Chronic consumption of contaminated feed with 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide in rodents: effects on male reproductive health

Consumo crônico de ração contaminada com herbicida ácido 2,4-diclorofenoxiacético (2,4-D) em roedores: efeitos na saúde reprodutiva masculina

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RESUMO

Introdução: O herbicida 2,4- ácido diclorofenoxiacético (2,4-D) é um dos agrotóxicos mais utilizados no mundo. Há evidências de que este herbicida pode induzir efeitos deletérios em organismos não-alvo, incluindo prejuízo na função reprodutiva. **Objetivo:** O objetivo deste estudo foi avaliar os efeitos reprodutivos do consumo crônico de ração contaminada com 2,4-D em ratos, utilizando simulação de pulverização ambiental de alimentos. **Métodos**: Animais expostos oralmente receberam ração nebulizada com solução de 2,4-D em diferentes concentrações por 180 dias: 0 (controle - GC), 20,69 (LCG), 34,63 (MCG) ou 51,66 ppm dia^{-1} (HCG). **Resultados:** A qualidade espermática foi prejudicada pelo 2,4-D. A porcentagem de espermatozoides com movimento progressivo, número de espermatozoides no testículo e produção diária de espermatozoides foram menores em todos os grupos expostos ao herbicida, quando comparados ao GC. A contagem de espermatozoides na cabeça/corpo e cauda do epidídimo foi reduzida em MCG e HCG, e o tempo de trânsito espermático atrasou no epidídimo em LCG. Houve impacto negativo na morfologia espermática e na integridade da membrana plasmática em MCG e HCG, respectivamente. Esfoliação de células germinativas no lúmen dos túbulos seminíferos e vacuolização epitelial no epidídimo foram encontradas em HCG. **Conclusão:** Este é o primeiro estudo a descrever o impacto negativo na morfofisiologia reprodutiva masculina após exposição crônica ao 2,4-D, utilizando nebulização de alimentos em concentrações ambientalmente relevantes, com base no uso agronômico do herbicida. As lesões reprodutivas identificadas levantam preocupações sobre os impactos da ampla exposição da população ao 2,4-D.

Palavras-chave: Exposição a pesticidas, Espermatozoides, Reprodução, Exposição ambiental, Ratos.

ABSTRACT

Introduction: The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most widely used pesticides in the world. There is evidence that this herbicide can induce deleterious effects in non-target organisms, including impairment of reproduction function. **Objective:** The aim of this study was to evaluate the reproductive effects of the chronic consumption of contaminated feed with 2,4-D in rats using food environmental spraying simulation. **Methods:** Animals orally exposed received nebulized chow with 2,4-D solution in different concentrations for 180 days: 0 (control - CG), 20.69 (LCG), 34.63 (MCG), or 51.66 ppm day−1 (HCG). **Results:** Sperm quality was impaired to 2,4-D. The percentage of sperm with progressive movement, number of sperm in the testis and daily sperm production were decreased in all exposed groups to the herbicide compared to CG. Sperm counts in the caput/corpus and cauda epididymis were reduced in MCG and HCG, and sperm transit time was delayed in the epididymis of LCG. There was a negative impact on sperm morphology and plasma membrane integrity in MCG and HCG, respectively. Germ cell exfoliation within the lumen of the seminiferous tubules and epithelial vacuolization in epididymis were found in the HCG. **Conclusion:** This is the first study to describe the negative impact on male reproductive morphophysiology after chronic exposure to 2,4-D using food nebulization in environmentally relevant concentrations, based on agronomic use of the herbicide. The reproductive injuries identified raise concerns about the impacts of wide population exposure to 2,4-D.

Keywords: Pesticide exposure, Spermatozoa, Reproduction, Environmental exposure, Rats.

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INTRODUCTION

The projection of a world population of nine billion by 2050[1] associated with global food waste[2] indicates the perspective of an increase in the demand for agriculture production. Food production keeps growing in several countries to the need for improved nutrition, even when ceasing population growth at the global level[1]. Faced with this scenario, it is difficult to decrease the use of pesticides and maintain production and crop yields[3].

2,4-Dichlorophenoxyacetic acid (2,4-D) is one of the most widely used pesticides in the world. It is a pre- and post-emergent systemic herbicide that controls broadleaf weeds in crops of sugarcane, wheat, soybean, maize, rice, fruit, and vegetable, lawns, forests and aquatic environments[4]. The higher level of exposure to 2,4-D occurs due to the occupational contact by manufactory and application in forestry and agriculture. However, the general population can also be affected due to the consumption of contaminated water and food or contact with soil or air containing herbicide residues[5].

This herbicide can cause negative effects on non-target organisms[6]. Studies of chronic consumption of contaminated feed with 2,4-D in rats, in the same exposure protocol used in the present study, showed cardiotoxicity[7], deleterious effects on the bone quality[8] and stimulus of proliferation and inflammatory response in oral mucosa[9]. In addition, there is evidence of a correlation between 2,4-D exposure and cancer, neurologic disease, immunotoxicity and reproductive disorder[10].

The 2,4-D exposure may change the metabolism of Sertoli cells via altering glucose metabolism and impairing spermatogenesis[11]. Moreover, the herbicide can act as a possible endocrine disruptor. The 2,4-D decreases cholesterol levels in Leydig cells with consequential reduction in testicular testosterone and degeneration of germ cells and Sertoli cells[12]. Although some mechanisms of reproductive toxicity are known, most studies focus on acute or subacute exposure and doses that do not mimic real environmental exposure.

This study evaluated the reproductive effects of chronic consumption of contaminated feed with 2,4-D in male rats. The exposure method (food nebulization) and different concentrations (adapted from agronomic use) chosen simulate environmental spraying and they are the great highlight of this study.

MATERIAL AND METHODS

Chemicals

For exposure of animals, it was used the commercial formulation of (2,4-dichlorophenoxy) acetic acid (2,4-D; Nortox S.A., Arapongas, Paraná, Brazil, registered in the Ministry of Agriculture, Livestock and Food Supply # 03009). The formulation constituted of 806g/L (80.6% m/v) of dimethylamine salt of (2,4-dichlorophenoxy) acetic acid (2,4-D); 670g/L (67.0% m/v) of the acid equivalent of 2,4-D and 424g/L (42.4% m/v) of inert ingredients. The 2,4-D formulation was diluted in 10 mL of 0.9% saline solution (sodium chloride solution – NaCl) in concentrations preconized to each experimental group.

Animals

Adult male Wistar rats (75 days, n = 40), supplied by the Central Animal Facility of the University of Western São Paulo (UNOESTE), Presidente Prudente, SP, Brazil. During the experiment, animals were allocated into polypropylene cages (43 cm×30 cm×15 cm) with laboratory-grade pine shavings as bedding. Rats were maintained under controlled temperature (22 ± 2 ºC) and lighting conditions (12L, 12D photoperiod). Rat chow (Supralab®, Alisul, Brazil) and filtered tap water were provided *ad libitum* to the animals. The experimental protocol was approved by the Ethics Committee for Use of Animals of the UNOESTE (Protocol # 4868-CEUA). It also complies with the principles of laboratory animal care formulated by the Brazilian College of Animal Experimentation (COBEA).

Experimental design

Rats were randomly assigned to four experimental groups (n = 10 animals per group) (Figure 1):

- Control group (CG): rats daily consumed feed previously nebulized with 0.9% saline solution (vehicle);
- Low concentration group (LCG): rats daily consumed feed previously nebulized with 2,4-D at concentration of 3.71×10^{-3} g of active ingredient per hectare (correspondent to 20.69 ppm).

- Medium concentration group (MCG): rats daily consumed feed previously nebulized with 2,4-D at a concentration of 6.19×10^{-3} g of active ingredient per hectare (correspondent to 34.63 ppm).
- High concentration group (HCG): rats daily consumed feed previously nebulized with 2,4-D at a concentration 9.28×10^{-3} g of active ingredient per hectare (correspondent to 51.66 ppm).

The different concentrations of the 2,4-D herbicide used considered environmentally relevant concentrations, according to the product application and its agronomic prescription. The 2,4-D concentrations used in agriculture (in grams of active ingredient per hectare - g.i.a) were adjusted to exposure box dimensions, according to Parizi et al.[9].

Exposure protocol to 2,4-D herbicide

Two plastic boxes (32 x 24 x 32 cm) connected to an ultrasonic nebulizer (Pulmosonic Star®, Brazil) were used[13] (Figure 1). The feed was exposed in the boxes connected to the nebulizer for 15 minutes [time required for the entire solution (10 mL) to be nebulized] to 2,4-D solution in the concentration of each experimental group. The exposure occurred one day before being offered to animals and the feed was changed every two days. The animals were exposed for a period of 180 days.

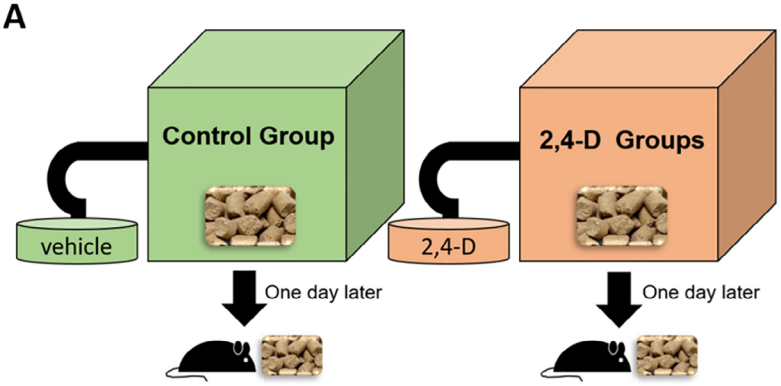
Tissue and organs collection

At the end of the exposure period, rats from each experimental group were anesthetized and euthanized by administering 100mg^{-1}kg of sodium thiopental (ip., Syntec, EUA). The right testis, epididymis and vas deferens, ventral prostate, seminal vesicle (without the coagulating gland and full of secretion), were removed and their weights were determined.

Sperm motility and morphology and plasma membrane integrity

Immediately after euthanasia, the left vas deferens was collected to obtain spermatozoa in 1.0mL phosphate-buffered saline at 34 ºC. Warmed Neubauer counting chamber was loaded with a small

A



B

Experimental Group	Group Acronym	Concentration of 2,4-D herbicide		
		g a.i. ha^{-1}	mg m^{-3}	ppm
Control group	CG	0	0	0
Low concentration group	LCG	3.71 x 10^{-3}	187.17	20.69
Medium concentration group	MCG	6.19 x 10^{-3}	313.31	34.63
High concentration group	HCG	9.28 x 10^{-3}	467.93	51.66

Figure 1. Exposure protocol to 2,4-D. A) Oral exposure protocol according to Mello et al. (2018). B) Experimental groups and concentration of 2,4-D exposure in different units. Exposure to only vehicle = solution of sodium chloride 0.9% (control group). Exposure to different concentrations of 2,4-D (LCG - low concentration group, MCG - medium concentration group and HCG - high concentration group).

aliquot of sperm solution. Sperm motility evaluation was assessed by visual estimation (200 spermatozoa per animal, in duplicate) under a microscope (Leica DMLS) at 200X magnification. Spermatozoa were classified as immotile, motile without progression and motile with progression, according to Perobelli et al.[14].

The right vas deferens was collected to obtain spermatozoa fixed in 1.0mL of saline formol. Sperm morphology analysis was performed with a microscope (400× magnification)[15]. Sperm were classified according to Filler[16].

Sperm viability (plasma membrane integrity) was assessed using an eosin-nigrosin staining test[17]. Two hundred spermatozoa were counted under a light microscope (1000× magnification), and classified in unstained (live sperm) and stained red (dead sperm).

Daily sperm production per testis, sperm number and transit time in the epididymis

Right testes were decapsulated and caput/corpus cauda segments of the right epididymis were separated. The tissues were frozen until sperm counts. Spermatids in stage 19 of spermiogenesis and spermatozoa in the caput/corpus and cauda epididymis were counted as described previously by Robb et al.[18], with adaptations of Fernandes et al.[19]. The number of spermatids at stage 19 was divided by 6.1 (which is the number of days of the seminiferous cycle when these spermatids are present in the seminiferous epithelium) to calculate daily sperm production (DSP). Sperm transit time through the epididymis segments was determined by dividing the number of sperm in each segment by the DSP[18].

Testis and epididymis histology

The left testis and epididymis were collected and fixed in buffered formalin (10%) for 24 hours. After this period, the organ was sectioned and returned to the buffered formalin for additional 24 hours. The pieces were embedded in paraffin wax and sectioned at 5µm. Tissue sections used for histological evaluation were stained with hematoxylin and eosin (HE), examined and photographed by light microscopy.

The organization of the cytoarchitecture of the interstitial tissue and seminiferous epithelium was examined and the occurrence of alterations such as the presence of intraepithelial vacuolization, acidophilic cells with a pyknotic nucleus, multinucleate germ cells, germ cell exfoliation, germ cells loss, atrophy tubular and spermatogenesis arrest. In the epididymis, it was investigated the presence of a cribriform alteration in the epithelium, epithelial vacuolization, inflammatory infiltrates in the interstitial tissue, quantity and aspect of the sperm in the lumen[20]. Histological changes were counted in 100 random sections of seminiferous tubules or epididymal ducts in each animal.

Statistical analysis

For comparison of parameters, ANOVA with *a posteriori* Tukey test or nonparametric Kruskal-Wallis test with *a posteriori* Dunn test were performed. A Kolmogorov-Smirnov test was applied to test for normal distributions before the statistical analyses. Differences were considered significant when $p < 0.05$.

RESULTS

The weights of the testis, epididymis, vas deferens, ventral prostate and seminal vesicle there presented no significant difference among experimental groups (Table 1).

The percentage of sperm with the progressive movement was reduced ($p < 0.05$) in the groups of the three concentrations of the herbicide in relation to the CG (Figure 2A). In addition, the percentage of immotile sperm was increased ($p < 0.05$) in HCG compared to CG (Figure 2C). However, the percentage of sperm without movement progression was similar among the experimental groups ($p > 0.05$) (Figure 2B).

There was a significant reduction ($p < 0.05$) in the percentage of morphologically normal spermatozoa, with a consequential increase of abnormalities of the flagellum in MCG, compared to CG (Table 2). Moreover, morphological abnormalities of the sperm head were increased in MCG compared to CG. Plasma membrane integrity was decreased in HCG compared to CG (Table 2).

The absolute and relative numbers of sperm in the testis and absolute and relative DSP were reduced ($p < 0.05$) in all exposed groups to the herbicide compared to the CG. (Table 3).

Table 1
Organ weights in rats from control and exposed to 2,4-D groups.

Parameter	CG	LCG	MCG	HCG
Testis (g)	1.67±0.09	1.65±0.12	1.60±0.06	1.63±0.09
Epididymis (mg)	702.70±26.99	676.70±60.57	699.10±106.83	730.66±93.72
Vas deferens (mg)	145.10±11.94	157.70±17.48	158.90±23.33	159.37±20.41
Ventral prostate (mg)	647.90±107.63	622.70±169.07	561.80±172.62	578.00±149.15
Full seminal vesicle (g)	1.43±0.26	1.24±0.36	1.12±0.33	1.37±0.24
Empty seminal vesicle (mg)	692.70±109.35	655.10±172.40	611.30±104.33	767.00±122.02

Values expressed as mean ± SD. ANOVA with a posteriori Tukey test ($p > 0.05$). CG = Control group. LCG = Low concentration group. MCG = Medium concentration group. HCG = High concentration group.

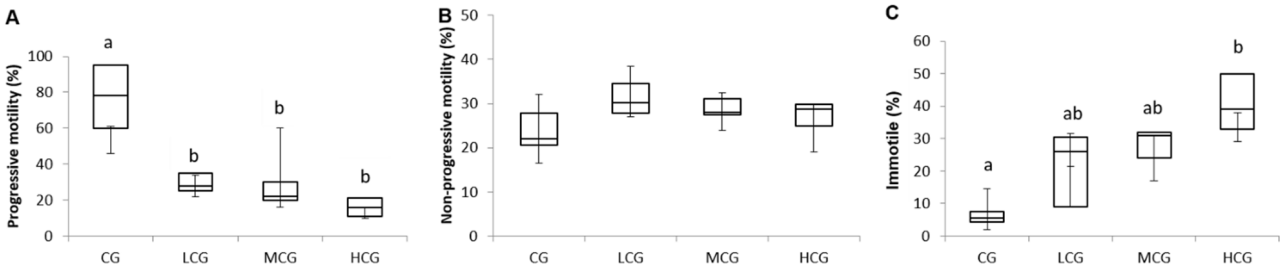


Figure 2. Sperm motility in rats from control and exposed to 2,4-D groups. A) motile with progressive movement. B) motile without progression. C) immotile.
Values expressed as median (Q1 – Q3). Kruskal–Wallis test, with *a posteriori* Dunn test. Different letters indicate a statistically significant difference ($p < 0.05$). CG = Control group. LCG = Low concentration group. MCG = Medium concentration group. HCG = High concentration group.

Table 2
Sperm morphology and plasma membrane integrity in rats from control and exposed to 2,4-D groups.

Parameter	CG	LCG	MCG	HCG
Normal sperm (%)	96.25 (95.25-97.50)a	94.50 (93.50-97.00)a	88.50 (86.25-89.25)b	92.75 (91.50-96.25)ab
Morphological abnormalities of the sperm head (%)	0.75 (0-1.25)a	1.50 (1.00-4.00)ab	4.50 (3.00-7.00)b	3.25 (2.37-4.75)ab
Morphological abnormalities of the flagellum (%)	2.25 (1.5-3.5)a	2.50 (2.00-4.00)ab	6.00 (3.50-8.50)b	3.75 (3.37-4.12)ab
Plasma membrane integrity (%)	91.25 (89.00-93.00)a	86.00 (81.75-88.75)ab	83.00 (77.62-89.00)ab	76.25 (69.87-82.12)b

Values expressed as median (Q1 – Q3). Kruskal-Wallis test with *a posteriori* Dunn. Different letters indicate a statistically significant difference ($p < 0.05$). CG = Control group. LCG = Low concentration group. MCG = Medium concentration group. HCG = High concentration group.

Table 3
Sperm counts in testis epididymis and in rats from control and exposed to 2,4-D groups.

Parameter	CG	LCG	MCG	HCG
Testis				
Sperm number ($x10^6$)	206.89±24.68a	150.82±15.56b	143.86±20.32b	146.53±9.89b
Sperm number per gram of organ ($x10^6/g$)	138.94±13.92a	110.84±10.65b	103.10±16.13b	104.30±8.59b

Daily sperm production (x10^6/testis/day)	33.91±4.04a	24.33±2.89b	23.57±3.33b	24.05±1.59b
Relative daily sperm production (x10^6/g/day)	22.78±2.27a	17.73±1.97b	17.10±2.56b	17.12±1.46b
Caput/corpus epididymis				
Sperm number (x10^6)	200.94±28.92a	209.92±14.13a	144.85±18.94b	144.50±17.98b
Sperm number per gram of organ (x10^6/g)	548.33±34.76a	547.99±29.93a	443.78±28.71b	430.62±25.05b
Transit time (days)	5.95±1.14a	8.78±1.01b	6.26±1.20a	6.11±0.87a
Cauda epididymis				
Sperm number (x10^6)	341.13±40.84a	319.52±45.75a	266.79±22.38b	262.46±33.34b
Sperm number per gram of organ (x10^6/g)	1401.00±62.73a	1373.47±67.70a	1135±46.95b	1134±36.63b
Transit time (days)	10.18±1.73a	13.13±1.74b	11.74±1.77ab	10.88±1.73a

Values expressed as mean ± SD. ANOVA with a posteriori Tukey test. Different letters indicate a statistically significant difference ($p < 0.05$). CG = Control group. LCG = Low concentration group. MCG = Medium concentration group. HCG = High concentration group.

The absolute and relative numbers of sperm in the caput/corpus and cauda epididymis were reduced ($p < 0.05$) in MCG and HCG compared to CG and LCG after oral exposure (Table 3). Moreover, there was a significant delay ($p < 0.05$) in the sperm transit time in the caput/corpus and cauda epididymis in LCG compared to CG (Table 3).

In the testis histology analysis, the seminiferous tubules showed 14 stages of the spermatogenic cycle and normal morphology in control rats (CG) (Figure 3A). The testicular histopathological changes observed were focal and showed interindividual variability. It was observed germ cell exfoliation with the presence of immature germ cells and cellular debris within the lumen of the seminiferous tubules in 60% of the rats of the HCG (Figure 3B –3E). However, these alterations appear in only 2% of the seminiferous tubule sections ($p > 0.05$).

Epididymis of the control group showed normal aspect of epithelium and lumen (Figure 3F). Epithelial vacuolization in the epididymis (Figure 3G) and immature germ cells in the lumen of the epididymis (Figure 3H) were found in 20% of the animals of the HCG and in 2% of the epididymis sections ($p > 0.05$).

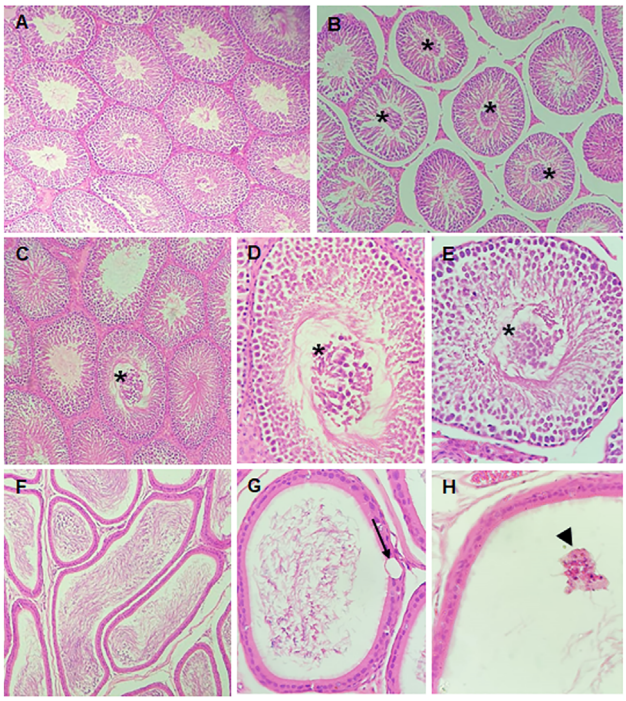


Figure 3. Testis (A – E) and epididymis (F – H) histology. A) Control group (CG). Observe the normal morphology of seminiferous tubules and interstitial tissue. B, C, D) Group exposed to high concentration of 2,4-D (HCG). E) Group exposed to low concentration of 2,4-D (LCG).
Observe the presence of germ cells exfoliation with the presence of immature germ cells and cellular debris within the lumen of the seminiferous tubules (*) in B – E. F) Normal aspect of epididymal epithelium and lumen in the control group (CG). G) Notice the epithelial vacuolization in the epididymis (arrow) in HCG. H) Observe the presence of immature germ cells and debris in the epididymis lumen (head of arrow) in HCG. A, B, C and F: 100X; D, E, G and H: 400X. H&E stain.

DISCUSSION

In the last decades, several studies have pointed to a decline in seminal quality with a consequential impact on fertility rates[21-23]. There is no consensus about the decrease in semen

parameters over time among men. However, relevant data raise concerns about this matter. Although the possible causes have not been established, several factors may be associated, including exposure to environmental contaminants[24].

The herbicide 2,4-D is one of the most used worldwide, so it is essential to understand its possible impacts on male reproduction, especially in chronic exposure and environmentally relevant concentrations. In the present study, chronic oral exposure to 2,4-D through consumption of contaminated feed caused deleterious effects on the male morphophysiology reproductive, especially in sperm quality.

Reproductive organ weights were not affected by herbicide exposure. The testicular alteration that caused a decrease in the numbers of sperm in the testis and daily sperm production was not enough to reduce the organ mass. However, Marouani et al.[25] observed a reduction in the weights of the testis, seminal vesicles and prostate of rats after oral by force feeding with 100 and 200 mg of 2,4-D/kg for 30 days. A decrease in the weights of testes and sex accessories glands was also found after oral exposure to 50, 100 and 150 mg/kg during 30 and 45 days [26].

One of the mechanisms of testicular toxicity suggested to 2,4-D is related to the disruption of cholesterol/testosterone homeostasis in Leydig cells[12]. The testosterone levels are correlated with the weights of testes, seminal vesicles and prostate[27]. Thus, the results of organ weights in the present study suggest the absence or reversible testosterone change during chronic exposure with physiological adaptations. Adaptive homeostasis describes transient changes in the homeostatic range that occur in response to sub-toxic exposure. Also, it can occur in exposures to xenobiotics at toxic levels as long as there is no irreparable harm[28].

The sperm quality of the groups exposed to all concentrations of 2,4-D was impaired. The reduction in progressive sperm motility observed in exposure groups to herbicide was mainly related to an increase in immotile sperm. Despite the reduced motility, there was no delay in sperm transit in the caput-corpus segment of the epididymis. Thus, other mechanisms involved in sperm maturation and/or sperm physiology can have been affected. The intense progression of sperm motility is generated through the energy produced by the mitochondria. The herbicide 2,4-D has been reported as a harmful agent to mitochondria[29], which can cause a reduction in sperm motility and cell death, observed as increase in immotile sperm and a decrease in vitality (plasma membrane integrity). Moreover, pesticides can decrease sperm motility by disrupting mitochondrial function and increasing the level of oxidative stress during sperm maturation[30].

According to Tan et al.[31], ejaculated human spermatozoa *in vitro* exposed to 2,4-D can have inhibition of the total and progressive motility, ability to penetrate viscous medium, capacitation and acrosome reaction rates, increasing the risk of infertility. Lerda and Rizzi[32] observed higher numbers of dead, abnormal and immotile spermatozoa in persons occupationally exposed to 2,4-D. Moreover, several studies identified sperm motility impairment after 2,4-D exposure in rodents[25,26]. Joshi et al.[26] observed severe impairment in sperm motility with an impact on fertility. Marouani et al.[25] showed decreased motility with an increased sperm abnormality in rats exposed to 2,4-D. In the present study sperm morphology also was changed in head and flagellum.

The transit time of spermatozoa in the epididymis varies between species and in the rat is 8 to 11 days[33]. In the present study, sperm transit was slower, including in control animals. However, other studies also observed this longer transit time in Wistar rats[34,35]. According to Kempinas et al.[36], a delay in transit time through the epididymis does not change the fertile capacity of gametes, but when it is accelerated, fertility can be compromised.

The testicular histopathological changes observed after 2,4-D exposure were focal and showed interindividual variability. Germ cell exfoliation and the presence of immature germ cells and cellular debris within the lumen of the seminiferous tubules were observed in the group exposed to higher concentrations. The exfoliated germ cells and cytoplasmic debris were detected in the epididymis lumen. This result was concordant with studies of Marouani et al.[25] and Zhang et al.[37].

Marouani et al.[25] identified that 2,4-D exposure caused a reduction of spermatozoa number in the lumen of the seminiferous tubules and the disappearance of the Sertoli cells in rats. Oral exposure (gavage) to 100 and 200 mg/kg/day of 2,4-D for 14 days caused spermatogenesis disruption with testis histology impairment in mice. Among the alterations found, the following stand out atrophy of seminiferous tubules, epithelium, depletion and detachment of germ cells and disruption of Leydig

cells[37]. These authors suggested the possible involvement of oxidative stress and apoptosis in testicular toxicity.

CONCLUSION

Chronic consumption of contaminated feed with 2,4-dichlorophenoxyacetic acid (2,4-D) in different concentrations caused deleterious effects on the spermatogenesis and sperm quality in adult male rats. This is the first study to describe the negative impact on male reproductive morphophysiology after chronic exposure to 2,4-D, using food nebulization in environmentally relevant concentrations, based on agronomic use of the herbicide. The exposure method that simulates environmental spraying is more relevant when compared to studies found in the literature, which usually use oral exposure by gavage (force-feeding) in acute or subchronic exposures and unreal doses.

The reproductive changes identified raise concerns about the impacts of widespread global exposure to 2,4-D. In addition, they generate the need for comprehensive epidemiological studies, which consider and correlate the geographical distribution of reproductive effects and local environmental contamination.

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Authors' contributions
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