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HIGH DOSES OF BISPHENOL-A PROMOTE A REDUCTION IN THE NUMBER OF MYENTERIC NEURONS IN RATS

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ABSTRACT
Bisphenol-A (BPA), found in plastics and food coatings, is known for its potential adverse health effects. While some studies have focused on BPA's endocrine effects, other research explores its impact on the myenteric plexus, which controls the motility of the digestive tract. The study investigated the effects of BPA on myenteric plexus neurons in rats. For the analysis of the effects on the myenteric plexus (MP), 32 male Wistar rats aged 40 days were divided into four groups (n=8) that received filtered water via gavage over 12 weeks for the control group (CG), dose group 1 (DG1) BPA solution at 0.05mg/kg, dose group 2 (DG2) BPA solution at 0.25mg/kg, and dose group 3 (DG3) BPA solution at 0.5mg/kg. The animals were weighed weekly to adjust the administered volume. At the end of the experimental period, a new weighing was performed, and the rats were euthanized. After laparotomy, the intestinal segments corresponding to the jejunum-ileum were collected to demonstrate the density of the population (Giemsa+), the subpopulations of active myenteric neurons (NADH-d+), and the nitrergic subpopulation (NADPH-d+). BPA exposure did not significantly affect the rats' weight gain, but, there was a reduction in the total density of myenteric neurons in DG3 (10.78mm²) compared to DG (18.88mm²) and DG1 (16.28mm²), and also a reduction in the NADH-d+ subpopulation between DG3 (8.50mm²) and the CG (14.45mm²) and DG1 (14.17mm²) groups. For the nitrergic subpopulation (NADPH-d+), there was no difference in the expression of these neurons. Based on the results obtained, it can be inferred that there was a reduction in cholinergic neurons (NADPH-d-), calculated from the total population of neurons. These findings suggest that BPA may compromise the neuronal function of the MP, possibly by inducing oxidative stress and apoptosis of subpopulations, acting as a xenoestrogen on the nuclear receptors of these neurons, and consequently affecting the regulation of gastrointestinal motility, interfering with cholinergic neuronal transmission and nitric oxide production, and also greater resistance of nitrergic neurons can be observed. Both studies highlight the importance of raising awareness about BPA, as well as the possible harmful effects on unique health. The need to understand the impacts of BPA on different biological systems is emphasized to better understand its effects on human health, especially considering its widespread presence in modern society.

Keywords: Bisphenol A. Enteric Nervous System (ENS). Chronic Exposure. Neurotoxicity. Auerbach's Plexus.

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INTRODUCTION

Bisphenol A (BPA) is a chemical compound widely used in the production of plastics and food coatings. However, its presence in these products has raised growing concern due to the leaching process of this compound in food, due to poor polymerization and exposure of these packages to high temperatures, and its potential health effects (Ma *et al.*, 2019).

Studies have focused primarily on the effects of BPA on the endocrine system, particularly the reproductive system, and evidence has suggested its role as a hormone disruptor, through estrogen agonist action (Costa *et al.*, 2014; Kabir; Rahman; Rahman, 2015; Osborne; Rudel; Schwarzman, 2015).

However, different studies have investigated the effects of BPA on other biological systems, including the central and peripheral nervous systems (Li *et al.*, 2023; Santoro, 2019). The enteric nervous system (ENS), also known as the "second brain", is composed of a complex network of neurons that control gastrointestinal function through clusters of nerve cells, ganglia, and nerve fibers, supplying the muscle of the intestinal wall (Furness; Costa, 1980). This intrinsic network is involved in the regulation of intestinal motility and circulation through the myenteric plexus and the submucosal plexus, responsible for the secretion of digestive enzymes, and absorption of nutrients (Furness, 2012; Furness; Costa, 1980) in addition to communicating with the central nervous system.

The presence of estrogen receptors on ENS neurons raises the possibility that BPA may affect enteric neural function due to its known estrogenic activity (Eusébio; Ramos, 2016). Li *et al.* (2023) describe that, in both *in vivo* and *in vitro* studies, BPA could modulate neural activity and influence the release of neurotransmitters and suggest that exposure to BPA is capable of compromising the homeostasis of ENS neurons.

Furthermore, previous studies have also indicated that BPA exposure has the potential to negatively affect synaptic development and plasticity in the central nervous system (Duclot; Kabbaj, 2017). Thus, it is plausible to infer that BPA may have similar effects on the ENS, compromising its normal function and resulting in gastrointestinal dysfunctions.

To provide a better understanding of the damage induced by BPA in the myenteric plexus and contribute to the current knowledge about the neurotoxic effects of this chemical compound, the present study aimed to investigate the potential effects of BPA on the myenteric plexus of male rats for 12 weeks via the oral route.

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METHODOLOGY

METHODOLOGY

bisphenol a formulation for gavage administration

The production of BPA in stock solution followed the methodology described by Facina et al. (2021) with adaptations, 149g of BPA (Bisphenol A, Sigma Aldrich, USA, catalog no. 239658) was used, diluting it in 0.250mL of ethanol. After this dilution, the solution was added to 500mL of filtered water under stirring and at a temperature of 69° C, remaining stored in an amber glass container for photoprotection and sealed with Parafilm®, kept at room temperature. The process was repeated weekly.

experimental animals

All procedures during the experiment were performed in accordance with the instructions and regulations of the Animal Use Ethics Committee (CEUA) of UNIPAR under protocol no. SPP2020031000113.

The present study involved 32 male Wistar rats, approximately 40 days old, which were evaluated and dewormed by a veterinarian prior to the start of the experiment. They were kept in controlled laboratory conditions at a temperature of 22±2°C, relative humidity of 50±10%, and a 12-hour light/dark cycle, with an air exhaust system. The animals were fed with standard feed appropriate for the species and age and had access to filtered water *ad libitum*.

After weighing the animals, they were divided into four groups, each group of eight animals. The first group, called control (group C), received filtered water (vehicle) by gavage. The other groups were designated as follows: dose group 1 (DG1) received the lowest dose, according to the Tolerable Daily Intake (TDI) of 0.05 mg/kg/day of BPA (Bisphenol A 50 g, Sigma Aldrich, USA, catalog no. 239658) (ANVISA, 2015; EFSA, 2010). Dose group 2 (DG2) was exposed to a dose five times higher than the TDI, totaling 0.25 mg/kg/day. At the same time, dose group 3 (DG3) was subjected to a dose ten times higher than the TDI, corresponding to 0.5mg/kg/day via gavage. All groups were treated once a day, daily, for 12 weeks. Weighing was performed weekly to adjust the dose per kilo for each animal along with monitoring the animals' weight gain.

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euthanasia and tissue collection

After the experimental period, the animals were euthanized by hypersaturation in an anesthetic chamber with 3% isoflurane, under the CONCEA Euthanasia Practice Guidelines, with Resolution no. 1000/2012 of the Federal Council of Veterinary Medicine.

Laparotomy was performed and segments corresponding to the jejunum-ileum of the animals were collected, which were processed to mark the total population of neurons, inhibitory subpopulation, and metabolically active subpopulation, using the techniques of basic staining (Giemsa) (Barbosa, 1978), histochemistry (NADPH-diaphorase) (SCHERER-SINGLER et al., 1983), and histochemistry (NADH-diaphorase) (Gabella, 1969), respectively.

basic giemsa staining - detection of the total population of myenteric neurons

The Giemsa technique, described by Barbosa (1978), was used to mark the total population of myenteric neurons (Giemsa+). Thirty-two animals had their jejunum-ileum removed, washed, and filled with 0.9% saline solution, before being immersed in fixative solution for 48 hours. The jejunum-ileum were tied at their ends with suture thread to maintain filling. For neuronal labeling, each previously microdissected membrane preparation was immersed in Giemsa staining solution containing methylene blue in Sorensen phosphate buffer (pH 6.9) for up to 12 hours at room temperature under continuous agitation.

nadph-diaphorase histochemistry for detection of nadph-diaphorase positive myenteric neurons (nadph-d+ neurons)

To highlight active NADPH-diaphorase-positive (NADPH-d+) neurons, the methodology of Scherer-Singler *et al*. (1983) was adopted. The jejunum-ileum fragments were washed with phosphate buffer solution (pH 7.4) and one of the ends was tied with suture thread, while its interior was filled with phosphate buffer solution (pH 7.4). The opposite end was also tied, and the segment was washed twice (10 minutes each) in Sodium Phosphate Buffer (PBS) and permeabilized in PBS containing 0.3% Triton X-100 diluted in sodium phosphate buffer (pH 7.3) for 10 minutes.

After permeabilization, the jejunum fragments were washed twice more (10 minutes each) in PBS and incubated for 90 minutes in a reaction medium containing 50 mg of Nitro Blue Tetrazolium (NBT), 100 mg of β-NADPH and 0.3% Triton X-100 in Tris-HCl buffer (0.1

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M, pH 7.6). After this period, the end ties were removed and the fragments were immersed in a 4% paraformaldehyde solution to stop the reaction, fixed, and stored.

evidence of nadh-diaphorase positive myenteric neurons (nadhd+ neurons) according to gabella (1969)

To highlight metabolically active neurons (NADHd+ neurons) the jejunum-ileum of thirty-two animals was used. The segments were washed and filled with a Krebs solution (pH 7.3), and their ends were tied with suture thread to maintain the filling.

Then, the segments corresponding to the jejunum-ileum underwent two washes of 10 minutes each, in Krebs solution, and were then immersed in Krebs solution containing 0.3% Triton X-100 for five minutes. Subsequently, two more washes of 10 minutes each were performed in Krebs solution. Then, each segment was incubated in a reaction medium composed of 25 mL of Nitro Blue Tetrazolium (NBT, with a concentration of 0.5 mg/mL) stock solution, 25 mL of sodium phosphate buffer (0.1 M; pH 7.3) and 0.05 g of β-NADH in 50 mL of distilled water, for 45 minutes. The reaction was stopped using a 10% formaldehyde solution in phosphate buffer (0.1 M, pH 7.3), which also served for tissue fixation and storage.

obtaining membrane preparations and photomicroscopy

To obtain the membrane and photomicroscopy preparations, the intestinal segments corresponding to the jejunum-ileum of 32 rats were sectioned transversely into a fragment approximately 8 mm long and subsequently opened along the longitudinal axis at the level of the mesentery insertion. These fragments were microdissected on a glass plate, with the aid of tweezers and a stereomicroscope with transillumination, to remove the mucous and submucosal tunics, preserving the muscular tunic, where the myenteric plexus is located.

After microdissection, they were dehydrated in an increasing series of alcohols (90%, 95%, and absolute alcohol), diaphanized with three consecutive immersions in xylene, and placed between a slide and a glass coverslip with synthetic resin.

Quantification of myenteric neurons (neuronal density/mm² of the jejunum-ileum)

The slides were viewed by an image analysis system, the “Motic Images Plus” software, using a Nikon microscope (Eclipse E200), coupled to a high-resolution camera (Moticam 5, 5.0 megapixels) (Carson; Hladik, 1997).

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Under microscopy at 400x magnification, images of 120 random microscopic fields were captured per membrane preparation. The jejunum-ileum fragment's mesenteric, intermediate, and antimesenteric areas were included.

The region of the intestine circumference corresponding to the insertion of the mesentery (0º) served as a reference to delimit the mesenteric (0° to 60° and between 300° and 360°), intermediate (between 60° and 120°, and between 240° and 300°) and antimesenteric (between 120° and 240°) regions of the intestine (Miranda-Neto; Molinari; Natali, 2001) and guided the capture of images, which was conducted to sample the three areas in an equivalent number of images.

This capture was performed to quantitatively analyze myenteric neurons stained by the Giemsa basic staining technique and NADH-d+ and NADPH-d+ histochemistry. The difference between the mean density of Giemsa-stained neurons and the density of NADPH-d+ neurons was used to estimate the density of NADPH-d- neurons, the cholinergic neurons (Hermes-Uliana *et al.,* 2011; Porto *et al.,* 2012; Phillips*;* Kieffer; Powley, 2003).

statistical analysis

Statistical analysis was conducted using Bioestat software. The normality of the data was verified using the Liliefors test. After confirming normality and homogeneity of variance using the Levene test, the morphometric analyses and quantification of myenteric neurons were subjected to analysis of variance (ANOVA). In cases of significance, Tukey's post-test was applied. In all tests, a significance level of 5% was adopted.

RESULTS AND DISCUSSION

Over the twelve weeks of the experiment, weight gain was observed in all animals, with no significant difference in weight gain between the experimental groups compared to the control group (Table 1).

Table 1 - Means and standard errors of the means of initial and final weight and weight gain of the control group and groups exposed to different doses of Bisphenol A.

Group	Initial weight (g)	Final weight (g)	Weight gain (g)
CG	196.38±14.30	378.56±8.69	182.18±13.30
DG1	191.25±16.60	318.38±12.60	170.00±8.20
DG2	184.63±23.80	336.60±5.50	151.97±19.70

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DG3	196.75±13.00	375.44±9.96	178.69±13.96

CG (received filtered water (vehicle) by gavage), DG1 (dose group 1, received 0.05mg/kg/day of BPA via gavage), DG2 (received 0.25mg/kg/day of BPA via gavage) and DG3 (received 0.5mg/kg/day of BPA via gavage). n=8. Not significant by ANOVA.

As described by Rubin and Soto (2009), early exposure to BPA can impact several mechanisms fundamental to the regulation of body weight, including the formation of adipose cells, glucose absorption, and homeostasis, in addition to the development and maturation of pathways and circuits crucial to energy balance.

Other authors observed changes in body weight in rats exposed to BPA at 50 µg (Angle *et al*., 2013) and 90 days of age, and at 10 µg at 120 days of age (Wei *et al*., 2014), also Angle *et al*. (2013), suggesting that exposure to BPA may be associated with an increase in body weight gain, due to effects on the endocrine system and adipocyte hyperplasia, affecting metabolism and potentially contributing to weight gain.

However, the results are variable and not conclusive in all studies, as described by Rubin and Soto (2009), who report that the specific effects on the pathways and mechanisms due to exposure to BPA may vary according to the dose, precise moment of exposure and other factors involved, which corroborates the study developed by Somm *et al*. (2009), who found no statistical difference in the body weight of rats exposed to BPA in the perinatal period, which encompasses the end of gestation, delivery and the first weeks of life after birth, and the control group, which was not exposed to this substance.

The arrangement and location of the myenteric plexus (Figure 1), present in all groups, coincides with the literature, where the plexus is formed by ganglia connected by nerve fibers forming a structure similar to a ganglionic mesh or network (Irwin, 1931; Serenini *et al*., 2020; Silverio *et al*., 2009), in vertebrates. Notably, there were no changes resulting from BPA exposure.

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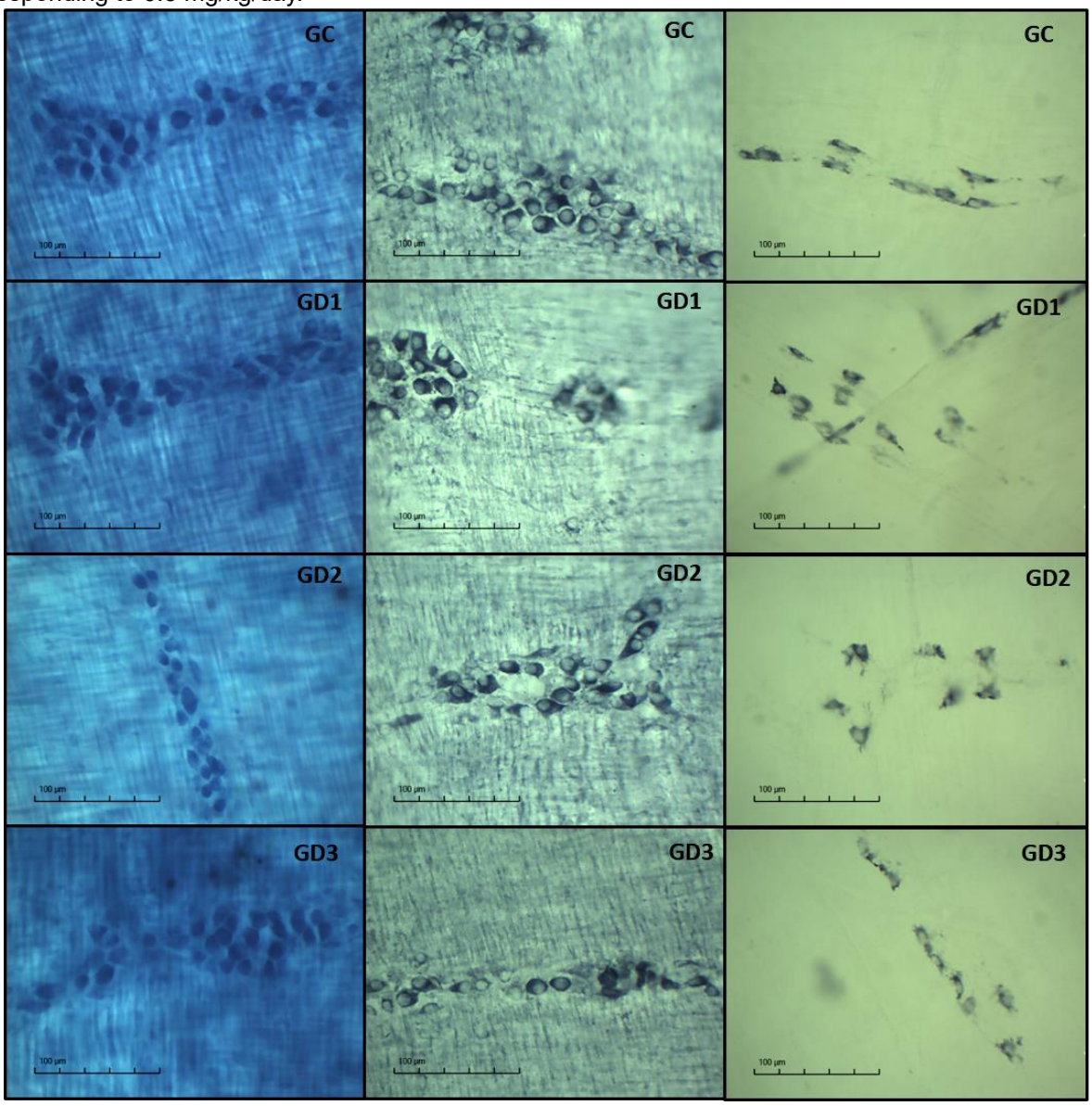
Figure 1 – Photomicroscopy showing ganglia of the myenteric plexus of the jejunum-ileum of rats from different groups stained by the Giemsa method, histochemistry of NADH-diaphorase and NADPH-diaphorase. (Scale bar 100µm. 40x objective). (CG) animals not exposed to Bisphenol A, (DG1) animals exposed to a dose corresponding to the IDT of 0.05 mg/kg/day, (DG2) animals exposed to a dose five times higher than the IDT corresponding to 0.25 mg/kg/day and (DG3) animals exposed to a dose ten times higher than the IDT corresponding to 0.5 mg/kg/day.



Source: The authors.

In the population of total neurons, stained by the Giemsa technique, a significant reduction was observed in neurons of the DG3 group to the CG and DG1 groups (Table 2), showing that exposure to high levels of BPA can be harmful to intestinal homeostasis and the reduction may be related to the neurotoxic profile that this compound can present, as described by Sarkar *et al*. (2013) who observed an increase in oxidative stress in neurons exposed to high doses of BPA (50mg/kg/day).

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Table 2 - Means and standard errors of the means of the number of Giemsa-positive neurons, NADPH-diaphorase positive, NADH-diaphorase positive and NADPH-diaphorase negative were present in mm² of jejunum membrane preparations from rats in the control group and groups of rats exposed to different doses of Bisphenol A (BPA).

Groups	GIEMSA	NADPH-d+/mm²	NADH-d+	NADPH-d⁻/mm²
	Total population	Nitrergic subpopulation	Metabolically active population	Estimated cholinergic subpopulation
CG	18.88±1.24[a]	7.10±1.77	14.45±0.24[a]	11.78
DG1	16.28±1.37[a]	5.42±1.13	14.17±0.41[a]	10.86
DG2	12.30±1.17[ab]	6.56±1.07	11.67±0.45[ab]	5.74
DG3	10.78±1.55[b]	6.42±0.30	8.50±0.57[b]	4.36

Averages followed by different letters in the column direction differ. CG (received filtered water (vehicle) by gavage), DG1 (dose group 1, received 0.05mg/kg/day of BPA via gavage), DG2 (received 0.25mg/kg/day of BPA via gavage) and DG3 (received 0.5mg/kg/day of BPA via gavage). n=8, statistical analysis by ANOVA test and Tukey post-test. P≤ 0,05. statistical analysis by ANOVA test and Tukey post-test.

Li *et al*. (2023), report that direct exposure to BPA in the CNS can cause disturbances in the energy metabolism of neuronal cells, inducing oxidative stress and impacting the mitochondrial oxidative respiratory chain, which can potentially inhibit the clearance of peroxides and reactive oxygen species (ROS), leading to abnormal morphological and functional changes and apoptosis of neuronal cells. Similar to what occurs in the CNS, after exposure to BPA, Sarkar *et al*. (2014) and Mandal, Sharma, and Dixit (2023), report that exposure to BPA in the ENS also increased oxidative stress, leading to neuron death, corroborating the reduction in the number of myenteric neurons in the jejunum-ileum of rats, found in the present study.

Neuronal labeling by NADPH-diaphorase histochemistry showed that the density of myenteric neurons, of the nitrergic subpopulation (inhibitory), did not present significant differences between the groups (Table 2), which can be attributed to the resistance of nitrergic neurons to oxidative stress, as already reported by Belai *et al*. (1995), Favetta *et al*. (2020), and Furlan *et al*. (2004). This result can be attributed to the protective effect that nitric oxide (NO) confers on these inhibitory neurons of the myenteric plexus, since neurons that constantly use NO develop an improvement in the defense mechanism against free radicals generated by oxidative stress, resulting, for example, from the effects of BPA (Cowen *et al.,* 2000; Mandal, Sharma, Dixit, 2023; Sarkar, *et al.,* 2014).

BPA can act on estrogen hormone receptors that are expressed by myenteric neurons; this interaction demonstrated an increase in the expression of the enzyme nitric

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oxide synthase (NOS) in the muscle layer, suggesting that BPA facilitates the production of nitric oxide (NO) in myenteric neurons, which in turn decreases intestinal contractility (Mandal; Sharma; Dixit, 2023).

In the study conducted by Sarkar *et al*. (2013) using the duodenum of rats orally exposed to BPA (50mg/kg/day) for 30 days, a significant reduction in the amplitude and frequency of duodenal contractions was observed. These results suggest that BPA may suppress duodenal contractions by stimulating NO synthesis in non-adrenergic non-cholinergic neurons and smooth muscle cells, thereby triggering a NO-mediated signaling cascade (Mandal, Sharma, Dixit, 2023; Sarkar *et al*., 2014; Szymanska; Makowska; Goncowski, 2018).

In the same study, the authors observed reduced antioxidant activity, indicating an increase in oxidative stress, where, as in the present study, the nitrergic neurons evidenced by the NADPH-diaphorase technique, no quantitative changes were observed, due to their ability to protect against free radicals (Mandal; Sharma; Dixit, 2023), however, there are no reports of such resistance by cholinergic neurons, the main excitatory neurons of the myenteric plexus, being considered more vulnerable to neuronal death (Cowen *et al*., 2020).

There was a change in the percentage between NADPH-d+ neurons and NADPH-d- neurons, where an increase to 59.55% was evidenced in the nitrergic subpopulation (NADPH-d+) and a decrease to 40.44% in the cholinergic population (NADPH-d-) in DG3, which was estimated based on the difference between the mean density of neurons stained with Giemsa and the density of NADPH-d+ neurons, as shown in Table 3.

Table 3 - Percentages (%) of NADPH-d+ and NADPH-d- myenteric neurons concerning total neurons stained by the Giemsa technique in the jejunum-ileum of groups CG, DG1, DG2, and DG3.

Group	NADPH-d+	NADPH-d-
CG	37.61%	62.39%
DG1	33.29%	66.71%
DG2	53.33%	46.66%
DG3	59.55%	40.44%

CG (received filtered water (vehicle) by gavage), DG1 (dose group 1, received 0.05mg/kg/day of BPA via gavage), DG2 (received 0.25mg/kg/day of BPA via gavage) and DG3 (received 0.5mg/kg/day of BPA via gavage).

For Phillips, Kieffer, and Powley (2003), approximately 98% of myenteric neurons are constituted by the combination of nitrergic and cholinergic populations, and the nitrergic population may represent around 34% of the total myenteric neurons, while cholinergic

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neurons make up the remainder (Wester; O'briain; Puri, 1999). Any disparity in this proportion, where one of the subpopulations prevails over the other, can cause changes in the functioning of the GIT.

Although no functional changes were observed in the digestive system over the 12 experimental weeks, such as diarrhea, intestinal stasis, or constipation, there was a quantitative increase in the nitrergic population, which suggests that these neurons are more tolerant to cell death after exposure to BPA and that cholinergic neurons do not present such resistance. Since NADPH-d- neurons are responsible for the contraction of intestinal muscle fibers, it can trigger a reduction in intestinal function in the long term (Sarkar *et al*., 2014).

In the myenteric plexus, neurotransmitters such as ACh and NO play a role in regulating several intestinal functions, including gastrointestinal motility, the secretory activity of the gastrointestinal tract, intestinal blood flow, and through their interconnections with the submucosal plexus, nutrient absorption, and other functions. The changes in subpopulations observed under the influence of BPA reinforce the adaptive capacity of the ENS in the face of pathological processes and exposure to toxins (Szymanska; Makowska; Goncowski, 2018).

BPA is a known xenoestrogen that can mimic the effects of the hormone estrogen due to its similar chemical structure (Mandal, Sharma, Dixit, 2023; Sarkar *et al*., 2014). Female sex hormones influence gastrointestinal motility through specific estrogen receptors, including nuclear, membrane, and G protein-coupled receptors (GPERs), in both rats (Pines *et al*., 1998) and humans (Hogan *et al*., 2009), inhibiting intestinal muscle contractions via estrogen receptor activation and involving nitric oxide (NO) production and inhibition of cholinergic neuronal activity. Bisphenol A (BPA), a potent estrogen agonist, can interact with GPER and receptors on myenteric neurons, suggesting a complex alteration in GI motility by affecting different neural pathways and neurotransmitters (Zielińska et al., 2017).

These observations corroborate the data found in the present study regarding the NADH-diaphorase technique, which quantifies metabolically active neurons (Miranda-Neto *et al*., 2001), where a significant reduction in neuronal quantification could be observed when comparing CG and DG1 with DG3, which may infer that, as they are less resistant to the effects of oxidative stress, cholinergic neurons may have undergone apoptosis (Table 2).

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More frequently, the modifications mentioned above derive from adaptive and/or neuroprotective processes, and these responses aim to preserve intestinal homeostasis in the face of altered conditions caused by different stimuli. These adaptations include variations in neuronal quantification and activity observed under the influence of BPA, strengthening the capacity to maintain homeostasis of the myenteric plexus in the face of disruptive stimuli.

Brazil has not yet established a specific IDT for Bisphenol A (BPA) through its regulations, however, BPA is subject to regulation by international health bodies. ANVISA (2015) follows the guidelines and directives proposed by entities such as the European Food Safety Authority (EFSA) and the United States Environmental Protection Agency (USEPA) that establish parameters for the IDT of BPA. EFSA, for example, has set a preliminary TDI of four micrograms per kilogram of body weight per day (µg/kg/day), while USEPA has set a TDI of 50 µg/kg/day (EFSA, 2010).

CONCLUSION

In this experimental model, it was observed that over 12 weeks of oral exposure, through daily gavage containing different concentrations of Bisphenol A (BPA), the dose reported by ANVISA of 0.05mg/kg (DG1) proved to be safe. However, in the group that received the dose of 0.5 mg/kg of BPA (DG3), there was a significant decrease in the neurons of the myenteric plexus, observed by the reduction in the total population of neurons (Giemsa+), when compared to the CG and DG1 groups, the same being found in the population of metabolically active neurons (NADHd+). However, no significant changes were observed in the amount of the nitrergic subpopulation (NADPH-d+) between the groups, suggesting a greater resistance of these neurons to the toxic effects of BPA.

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