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Glyphosate Alters Redox Balance and Induces Histomorphological Alterations in the Testes of Fruit-Eating Bats (*Artibeus lituratus*)

Ospina EG[1], Pereira de Freitas RM[1], Fonseca Destro AL[1], Oliveira JM[2], Alves de Melo FCS[1], Pinto da Matta SL[3], Mariáurea MS[1], Castro DJ[1], Fernanda Carolina DR[4], Gonçalves RV[1] and Freitas MB[1]*

[1]Department of Animal Biology, Federal University of Viçosa, Brazil
[2]Department of Biological Sciences and Health, Federal University of Alagoas, Brazil
[3]Department of Cellular and Structural Biology, Federal University of Viçosa, Brazil
[4]Department of Structural Biology, Federal University of Triângulo Mineiro, Brazil

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***Corresponding author:** Freitas MB, PhD in Animal Biology-Federal University of Viçosa, Avenida Peter Henry Rolfs, Campus Universitário, Viçosa, Minas Gerais, CEP: 36570-000, Brazil; Email: mariella.freitas@gmail.com

Abstract

We investigate the effects of Glyphosate (GLY) on the testes of fruit-eating bats (*Artibeus lituratus*). Our study aimed to evaluate the effects of short-time exposure to GLY herbicide on adult male bats testes. Adult male bats were exposed for three days: Control (fed fresh fruits) and GLY (fed 4 mL / 100 mL GLY-pulverized fruit). Animals fed with contaminated fruits showed a reduction in body mass. Antioxidant enzyme catalase (CAT) activity and carbonylated protein (PCN) levels increased in the teste's GLY-exposed bats. Histomorphometry revealed an increase in the luminal parameters as well as in the epithelium tubule ratio, which is indicative of a reduction in epithelium area. There was also an increase in the percentage of macrophages and a decrease in the percentage and number of Leydig cells. The results indicate that short term exposure to GLY in frugivorous bats may cause oxidative stress of the tissue that possibly induces testicular morphological changes that may compromise the reproduction and maintenance of the species.

Keywords: Antioxidant Enzymes; Herbicide; Leydig Cells; Oxidative Stress

Abbreviations: TER: Tubule Epithelium Ratio; GLY: Glyphosate; PCN: Carbonylated Protein; ROS: Reactive Oxygen Species; GST: Glutathione S-Transferase; SOD: Superoxide Dismutase; TSI: Tubule Somatic Index; TBARS: Thiobarbituric Acid Reactive Substance; BW: Body Weight; STL: Seminiferous Tubules Length; SEC: Seminiferous Epithelium Cycle; NSC: The Number of Sertoli Cells; LSI: The Leydigo Somatic Index.

Introduction

Among the various herbicide groups, Glyphosate (GLY) has been used for over 40 years [1] and is currently most used worldwide [2]. Like ammonium or sodium salt, GLY is an organophosphate that is still cited as medially toxic, however there is evidence of its deleterious effects on the environment, wildlife and humans [3-5]. GLY residues and their metabolite, aminomethyl phosphonic acid, are found in the harvest of young foliage, in farm animals, soil and water [6,3].

This herbicide is an endocrine disruptor, as it reduces testosterone synthesis, induces germ cell and Sertoli cell apoptosis, inhibits antioxidant defense systems promoting oxidative stress, and has high carcinogenic potential [3,7-9]. Male germ cells are more susceptible to free radical action

due to the high concentration of polyunsaturated fatty acids present in their cell membranes and their low antioxidant capacity [10]. These characteristics make this tissue more susceptible to oxidative stress, mainly due to the imbalance between the production of reactive oxygen species (ROS), nitrogen (RNS) and antioxidant defenses [11]. Antioxidant defenses may be enzymatic and non-enzymatic [12]. Among the enzymatic we can mention the superoxide dismutase (SOD), catalase (CAT) and Glutathione S-transferase (GST) [12], while the non-enzymatic are vitamins, carotenoids and flavonoids [13].

Despite being one of the most used in the world, there are no studies evaluating the effects of this herbicide on reproduction bats [2]. A recent study found 4505.2 pg/mg of GLY in bat's hair pool, this pesticide was the most common in the bats among 8 others pesticides-2,4-D, Atrazine, Carbaryl, Clothianidin, Dicamba, Imidacloprid and Thiamethoxam [14]. The dispersal of seeds by fruit-eating animals is a crucial interaction for the dynamics of plant populations and the regeneration of degraded areas [15]. Fruit-eating bats are important seed dispersers participating in the regeneration of forest ecosystems [16]. This fruit-only diet can also make fruit bats such as *Artibeus lituratus* good indicators of the presence and magnitude of pesticide contamination [17].

Artibeus lituratus is a large bat (Phyllostomidae: Stenodermatinae) found throughout Neotropic [15]. This species forms polygamous groups that shelter in the tree tops. The reproductive period of the species may vary according to geographical distribution. In Brazil, for example, they may have a bimodal polystria pattern, which is characterized by continuous reproduction between seasons [18]. Herbicides and other chemical components can be transported by leaching, runoff, evaporation, erosion and through food, reaching regions far from application areas and impacting non-target organisms such as bats [19]. Changes such as oxidative stress, reduced energy reserves and histopathological changes have been observed in bats exposed to different environmental contaminants, especially testicular, hepatic and metabolic modifications [20-23]. Based on the above, our study aimed to evaluate the effects of short-time exposure to GLY herbicide on adult male *Artibeus lituratus* testes.

Material and Methods

Animals

Male adult bats (*Artibeus lituratus)* (n = 13) were captured with mist nets near the campus of the Federal University of Viçosa (20° 45'S e 42° 52' W) and identified with the identification key of Brazilian Bats [24]. Adult animals were identified based on the fusion of the epiphyseal cartilage of the fourth finger, at the metacarpal-phalangeal junction, according to Kunz TH, et al. [25].

The animals were housed in individual cages (45 × 22 cm) with temperature and natural light and dark cycles in the University Museum of Zoology João Moojen. The animals passed through three days of acclimatization and were then randomly separated in experimental groups: Control (n=7) received papaya (*Carica papaya*) in natura; GLY (Roundup Original DI®) (n=6) receiving the fruit contaminated with the commercial GLY formulation ($C_3H_8NO_5P$) at the concentration of 4% (v/v) diluid in water. This concentration was chosen based on the commercial recommendation for the use of this herbicide. The fruits were sprinkled with GLY-containing syrup and then kept in suitable boxes until the pesticide layer dried. The fruits were then split, weighed and offered (200 g) to the animals, with the bark facing upwards, for 3 days at 6:00 p.m., and water *ad libitum* was offered. The leftovers were heavy in the morning. After treatment, the animals were weighed and then euthanized by cervical dislocation followed by decapitation. The testes were removed and weighed, the left one being for histomorphometric analyzes and the frozen right (-80ºC) for the evaluation of oxidative stress. The procedure was previously approved by the Brazilian Government (SISBIO, registration nº 55798-1) and Ethics Committee of Animal Use of the Federal University of Viçosa (CEUA/UFV 85/2016).

Analysis of Oxidative Markers and Antioxidant Enzymes

Oxidative Markers: Testes fragments for oxidative analysis were homogenized in 0.1M phosphate buffer, pH 7.4 and centrifuged at 4°C. Malondialdehyde (MDA) production, a lipidic peroxidation marker, were analyzed in the supernatant (homogenate). The homogenate was mixed with thiobarbituric acid and the formation of thiobarbituric acid reactive substance (TBARS) was mensured at 535nm, according to the protocol previously described by Buege JA, et al. (1978) [26]. The protein carbonyls assay was performed through the method based on carbonylation reaction of proteins with 2,4-dinitrophenylhydrazine (DNPH) forming dinitrophenylhydrazone [27]. The content of carbonylated proteins was calculated using the molar extinction coefficient (21×10^{31} mol cm). The results of TBARS and total proteins were normalized according to the total protein levels in the supernatant and expressed in (nmol/mg) of protein [28]. These analysis were measured in a microplate scanning spectrophotometer (Multiskan GO, Thermo Scientific).

Antioxidant Enzymes: The activity of superoxide dismutase (SOD) was determined based on the ability of this enzyme to catalyze the reaction of superoxide

(O^{-2}) to hydrogen peroxide, thereby reducing the auto-oxidation rate of pyrogallol [29], the reaction mixture was measured by absorbance at 570 nm in a microplate scanning spectrophotometer. The catalase (CAT) activity was evaluated according to the protocol described by Aebi H, et al. [30] in which the rate of decomposition of H_2O_2 is measured in 60 seconds. Glutathione S-transferase (GST) activity was assayed through the formation of glutathione-conjugated 2,4-dinitrochlorobenzene (CDNB) [31]. CAT and GST were measured in spectrophotometer (UV-Mini 1240, Shimadzu). The total protein dosage in the homogenates was performed by the method of Lowry [28]. The data were normalized according to the total protein levels in the supernatant and the results were expressed in U CAT/ SOD. The results of GST were expressed in μmol/min/g.

Testicular Histomorphometry

Tubular Histomorphometry: The left testes were fixed in Karnovsky solution (paraformaldehyde 4%, glutaraldehyde 4%, 1:1 in phosphate buffer 0.2 M, pH 7.3) for 24 h and transferred to 70% ethanol. Testicular fragments were dehydrated in a growing series of ethanol and embedded in glycol-methacrylate (Historesin®, Leica). Semi-serial sections (3μm) were made using a rotary microtome (RM 2255, Leica), with a minimum of 40μm between sections, and stained with toluidine blue/sodium borate 1%. Morphometry and stereology were performed using digital images captured with the light microscope (Olympus BX-60®, Tokyo, Japan) connected to a digital camera (Olympus QColor-3®, Tokyo, Japan), and analyzed using the Image-Pro Plus 4.5® software.

Both testes and the tunica albuginea were removed, weighed, and the albuginea weight was subtracted from the total testicle weight, providing the testicular parenchyma weight. The gonadosomatic index (GSI) was calculated by dividing the testicular weight by body weight and multiplied by 100.

The volumetric density of all seminiferous tubule's components (tunica propria, seminiferous epithelium and lumen) and interstitium was estimated after counting 2,660 intersection points, per animal, in 10 square grids (266 intersections/points each), placed randomly over digital images (200x magnification). The volume of each component was estimated by multiplying its percentage within the testis by the testicular parenchyma volume and dividing the result by 100. Since the mammalian testis density is around 1 [32], its weight was considered as the same as the volume.

The tubule somatic index (TSI) was calculated to quantify the investment in seminiferous tubules in relation to body weight (BW) by the formula TSI = STV/BW × 100, where STV = seminiferous tubule volume [33].

The mean tubular diameter was obtained after measuring 30 random circular seminiferous tubules cross sections from each animal, regardless of the tubular stage (200x magnification). The seminiferous epithelium height was measured in the same tubular sections in which the tubule diameter was obtained (as the mean of two diametrically opposed measurements).

The tubular (STAr), luminal (LAr) and epithelial (EAr) areas were calculated according to the formulas: STAr = TR^2, where TR = tubular radius; LAr = LR^2, where LR = luminal radius; EAr = STAr – LAr. The tubule epithelium ratio (TER) was calculated by dividing STAr/EAr.

The seminiferous tubules length (STL), in meters, was calculated as described by Morais DB, et al. [34] using the formula STL = STV/πR^2, where STV = seminiferous tubule volume, R^2= transverse section area of the seminiferous tubule, and R= tubular diameter/2. The STL was divided by the testicular weight in order to calculate the seminiferous tubules length per gram of testis (STL/g).

The stages of the seminiferous epithelium cycle (SEC) were characterized according to the tubular morphology method [35]. The number of each cell type found at Stage 1 of the SEC was estimated by counting their nuclei (germ cell) or nucleoli (Sertoli cell) in 10 random tubular cross sections per animal. Thirty nuclear diameter of each germ cell type and nucleolar diameter of the Sertoli cell were measured for each animal. The results were corrected due to variations in the size of the cells and the section thickness as described by Amann RP [36]. The following indexes were calculated from the corrected numbers of germ and Sertoli cells: spermatogonial mitosis (mitotic index), meiotic index, spermatogenic yield, Sertoli cell index, and the total support capacity of the Sertoli cell [34]. The number of Sertoli cells (NSC) was determined as described by Morais DB, et al. [34], and the obtained results were divided by the testicular weight in order to calculate the number of Sertoli cells per gram of testis (NSC/g).

Intertubular Histomorphometry: One thousand coincident points over the intertubular components were recorded (400x magnification): nucleus and cytoplasm of Leydig cell, blood vessels, lymphatic space, and connective tissue, and the percentages of these components were estimated. The volume of each intertubular component in the testicular parenchyma was calculated by the following formula: (percentage of each component in the testis x gonadal weight) / 100.

The mean diameter of the Leydig cell nucleus was obtained after measuring 30 cells per animal and selecting those with the most spherical nuclei and evident nucleoli. The

nuclear volume was obtained by using the formula $4/3\pi R^3$, where R = nuclear diameter/2. The cytoplasmic volume was estimated by multiplying the percentage of cytoplasm by the nuclear volume, divided by the nuclear percentage. The single cell volume was estimated by adding the nuclear and cytoplasmatic volume.

The number of Leydig cells was estimated from the Leydig cell individual volumes and the total volume occupied by these cells in the testicular parenchyma [37]. The result was divided by the gonadal weight to estimate the number of Leydig cells per gram of testis, allowing comparisons between different species. The Leydigo somatic index (LSI), which quantifies the investment in Leydig cells related to body mass, was estimated by dividing the Leydig cell volume in the testicular parenchyma by the body weight and multiplying by 100.

Statistical Analysis

Results were reported as mean + standard error of the mean. Data distribution was determined by the Shapiro-Wilk test. The data were submitted to Student's t test for multiple comparisons using the program GraphPad Prism (version 6.0, Graph Pad Software Inc., San Diego, CA, USA). Statistical significance was set at $p \leq 0.05$.

Results

Animals and Food Consumptive

The GLY-treated bats did show increase in weight loss (p=0.0006) (Table 1), but did not show difference in other parameters of consumption.

Treatments		
	Control (n=7)	GLY (n=6)
Papaya consumption (g/body mass/day)	1.47±0.11	1.64 ±0.04
Weight (g) preexposure	66.00±2.91	76.50±5.97
Weight (g) postexposure	65.15±2.81	72.34±4.98
Weight loss (%)	0.85±0.07	4.16±0.76*
Testis weight (g)	0.48 ± 0.04	0.41 ± 0.07
Albuginea weight (g)	0.15 ± 0.07	0.05 ± 0.01
Parenchyma weight (g)	0.33 ± 0.09	0.36 ± 0.06
Gonadosomatic index (%)	0.74 ± 0.07	0.56 ± 0.08

Data are expressed as mean ± SEM. * In the same line indicates difference ($p \leq 0.05$).
Table 1: Consumption of fruits, body and testis weight from *A. lituratus* exposed to GLY.

Oxidative Markes and Antioxidante Enzymes

The testicles of GLY-treated bats did show increase in PCN levels (p=0.0158) in the herbicide treated group (Figure 1B) CAT activity (p=0.0255) also increased in herbicide treated group (Figure 2B). The others biomarker did not show difference (Figure 1A, Figure 2A & 2C).

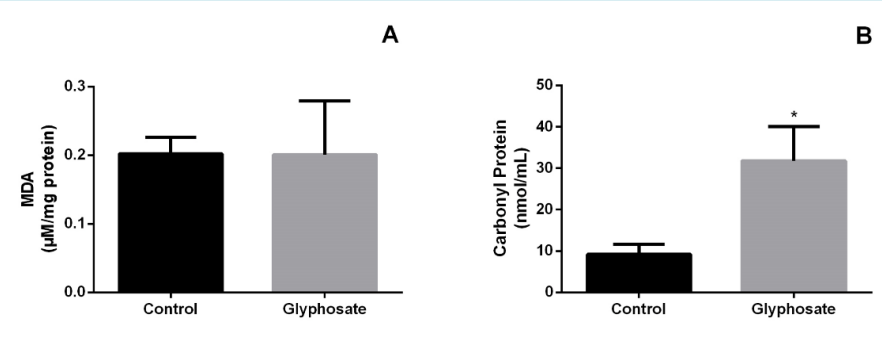

Figure 1: Levels of malondialdehyde (MDA) (A) and carbonyl protein (PCN) (B) in testes from *A. lituratus* exposed to GLY. Control (water); GLY (Roundup 4mL/100 mL of water). *Significant difference. Data expressed in mean±SEM.

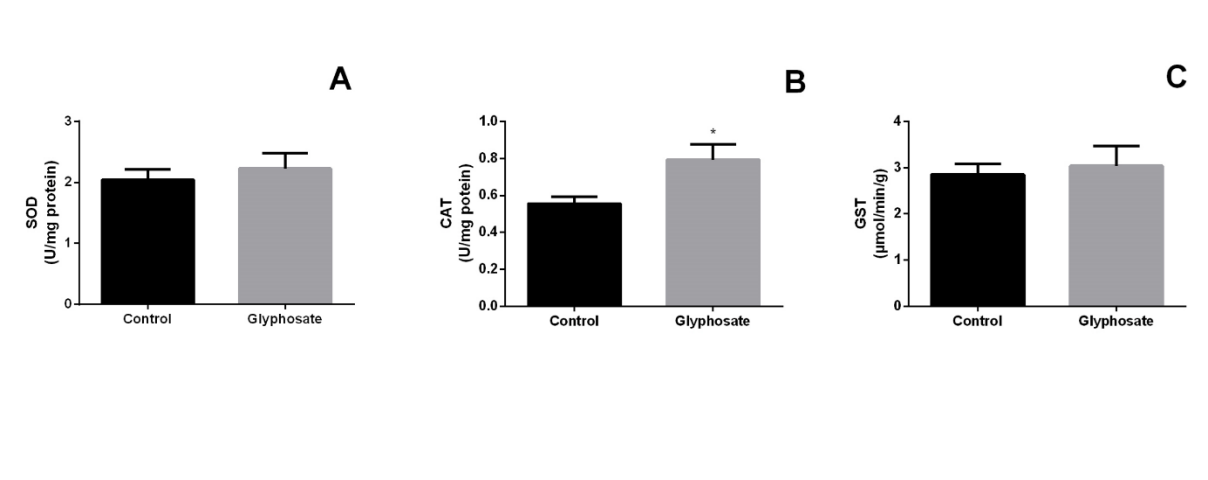

Figure 2: Levels of superoxide dismutase SOD (A), catalase CAT (B) and Glutathione S- tranferase (C) in testes from *A. lituratus* exposed to GLY. Control (water); GLY (Roundup 4mL/100 mL of water). *Significant difference. Data expressed in mean±SEM.

Testicular Histomorphometry

In the morphometric and stereological parameters, the testicles of GLY-treated bats shown increase in percentage of lumen (p=0.0443) and tunica propria (p=0.0013), also in area (p=0.0159) and diameter of lumen (p=0.0159). There was an increase in tubule epithelium ratio (p=0.0159) (Table 2). The others parameters did not show difference.

Treatments		
	Control (n=7)	GLY (n=6)
Seminiferous tubule (%)	91.02 ± 1.05	88.73 ± 0.94
Seminiferous epithelium (%)	79.11 ± 0.88	77.59 ± 0.86
Lumen (%)	3.71 ± 0.98	6.59 ± 0.44*
Tunica propria (%)	8.19 ± 0.57	4.56 ± 0.29*
Intertubular compartment (%)	8.98 ± 1.05	11.27 ± 0.94
Seminiferous tubule volume (mL)	0.31 ± 0.08	0.32 ± 0.05
Seminiferous epithelium volume (mL)	0.27 ± 0.07	0.28 ± 0.04
Lumen volume (mL)	0.01 ± 0.01	0.02 ± 0.01
Tunica propria volume (mL)	0.03 ± 0.004	0.02 ± 0.004
Intertubular compartment volume (mL)	0.028 ± 0.017	0.041 ± 0.021
Tubulessomatic index (%)	0.47 ± 0.12	0.43 ± 0.06
Seminiferous tubules diameter (µm)	311.05 ± 14.93	337.05 ±9.75
Height of seminiferous epithelium (µm)	125.96 ±7.27	118.94 ±4.66
Lumen diameter (µm)	59.13 ± 4.96	99.17 ± 5.25*
Seminiferous tubule area (µm2)	76648.83 ± 7164.50	89458.21 ± 5221.10
Lumen area (µm2)	2822.11 ± 509.90	7801.49 ± 791.25*
Epithelium area (µm2)	73826.72 ±70433.19	81656.72 ±5046.14
Tubule epithelium ratio	1.24 ± 0.02	1.42 ± 0.03*
Seminiferous tubules length (m)	3.79 ± 0.90	3.51 ± 0.42
Seminiferous tubule length/g of testis (m)	7.82 ± 1.83	8.79 ± 0.48

Data are expressed as mean ± SEM. * In the same line indicates difference (p ≤ 0.05).
Table 2: Morphometric and stereological parameters of the testes of *A. lituratus* exposed to GLY.

In the number of germ cells per tubule cross section at Stage I, there was a decrease in Type A Spermatogonia, Pre-leptotene/leptotene Spermatocytes (p=0.0687) and Sertoli cells (Table 3). The others parameters did not show difference.

Treatments		
	Control (n=7)	GLY (n=6)
Type A Spermatogonia	3.33 ± 0.83	1.79 ± 0.26*
Pre-leptotene/leptotene Spermatocytes	2.97 ± 0.25	1.73 ± 0.60*
Pachytene Spermatocytes	11.35 ± 3.00	9.85 ± 2.48
Round Spermatids	18.27 ± 2.19	15.03 ± 6.52
Sertoli cell	58.40 ± 7.96	42.01 ± 4.16*
Mitotic index (%)	0.90 ± 0.25	0.95 ± 0.24
Meiotic index (%)	1.64 ± 0.36	1.49 ± 0.25
Spermatogenic Yield	5,64 ± 1,85	8,63 ± 4,66
Sertoli cell index (%)	0.31 ± 0.01	0.36 ± 0.14
Total support capacity of the Sertoli cell	0.61 ± 0.04	0.68 ± 0.17
Number of Sertoli cells (x105)	7.6 ± 4.5	4.9 ± 1.7
Number of Sertoli cells per gram of testis (x105)	21.00 ± 20.00	12.00 ± 20.00

Data are expressed as mean ± SEM. * In the same line indicates difference (p ≤ 0.05).

Table 3: Corrected number of germ cells per tubule cross section at Stage I of the seminiferous epithelium cycle, efficiency of spermatogenesis, and Sertoli cell index in *A. lituratus* exposed to GLY.

Regarding intertubular and leydig cell morphometry, there was an increase in percentage of lymphatic space (p=0.1934), macrophages (p=0.0008), connective tissue volume (p<0.0001) and number of leydig cells per gram of testis (p=0.0147). Also, it was a decrease in percentage of leydig cells (p<0.0001) and lymphatic space volume (p=0.0344) (Table 4). The others parameters did not show difference.

Treatments		
	Control (n=7)	GLY (n=6)
Intertubule		
Lymphatic space (%)	0.220 ± 0.150	0.500 ± 0.130*
Connective tissue (%)	1.17 ± 0.12	1.01 ± 0.09
Macrophages (%)	0.31 ± 0.12	1.02 ± 0.09*
Blood vessels (%)	0.43 ± 0.08	0.32 ± 0.10
Leydig cells (%)	6.58 ± 0.86	1.01 ± 0.9*
Lymphatic space volume (mL)	0.013 ± 0.0005	0.006 ± 0.0031*
Connective tissue volume (x103 mL)	0.004 ± 0.0013	0.03 ± 0.0031*
Macrophages volume (x103 mL)	0.0009 ± 0.0003	0.003 ± 0.0002
Blood vessels volume (mL)	0.001 ± 0.00003	0.0041 ± 0.00005
Leydig cells volume (mL)	0.002 ± 0.005	0.03 ± 0.008
Leydig Morphometry		
Nuclear diameter (µm)	6.43 ± 0.27	6.31 ± 0.11
Nuclear volume (µm3)	142.01 ± 19.39	131.97 ± 6.77

Cytoplasmic volume (µm3)	441.99 ± 58.80	327.63 ± 10.80
Leydig cell volume (µm3)	584.00 ± 76.97	459.60 ± 11.15
Number of Leydig cells (x107)	4.87 ± 1.58	9.76 ± 2.80
Number of Leydig cells per gram of testis (x107)	9.91 ± 3.09	22.33 ± 2.92*
Leydigossomatic Index (%)	0.003 ± 0.009	0.006 ± 0.010

Data are expressed as mean ± SEM. * In the same line indicates difference ($p \leq 0.05$).
Table 4: Intertubular and Leydig cell morphometry in *A. lituratus* exposed to GLY.

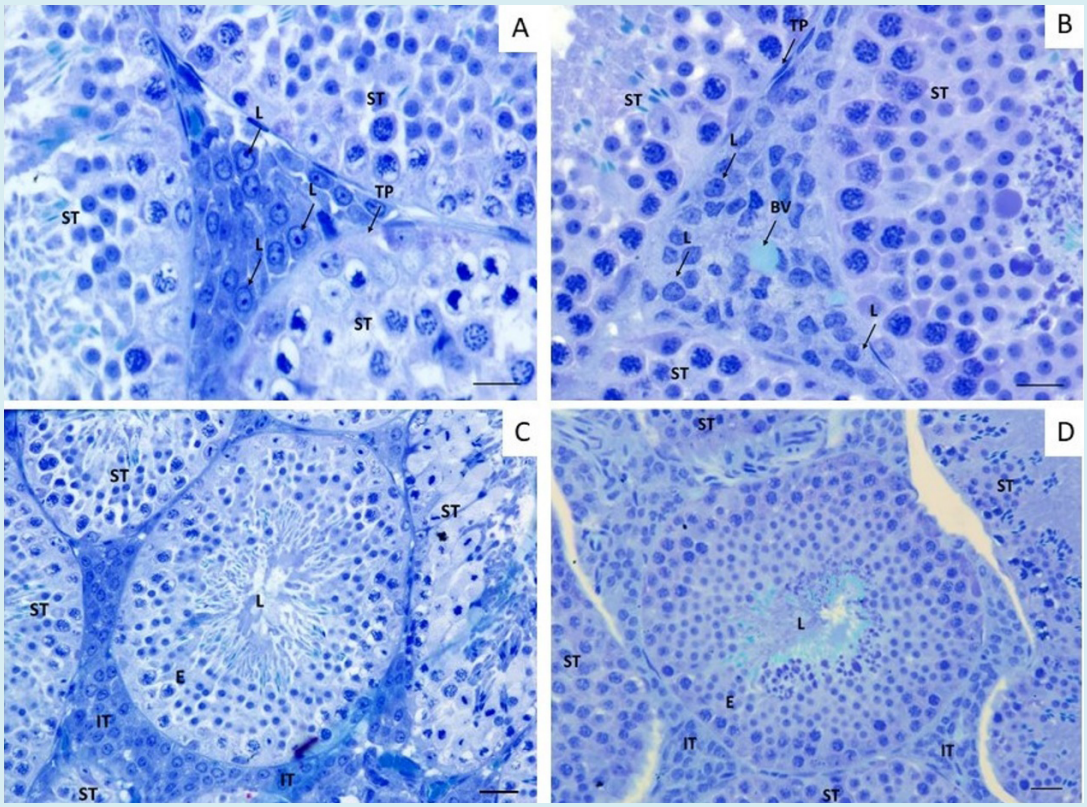

Figure 3: Intertubular and Tubular compartment in *A. lituratus* testis following 3 days exposure to GLY. **a** Intertubular compartment of control bats; **b** Tubular compartment of control bats; **c** Tubular compartment of bats exposed a 4 mL/ 100 mL of GLY; **d** Tubular compartment of bats exposed a 4mL / 100 mL of GLY. BV Blood vein; L Leydig Cell, IT Intertubular compartment; TB Tubular compartment; L Lumen; TP Tunica propria; E Epithelium. Toluidine blue. Scale bar = 20 µm.

Discussion

Animals exposed to GLY showed a reduction in final body mass compared to animals in the control group (Table 1). Similar results were found in bats exposed to lindane organochlorine for three days and pyrethroid deltamethrin for seven days [38,39]. Possibly these findings may be justified by the metabolic stress caused by exposure to pesticides such as organochlorines and organophosphates. In addition, these compounds induce greater consumption of body energy reserves with consequent loss of body mass [40]. In addition to loss in body mass, changes in target organs such as glands, nervous and liver tissue are common [21,41,42]. This is because many pesticides cause disturbance in the cognitive and reproductive capacity of mammals due to their characteristic endocrine disruption [43,44]. In our study, no significant changes in testis weight and gonadosomatic index were observed after pesticide exposure (Table 1), possibly due to short-term exposure which generally promotes microstructural changes in tissues that are macroscopically perceived later.

Regarding the oxidative profile, our findings showed that the testicles of GLY-treated bats did not show changes in MDA levels. However, PCN levels were increased in the herbicide treated groups (Figure 1A & 1B). MDA is the main

marker of cell lipid peroxidation, especially membrane triglycerides [45]. High concentrations of this marker indicate that free radicals are acting and promoting lipid oxidation, compromising the cell's electrolyte balance [45]. Despite the high amount of this marker inside the cell, there was no significant difference between the groups, possibly due to the increase of the antioxidant enzyme CAT that acts by accelerating the passage of electrons and consequently preventing the action of these radicals in cell membranes. As more free radicals and ROS are produced in the tissue, greater the oxidative stress and, consequently, the greater cellular and tissue damage [46]. To try to eliminate these highly reactive compounds, cells increase the expression of antioxidant enzymes to protect tissues from the devastating action of these molecules [47].

However, only the protective action of the CAT enzyme was not sufficient to prevent damage to intra and extracellular proteins in the testicular tissue as the amount of carbonylated proteins (PCN) increased after GLY exposure (Figure 1B). The increase in PCN is directly linked to protein oxidation and consequently its denaturation, compromising important processes within the cell such as gene translation and transcription, as well as altering all cellular metabolism [48]. These findings show the negative effects of GLY on maintaining the redox balance of the cell indicating toxicity and detrimental ability in testicular tissue. Similar results were found by Astiz M, et al. [49] who analyzed the effect of pesticide GLY on liver tissue concentrations 1/50 and 1/250 and observed increased MDA and PCN after exposure compared to animals receiving water alone.

The role of these enzymes is critical in maintaining the redox balance of cells and maintaining their functionality. The enzyme SOD catalyzes the transformation reaction of the superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and CAT catalyzes the reaction of transforming H_2O_2 to water and molecular oxygen, decreasing the oxidizing action of superoxide ion and H_2O_2, regulating the cellular homeostasis [50]. GST is responsible for the second phase biotransformation, i.e. detoxifying toxic compounds and / or the metabolites produced in the SOD and CAT phase I biotransformation [51]. In our study, CAT enzyme activity was higher in the treated group than in the control group (Figure 2A), protecting against oxidative damage, and the levels of the SOD and GST antioxidant enzymes in the treated group did not differ from control (Figures 2B & 2C), probably due to the short exposure time to the GLY for bats. Possibly the detrimental effect of GLY is associated with fast cell penetrating surfactant compounds which increase the influx of the enzyme lactate dehydrogenase (LDH) and the release of Ca^{2+}. The Ca^{2+} ions are considered one of the greatest mitochondrial ROS stimulants, so excess of intracellular Ca^{2+} causes overload and disruption of mitochondrial membranes and decreased synthesis of ATP, altering redox balance, causing disturbances in signaling pathways and consequently cell death in the cells that make up the testicular tissue [52,53].

Testis of bats exposed to GLY showed changes in histomorphometric parameters (Figure 3). Parameters such as diameter and luminal area increased in pesticide-exposed bats when compared to control (Table 2). Increased lumen may be associated with fluid accumulation following aggression by Sertoli cells. Sertoli cells participate in the formation of the occlusion junctions that make up the blood-testicular barrier, in addition to secreting fluid into the tubular lumen [9]. Although no significant changes in tubule diameter and epithelium height were observed, in the group receiving GLY the tubule diameter was larger and the epithelium height was smaller, resulting in a significant lumen increase. The tubule epithelium ratio (TER) showed a significant increase (Table 2) in animals exposed to GLY. The closer to 1 the value of TER is the greater the area of the seminiferous epithelium. Therefore, GLY treatment resulted in a smaller area of the epithelium in relation to the tubular area, probably due to the endocrine disrupting effect of this herbicide. Similar results were found in rats receiving GLY doses of 5, 50 and 250mg / kg during the prepubertal period. The authors reported increased luminal diameter and reduced germinal epithelium due to the anti-androgenic effect of this herbicide [9].

In the testicles of bats exposed to herbicide there was a reduction in the number of type A spermatogonia, primary pre-leptotene / leptotene spermatocytes and Sertoli cells (Table 3). However, the reduction in cell count of the initial spermatogenic lineage did not change the spermatogenic indices (mitotic, meiotic and general yield) and Sertoli indices evaluated. Probably this reduction is related to the location of these cells in the basal environment of the seminiferous epithelium, below the hematotesticular barrier, being more exposed to xenobiotic contamination. Sertoli cells, when mature, participate in primary spermatocyte proliferation, maintenance of spermatogenic lineage cells, and hematotesticular barrier formation [54]. Similar results were presented by nonylphenol surfactant, a contaminant present in the commercial formulation of GLY, which also drastically altered the morphology of Sertoli cells leading to apoptosis of this cell, in addition to altering the homeostasis of Sertoli Ca^{2+} and hydroelectrolytic balance in rats [55]. In our study, there were no changes in the most advanced spermatogenesis cell lines or changes in the overall spermatogenic process yield, probably due to the exposure time of only 3 days. Chronic GLY exposure has been shown to promote significant morphological changes in all spermatogenic germ line cells [56]. Based on this and considering that the animal may be exposed to these contaminants daily in its natural habitat, it

is believed that the effects of chronic exposure could be more severe and affect all cell lines. Regarding the intertubular parameters, there was an increase in the percentage of macrophages, percentage and volume of lymphatic space and decrease in the percentage and number of Leydig cells per gram of testis in animals exposed to GLY (Table 4). The increase in macrophage percentage may have been the result of a proinflammatory process caused by the increase in GLY-induced EROS. The increase in EROS stimulates the release of proinflammatory by macrophages and may cause a decrease in the proportion of Leydig cells, as observed in our study [57,58]. The decrease in Leydig cell number also can be associated with possible changes caused by GLY in proteins involved in the cell cycle. GLY exposure is known to inhibit cell cycle check proteins [7]. In general, changes in this group of proteins cause an increase in cell duplication and consequently cell numbers, but decrease their metabolic function [59]. GLY-exposed animals show up to 35% drop in plasma testosterone concentrations [60-62]. This herbicide can mimic the function of a natural hormone in living things or inhibit the normal activity of the hormone itself [63]. Low testosterone levels promote drop in epithelium height and lumen diameter, directly affecting epididymis, seminal vesicle and maintenance of sperm nutrition. These changes may result in damage to the male reproductive system including malformations and epigenetic changes in future offspring [9,64].

Conclusion

After evaluating the effects of GLY exposure on *Artibeus lituratus* fruit-eating bats for three days, there was presence of testicular histomorphometric changes, such as lumen enlargement, reduction of initial spermatogenic cells and Sertoli cells, and percentage increase and Leydig cell number. We believe that these findings are directly linked to the free radical generation capacity of this pesticide and consequently its high damaging power to and cellular proteins. The increase of the oxidative marker PCN accompanied by the increase of the antioxidant enzyme CAT shows that GLY causes testicular oxidative stress. However, further studies, especially dose response, are needed to identify the minimum dose of exposure to this pesticide capable of promoting significant morphological changes during short-term exposure. Thus, it can be concluded that acute exposure to GLY herbicide is enough to induce cellular, morphological and imbalance redox changes in the testicles of fruit-eating bats and, in the long run, may cause damage to the reproduction and maintenance of the species.

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