 2,4-D alters the cell body area of myenteric neurons, and the responses of myenteric neurons are not uniform over different intestinal segments, it would be interesting to investigate the behaviour of myenteric neurons and their sub-populations when other doses and treatment times are used, as well as to determine the response of the myenteric plexus in other bowel segments.

Thus, the results suggest that administration of 5 $mg.kg^{-1}$ of 2,4-D for 15 days, while it does not alter the spatial organisation and neuronal density of the myenteric plexus in the muscular wall of the jejunum, does lead to atrophy of the cellular body in the general population of neurons and hypertrophy of the cell body of NADPHd+ neurons that produce NO without promoting cell death.

4.1 Conflict of interest

All authors declare they do not have any conflict of interest.

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