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Environmental spray simulation: Reproductive effects of glyphosate in an animal model

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ABSTRACT
The aim of the present study was to evaluate the reproductive effects caused by chronic inhalational exposure to glyphosate in animals. For this, a model that simulates real environmental spraying with environmentally relevant concentrations of glyphosate was used. Wistar rats were divided into 4 groups, exposed to 0 (control group - GC), 20.69 (low concentration group - LCG), 34.63 (medium concentration group - MCG) or 51.66 ppm/day (group of high concentration - HCG). The animals were exposed for 15 minutes for 180 days. After the exposure period, the animals were euthanized and the testes and epididymis were collected for analysis of sperm counts, motility, morphology and vitality. The absolute and relative numbers of spermatozoa in the testis and epididymis and daily sperm production were reduced in all groups exposed to the herbicide, indicating an impact on spermatogenesis. Progressive motility was reduced in GAC, with a consequent increase in non-progressive motility and immobility. However, there was no delay in sperm transit time in the epididymis. Plasma membrane integrity and the percentage of morphologically normal spermatozoa were reduced in the exposed groups, with an increase in head and tail abnormalities. Exposure to glyphosate through an environmental spray simulation model affected sperm quantity and quality in an animal model. This warns of risks to male fertility, demanding strict regulation on the use of pesticides to prevent damage to reproductive health and the environment. In this way, more sustainable agricultural measures are crucial to balance production and global health.

KEYWORDS: Herbicide. Reproduction. Environmental health.

1 INTRODUCTION

Exposure to pesticides has been associated with a range of adverse health impacts in humans, including central nervous system disorders, congenital malformations, cardiac diseases, cancer, and various reproductive alterations (Hoppin et al., 2007; Riquinho, Hennington, 2012; Upadhyay et al., 2020; Lombardi et al., 2021; Wadani et al., 2022; Gilden et al., 2023). Studies have linked exposure to these chemicals to changes in sperm count and motility, hormonal imbalances, and an increased prevalence of male infertility (Jurewicz et al., 2009; Longo et al., 2021; Kapeleka, Sauli, Ndakidemi, 2021).

Among pesticides, glyphosate (N-phosphonomethyl glycine) is noted for its potential toxicity (Zhang et al., 2017; Islam et al., 2018; Van Bruggen et al., 2018; Zhang et al., 2019) and is extensively used in global agriculture (Todd et al., 2020). It serves to control the growth of unwanted vegetation in crops and other agricultural areas (Lozano et al., 2018). Glyphosate features a broad-spectrum, post-emergence, non-selective action and is used in pastures and in the cultivation of corn, rice, and soy, among others (Amarante, Santos, 2002; Roquetti, Takeda, Kuno, 2011).

When present in soil, glyphosate is rapidly degraded into carbon dioxide by microbial activity, while adsorbed glyphosate has a slower degradation rate or may remain inactive for long periods in the environment (Amarante et al., 2002). Exposure to glyphosate and other pesticides can occur during application or handling, as well as through environmental contamination (Mamane et al., 2015). Inhalation toxicity can happen in agricultural and industrial settings (Ye et al., 2013; Weiler et al., 2018; Tarmure et al., 2020; Ratanachina et al., 2022), with suggestions that this route might aggravate these effects (Williams, Watson, DeSesso, 2012; De Maria Serra et al., 2021; Strilbyska et al., 2022).

The World Health Organization (WHO, 2020) emphasizes the importance of safety measures during the use and handling of pesticides, along with the development of public

policies to mitigate environmental exposure to these products and their potential health risks (Damalas, Eleftherohorinos, 2011; Buralli et al., 2018; Silva et al., 2019). Considering this scenario, a profound understanding of the effects of glyphosate in various contexts is crucial for a comprehensive assessment of its benefits and risks in order to provide an informed perspective on this herbicide and its implications for agriculture, human health, and the environment. By adopting an inhalation exposure method employing concentrations that reflect environmental conditions as per the agricultural use of the herbicide and considering a prolonged (chronic) exposure period, our approach aims to simulate occupational exposure to evaluate potential effects on reproduction.

2 OBJETIVES

This study aimed to assess the potential male reproductive impacts caused by chronic inhalational exposure to the herbicide glyphosate through an experimental model that simulates real environmental spraying.

3 METODOLOGY

3.1 Chemical Agents

For glyphosate exposure, a commercial formulation of glyphosate (Roundup®), registered under number 09106, was used, containing 480 g/L (360 g/L acid equivalent) of glyphosate isopropylamine salt. The formulation was diluted in 10 mL of 0.9% saline solution (sodium chloride – NaCl) at the prescribed concentrations for each experimental group.

3.2 Animal Study Model

Adult male Wistar rats (total of n = 40) were acquired from the Central Vivarium of the University of Western São Paulo (UNOESTE), Presidente Prudente, SP, Brazil. Throughout the experiment, these animals were housed in polypropylene cages (43 cm x 30 cm x 15 cm) containing laboratory-grade wood shavings as a substrate. The maintenance conditions involved a controlled environment with a temperature maintained at 22 ± 2 ºC and a 12-hour light/dark cycle. A diet based on rat feed (Supralab® Alisul, Brazil) and unrestricted access to filtered water were provided. The experimental protocol was submitted to and approved by the UNOESTE Animal Use Ethics Committee (Protocol No. 6063-CEUA) and strictly followed the animal welfare guidelines established by the Brazilian College of Animal Experimentation (COBEA).

3.3 Experimental Groups and Herbicide Exposure Protocol

The rats were randomly divided into four experimental groups, totaling 10 animals per group, and exposed to different concentrations of glyphosate as shown in Figure 1.

The control group (GCl) was exposed only to 0.9% saline solution (vehicle). The selected concentrations for the herbicide exposure groups reflect environmentally relevant

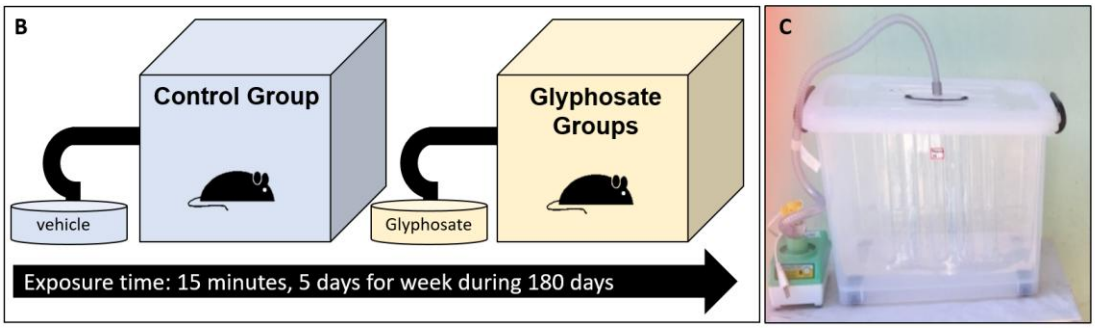
levels in line with the application dosages of the products and their agronomic recommendations. The glyphosate concentrations used in agriculture (expressed in grams of active ingredient per hectare - g.a.i) were adapted to the dimensions of the exposure boxes as mentioned by Parizi et al. (2020) (Figure 1).

For the exposure of the rats, a system composed of two plastic boxes (32 x 24 x 32 cm) connected to an ultrasonic nebulizer (Pulmosonic Star®, Brazil) (Figure 1) was adopted following the methodology of Mello et al. (2018). The animals underwent a 180-day exposure period. The duration of daily inhalation exposure was approximately 15 minutes per animal. This time interval ensured the complete nebulization of the solution.

Figure 1 – Exposure Protocol. Experimental groups and glyphosate exposure concentrations in different units (A). Simulation model of exposure for the control group and groups exposed to glyphosate (B). Exposure box connected to an ultrasonic nebulizer (C).

A

Experimental group	Group	Exposure concentration		
		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
Media 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



Source: Developed by the authors, adapted from Parizi et al. (2020) (A) and Mello et al. (2018) (C).

3.4 Collection of Biological Materials

At the end of the exposure period, the rats from each experimental group were anesthetized and euthanized by intraperitoneal administration of sodium thiopental at a dose of 100 mg/kg. The right testis and epididymides were collected for sperm analyses.

3.5 Evaluation of Sperm Motility, Morphology, and Integrity

Immediately following euthanasia, the left vas deferens was collected to obtain sperm in a phosphate-buffered saline solution at 34°C. Using a heated Neubauer counting chamber, sperm motility was assessed by visual observation (200 spermatozoa per animal in duplicate) under a microscope (Leica DMLS) at 200X magnification. Spermatozoa were categorized as immotile, motile without progression, and motile with progression according to the methodology of Perobelli et al. (2012).

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For sperm morphology analysis, the right vas deferens was collected for sperm fixation in saline formalin (10 mL). The evaluation was conducted using a microscope (magnification 400X) according to the classification proposed by Filler (1993).
Plasma membrane integrity (sperm vitality) was determined through eosin-nigrosin staining (WHO, 1999). After counting 200 spermatozoa under a light microscope (magnification 1000X), the cells were divided into unstained (indicative of live spermatozoa) and red-stained (indicative of dead spermatozoa).

3.6 Evaluation of Daily Sperm Production, Sperm Counts, and Epididymal Transit Time

The right testes were decapsulated, and the segments of the right epididymal head/body and tail were separated. These tissues were frozen for later sperm counting. Spermatids at stage 19 of spermiogenesis and spermatozoa present in the head/body and tail of the epididymis were quantified according to the protocols described by Robb et al. (1978) with adjustments by Fernandes et al. (2007). Daily sperm production (DSP) was calculated by dividing the number of stage 19 spermatids by 61 (representing the number of days these spermatids are present in the seminiferous epithelium). The transit time of spermatozoa in the segments of the epididymis was determined by dividing the total number of spermatozoa in each segment by the DSP according to the methodology of Robb et al. (1978).

3.7 Statistical Analysis

For the comparison of the evaluated parameters, analyses of variance (ANOVA) were conducted, followed by Tukey's post-test or non-parametric Kruskal-Wallis tests followed by Dunn's test. A Kolmogorov-Smirnov test was applied to verify the normality of distributions before statistical analyses. A p-value < 0.05 was considered statistically significant.

4 4. RESULTS AND DISCUSSION

Glyphosate is widely used in agriculture to facilitate and increase food production. While this pesticide plays a crucial role in agricultural production, its excessive use can have various adverse consequences for human and environmental health (ARL, 2021). In recent years, a progressive decline in male reproductive function has been observed, possibly correlated with exposure to environmental contaminants, particularly pesticides (Gorga et al., 2020). In this study, the different concentrations of glyphosate in inhalational exposure led to alterations in the quantity and quality of sperm in an animal model, indicating a potential risk to male fertility.

The most commonly observed reproductive toxic effects after exposure to pesticides include: reduced ejaculate volume, decreased testosterone concentration, reduced sperm production, lower sperm concentration, and alterations in sperm morphology and motility (Romano et al., 2012; Dai et al., 2016; Owagboriaye et al., 2017; Cai et al., 2017; Anifandis et al., 2018; Vanlaeys et al., 2018; Pham et al., 2019; Gorga et al., 2020; Jarrell, Ahammad, & Benson, 2020; Bwana Mutwedu et al., 2021), which is consistent with the findings of our study.

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The absolute and relative numbers of spermatozoa in the testis and the absolute and relative daily sperm production (DSP) were reduced in all groups exposed to the herbicide compared to the CG (control group) (Table 3), indicating an impact on spermatogenesis. However, the weight of the testes was not altered by the herbicide (Table 1).

Table 1 - Sperm counts in the testis of rats from the four experimental groups: Control (CG) and exposed to glyphosate via inhalation at different concentrations (LCG, MCG, HCG).

Testicular counts	**CG**	**LCG**	**MCG**	**HCG**
Testicular weight (g)	1.58±0.10	1.59±0.11	1.59±0.13	1.60±0.12
Sperm number (x10^6)	196.15±20.43a	181.08±19.98b	178.49±11.31b	176.70±18.18b
Sperm number/g (x10^6/g)	127.11±12.71a	118.55±19.20b	117.00±13.77b	116±15.77b
DSP (x10^6/testículo/day)	31.44±3.12a	22.12±2.67b	22.10±1.89b	21.91±1.76b
Relative DSP (x10^6/g/day)	21.88±2.44a	17.01±2.52b	16.23±2.61b	16.68±2.30b

Different letters indicate a statistically significant difference between the groups ($p < 0.05$).
Source: Authors.

Dai et al. (2016) observed changes in sperm counts and a reduction in the absolute and relative number of spermatozoa in the testis and total and per gram testis daily sperm production in rats exposed to concentrations of 5, 50, and 500 mg/kg of the herbicide glyphosate. The results are similar to those found in this study. Signs of reproductive toxicity were also found in the study by Romano (2012), where a decrease in the number of spermatozoa, daily sperm production, and an increase in the percentage of abnormal spermatozoa were observed.

The absolute and relative numbers of spermatozoa in the head/body and tail of the epididymis were reduced ($p < 0.05$) in the three groups exposed to glyphosate compared to the CG (Table 2). However, there was no significant delay ($p < 0.05$) in sperm transit time in the head/body and tail of the epididymis compared to the CG (Table 2), despite the observed motility changes (Figure 2) indicating a potential impact on sperm maturation.

Table 2 - Sperm counts in the epididymis of rats from the four experimental groups: Control (CG) and exposed to glyphosate via inhalation at different concentrations (LCG, MCG, HCG).

Epididymal counts	**CG**	**LCG**	**MCG**	**HCG**
Caput/corpus				
Sperm number (x10^6)	136.74±22.60a	114.19±19.97b	109.78±21.09b	107.50±22.06b
Sperm number/g (x10^6/g)	345.39±22.70a	321.30±29.82b	319.31±22.83b	318.76±21.81b
Transit time (days)	4.95±1.14	4.87±1.01	4.73±1.05	4.11±1.04
Cauda				
Sperm number (x10^6)	224.25±41.33a	202.06±32.90b	198.37±42.34b	197.04±32.08b
Sperm number/g (x10^6/g)	984.06±89.01a	976.93±68.77a	921.83±102.97b	922.97±77.24b
Transit time (days)	6.56±1.57	7.52±2.46	7.72±1.62	7.09±2.03

Different letters indicate a statistically significant difference between the groups ($p < 0.05$).
Source: Authors.

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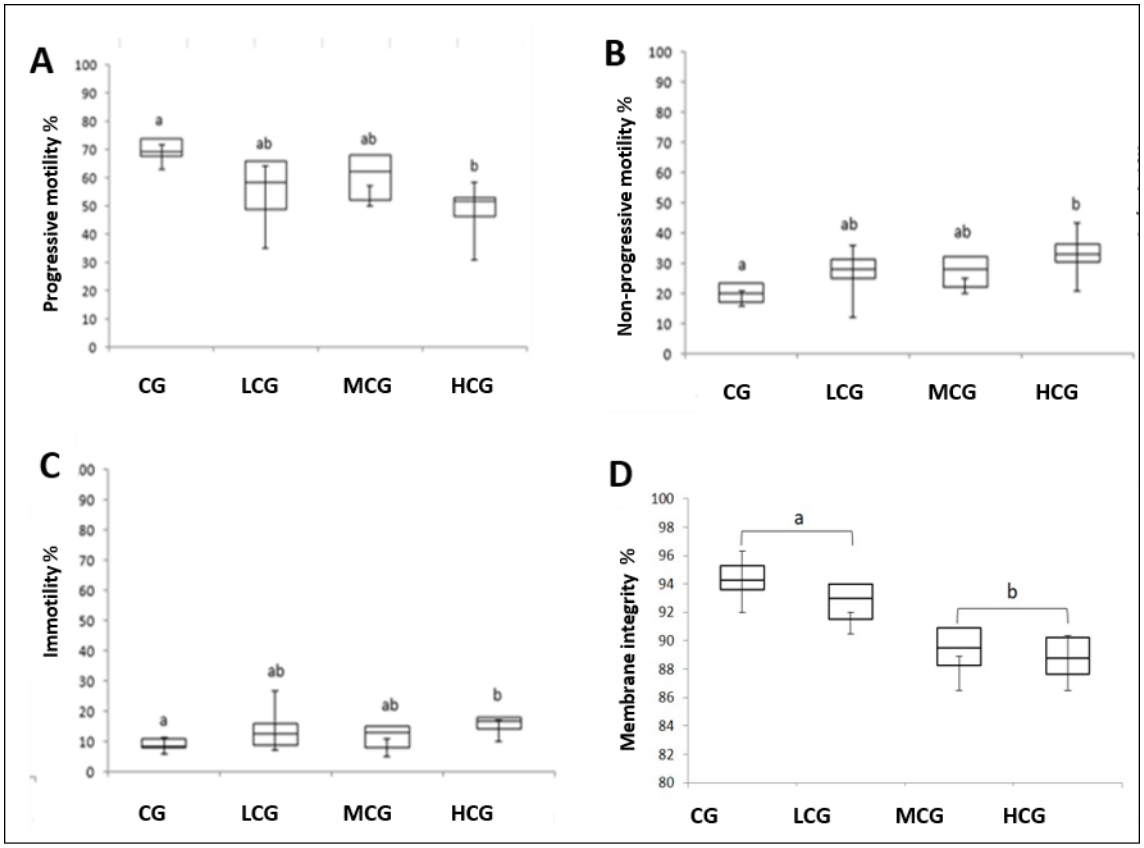
Sperm motility is a fundamental aspect in relation to fertilization because only motile spermatozoa can reach the oocyte for fertilization. Therefore, a decrease in motility directly affects the reproductive capacity of spermatozoa (Cai et al., 2017). Numerous investigations have illustrated the effects of pesticides on this specific parameter. Anifandis et al. (2018) conducted a study with sperm samples exposed to 1 mg/L of glyphosate after 1 hour and observed a decrease in progressive motility.

In the present study, the percentage of spermatozoa with progressive movement (Figure 2A) was significantly reduced ($p < 0.05$) in the HCG compared to the GCI and similar between the groups exposed to glyphosate ($p > 0.05$). Consequently, the percentage of spermatozoa with non-progressive motility (Figure 2B) and immotile (Figure 2C) was increased in animals exposed to the highest concentration of the herbicide. This demonstrates that there was a tendency to reduce the motile capacity of spermatozoa at all exposure concentrations of glyphosate; however, only at the highest concentration was the difference statistically significant ($p < 0.05$). The medium and high exposure concentration groups were the ones that most showed alterations, indicating that the dose of exposure is directly related to spermatic damage as studied by Joshi et al. (2012).

Figure 2 - Sperm motility (A-C) and plasma membrane integrity (D) of the four experimental groups: Control (CG) and exposed to glyphosate via inhalation at different concentrations (LCG, MCG, HCG).



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The analysis of sperm morphology also plays a significant role not only in assessing the functionality of the testes but also as an indicator of stress induced by the environment. Anomalies in sperm shape may indicate changes that occurred during the process of spermatogenesis, directly affecting their fertilization capacity (Najafi et al., 2015). In the present study, there was a significant reduction ($p < 0.05$) in the percentage of morphologically normal spermatozoa, with a consequent increase in head and tail abnormalities in the groups exposed when compared to the GCI (Table 3). Additionally, the integrity of the plasma membrane was reduced ($p < 0.05$) in the MCG and HCG groups exposed to glyphosate compared to the CG and LCG (Figure 2D).

Table 3 - Sperm morphology in rats from the four experimental groups: Control (CG) and exposed to glyphosate via inhalation at different concentrations (LCG, MCG, HCG).

Morphology	**CG**	**LCG**	**MCG**	**HCG**
Normal (%)	96.50 (94.00-99.00)a	90.00 (83.00-95.00)b	88.60 (78.00-94.00)b	86.30 (76.00-91.00)b
Abnormalities of sperm head (%)	2.00 (1.00-11.00)a	4.50 (1.00-14.00)a	5.50 (1.00-17.00)ab	8.00 (2.00-19.00)b
Abnormalities of sperm flagellum (%)	0.27 (0.00-1.00)a	0.70 (0.00-3.00)ab	1.07 (0.00-3.00)b	1.72 (0.00-8.00) bc

Different letters indicate a statistically significant difference between the groups ($p < 0.05$).
Source: Authors.

The reduction in morphologically normal spermatozoa and the increase in head and tail abnormalities of spermatozoa are similar to alterations found in the study by Owagboriaye et al. (2017). These authors observed a significant reduction in the number of morphologically normal spermatozoa in rats exposed to glyphosate. The authors propose that such alterations have a negative impact on the reproductive health of rats resulting from exposure to the herbicide. Romano et al. (2010) also found a significant reduction in testosterone concentrations and changes in testicular morphology in male rats exposed to different doses of glyphosate. As in this study, the occurrence of morphological alterations and the reduction of normal spermatozoa with an increase in abnormalities of the head and tail in the exposed groups indicate that the use of glyphosate can alter sperm quality.

5 CONCLUSION

The results revealed that inhalational exposure to glyphosate at environmentally relevant concentrations, conducted with a model simulating real environmental spraying, led to significant alterations in both the quantity and quality of sperm in animals. The different concentrations of exposure demonstrated gradual effects, where higher concentrations presented greater damage. Particularly, a decrease in sperm motility, a crucial factor for successful fertilization, as well as changes in sperm morphology and plasma membrane integrity, were observed. These alterations indicate a potential risk to male fertility, reinforcing the importance of strict regulations in the use of pesticides to minimize adverse impacts. In

summary, the results of this study emphasize the need for further research and careful analysis of the risks associated with the use of glyphosate and other pesticides, considering their impact on male reproductive health and the potential for transmitting negative effects to future generations. Regulatory measures and more sustainable management strategies in agriculture may be necessary to balance food production with the preservation of human and environmental health.

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