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Peripubertal Exposure to Glyphosate-Based Herbicides Promotes Histopathological Impairment in the Structure of the Diaphragm Muscle of C57BL/6 Mice

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SUMMARY

Introduction. Glyphosate is an organophosphate herbicide most used in Latin America, with multisystemic effects, including the respiratory system. In this sense, the objective of this research was to analyze the muscle fibers and neuromuscular junctions (NMJs) of the diaphragm muscle of adult mice exposed to the glyphosate-based herbicide in the peripubertal period.

Methods. Twelve male mice were used, divided into a control group (CTL, n = 6), which received water and a glyphosate-based herbicide group (GBH, n = 6), which received 50 mg/Kg/day of Roundup®, both by gavage from 30 to 60 days. At 150 days, the animals were euthanized, and the diaphragm was collected for analysis of the muscle fibers through hematoxylin-eosin, Masson's trichrome and Picrossirius Red and the NMJs through the nonspecific esterases reaction.

Results. Rounded fibers, hypereosinophilic sarcoplasm and enlarged nuclei were found predominantly in GBH. A reduction in body weight, an increase in muscle fiber morphometry, an increase in type III collagen, a decrease in the overlapping of type I and III collagen, and an increase in the area and greater diameter of the NMJs in GBH were observed.

Conclusions. Peripubertal exposure to glyphosate-based herbicides showed morphological changes characteristic of muscle degeneration and altered the morphometry of muscle fibers and NMJs of the diaphragm of adult mice.

KEY WORDS
DOHaD; morphology; neuromuscular junction; pesticide; skeletal muscle.

INTRODUCTION

Glyphosate or N-(phosphonomethyl)glycine is an organophosphate pesticide, with systemic, non-selective and broad-spectrum action (1, 2), is the main active ingredient contained in Roundup® (3). Glyphosate-based herbicides are widely used worldwide (4), especially in South America, where it is the most commercialized herbicide in Brazil, reaching approximately 63% of the pesticide market in 2020 (5). In this sense, its exacerbated use in agriculture has left residues in soil, water and food (1).

Contact with pesticides can occur through mucous membranes, skin, breathing or ingestion (6) and this exposure can trigger different respiratory problems (7). The impairment of the respiratory muscles, caused by organophosphate intoxication, possibly contributes to the high morbidity rate in intoxications (8). The association of exposure to GBH, and other organophosphates, to respiratory changes has received attention due to the increased incidence of respiratory problems in regions of exposure to pesticides (7, 9).

GBH has a potential endocrine disruptor effect (10), with these exogenous substances altering the functions of the endocrine system and, consequently, causing deleterious effects on the health of the organism or its offspring (11). Exposure to these xenobiotics during critical periods of development and maturation, such as fetal, perinatal and puberty, may be related to changes in gene expression patterns since it is during these periods that the organism establishes the main epigenetic patterns (12, 13). Since the consequences of this early exposure to glyphosate-based herbicides may only manifest in later stages of development and/or in adulthood (14).

Puberty is the period of childhood growth that precedes the onset of adolescence (15), it is a process of physical growth, emotional development and great brain plasticity (15). At this stage, there are structural and functional changes that can interfere with metabolism in adulthood (16), thus being a modulation window, where changes propitiated by the environment can lead to damage to normal development and affect the health of the individual, as is the case of exposure to pesticides.

The diaphragm is considered the main muscle of the mammalian respiratory system (17). It is innervated by the phrenic nerves (18) and is composed of different types of fibers, therefore it is classified as a mixed muscle (19). It has some peculiarities such as greater resistance to fatigue, greater blood flow, greater oxidative capacity and greater capillary density. These properties allow for the necessary resistance to high-demand activities throughout life (20).

Due to its association with respiratory capacity, the diaphragm muscle was chosen for this study, where we sought to evaluate the structure of the muscle after chronic exposure to GBH. Thus, the objective of this research was to analyze the histomorphometric and histopathological characteristics of the muscle fibers and the structure of the neuromuscular junctions (NMJs) of the diaphragm of adult mice exposed to GBH during the peripubertal period.

MATERIALS AND METHODS

Ethical approval

The Ethics Committee for Animal Use from the #05/2018 (CEUA - UNIOESTE) approved all the procedures involving animals (date of approval May, 2018). The procedures were in agreement with the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching purposes established by the Conselho Nacional de Controle de Experimentação Animal (CONCEA) and with the international guidelines for animal research (21).

Animals

For the present study, 12 male mice of the C57BL/6 lineage, 21 days old and with a body weight of 20 ± 2 g, were used. The animals were acquired from the central vivarium of the UNIOESTE and were kept in the sectoral vivarium of the Laboratory of Endocrine Physiology and Metabolism - LAFEM, under controlled conditions of temperature (28 ± 2 °C) and luminosity (12 hours light/dark). The animals were randomly divided into two groups, with only two offspring from the same mother allocated to the same group: the control group (CTL, n = 6) and the glyphosate-based herbicide group (GBH, n = 6). All mice received water *ad libitum* and were fed a Nuvilab® regular chow (Quimtia S.A., Colombo, Paraná, Brazil) from weaning until 150 days of age.

Exposure to GBH

From 30 to 60 days of life, daily by gavage, the GBH group received 50 mg/Kg/day of (22) Roundup® Original DI solution (Monsanto, Brazil), containing 445 g/L of diammonium salt of N-phosphonomethylglycine (37.0% m/v), of the active component glyphosate and the CTL group received water. The GBH solution and the water had the pH corrected between 5 and 5.5 with hydrochloric acid.

Euthanasia and tissue collection

After 150 days of life, the animals were weighed and measured to obtain the nasoanal length. Then, the mice were anaesthetized with a mixture of 9 mg/Kg xylazine hydrochloride (Anasedan®, Vetbrands, Brazil) and 90 mg/Kg ketamine hydrochloride (Dopalen®, Vetbrands, Brazil). Then, laparotomy was performed to collect the organs. Retroperitoneal and peritoneal fat were collected and weighed and standardized by body weight.

For the collection of the diaphragm muscle, the animals were kept in a ventral position and an incision was made in the median region just below the thorax, with subsequent reflection of the skin and muscles. The removal of the diaphragm consisted only of its costal part, being divided into right and left hemicupules. Only the right hemicupula was weighed. Then, each hemicupules was fragmented into two segments, and in the right hemicupules, the upper and lower fragments were destined for histological processing in paraffin. In the left hemicupula, the upper and lower segments were destined for histochemical study of the NMJs. Then, the fragments were stored in specific fixatives to carry out the histological and histochemical study.

Histological procedures

For the histomorphological analysis of the diaphragm muscle, cross sections of the proximal portions of the right cupula were fixed in Methacarn (60% Methanol, 30% Chloroform and 10% Glacial Acetic Acid), and then destined for histological processing for embedding in paraffin. Subsequently, 7 µm-thick cross-sections of the muscles were obtained, which were collected on glass slides, and used for histomorphological and histopathological analysis. One slide from each animal was stained using Hematoxylin and Eosin and destined for histomorphometric analysis. The other two slides were used for the analysis of the connective tissue, one stained with the Masson's Trichrome technique, and the other stained with the Picrosirius Red technique.

Histochemical procedures

For the morphological and morphometric analysis of the NMJs, the middle portions of the left antimeres were longitudinally sectioned with the aid of a stainless-steel blade to obtain several muscle fragments. These fragments were submitted to the Unspecific Esterase reaction and after the reaction mounted on a glass slide for analysis under light microscopy.

Images obtention

To acquire images for subsequent morphometric analysis, the slides stained by the pricosirius red technique were photographed in a polarized light microscope (Carl Zeiss™ AxioImager™). The slides obtained from the other techniques were photographed under a light microscope (Carl Zeiss™ Primo Star™). Both microscopes were coupled to the camera (Carl Zeiss™ AxioCam ERc 5s) and connected to the ZEN 3.1 program (Carl Zeiss™). According to the magnification used for the analyses, the following were used: 2 microscopic fields, for 40x magnification; 5 microscopic fields for 100x magnification; 10 microscopic fields for 200x magnification and 20 microscopic fields for 400x magnification. For image analysis, Image-Pro Plus 6.0® software (Media Cybernetics, MD, Rockville, USA) was used. For the analysis of connective tissue, collagen, lipid deposits and immunohistochemistry, pixel quantification was used, using the GNU - GIMP 2.10.30 program (GNU General Public License®, Berkeley, California, United States).

Histomorphometric measurements

For histomorphometric analysis, images containing whole muscle fibers were used and the relative area of each image was measured. Fibers, nuclei and capillaries were counted. From these data, it was calculated: fiber density (DENS = amount fibers per mm^2), the ratio of capillaries per fiber (ratio CF = amount of capillaries/amount of fibers), the ratio of nucleus per fiber (ratio NF = amount of nucleus/amount of fibers) and percentage of the central nucleus (NC%). The cross-sectional areas (CSA) of muscle fibers (n = 150 events per animal) were also measured, as well as the largest (LD) and smallest (SD) diameters of muscle fibers (n = 150 per animal). These data were used to calculate the myonuclear domain (muscle fiber CSA/NF ratio).

The quantification of connective tissue was performed on slides stained with Masson and based on this total amount of connective tissue, the value for each envelope (epimysium, perimysium and endomysium) was calculated. Collagen quantification was performed similarly, quantifying the pixels referring to type I collagen (in red) and type III collagen (in green) and the overlapping areas of both (in yellow). To measure the NMJs, the CSA, LD and SD were measured in 150 neuromuscular junctions. The diameter ratio (LD/SD) was calculated to verify the possible roundness of the NMJs, and the Ratio of CSA Fiber to CSA NMJ.

Histopathological Index Analysis

For the characterization of the structural alterations of the muscular tissue, five patterns of reaction (23) were used. Based on the previously described reaction patterns, the slides were evaluated blindly, classifying the reaction patterns and their extensions in the sampling areas. The results of each evaluator were used to construct the index. For the quantitative evaluation of the histopathology, two values were used: 1) importance factor (w), previously tabulated and which takes into account the pathological importance of the alteration: 1 – Minimal importance, 2 – Moderate importance, 3 – Great importance; 2) score value (a), which varies according to the extent of the alteration, being: (0) none, (2) minimal, (4) moderate and (6) extensive occurrence. From these values, the mathematical calculation for the injury rates was developed, which can be used both for the quantification of the reaction patterns and for the total evaluation of the tissue.

Statistical analysis

Normality (Shapiro-Wilk test) and homoscedasticity tests (Barttlet's test) were performed for all variables and those that were following these assumptions were analyzed by the Student's t-test to examine the effect of glyphosate. When the premises were not in agreement, the Mann-Whitney U test was performed. All analyses were performed with alpha significance level $\alpha = 0.05$.

Then, the matrices of the variables were standardized and analyzed using the principal component analysis (PCA). With the PCA, factorial loads are established for each variable and analyzed in response components. The data provided by the PCA is reduced, the data overlays are removed, and the most representative linear units of the data are known. The factor loads of the main components were evaluated in terms of statistical significance using a student t-test. As the main components (PC) are ordered in decreasing order of importance for a structure of variance of the data set, the greater the retention of the total variance in a smaller number of linear formulas, the better the application of the procedure to the experimental data. All procedures were performed in software R version 4.3.0.

RESULTS

Body characteristics

Regarding body characteristics (**table I**), GBH animals showed a reduction of approximately 9% in body weight, when compared to CTL (t = 2.9216, df = 6.943, P-value = 0.0225). However, there was no difference between groups in nasoanal length (t = 0.8613, df = 6.7846, P-value = 0.4185), Lee's index (t = 1.5126, df = 8.1398, P-value = 0.1682), retroperitoneal (t = -0.43171, df = 9.892, P-value = 0.6752) and peritoneal (W = 13, P-value = 0.4848) fat weight, body adiposity (W = 14, P-value = 0.5887) and diaphragm muscle (t = -0.16552, df = 9.2047, P-value = 0.8721) weight.

Diaphragm muscle morphology

The morphological characteristics of the diaphragm muscle were standard (**figure 1**). The morphology of the radial sections of the right hemicupule showed elongated and multinucleated polygonal fibers with nuclei displaced immediately below the sarcoplasmic membrane (**figure 1A-H**). The muscle fibers are individually enveloped by the connective tissue of the endomysium, organized into muscle fascicles delimited by the perimysium, with blood capillaries associated with these connective envelopes. However, fibers with increased nuclei and some with a basophilic halo were observed in greater numbers in the GBH group (**figure 1D,F**). Central nuclei were also found in both groups equally and fibers with a rounded shape and hypereosinophilic sarcoplasm were found in the CTL and GBH groups, but predominantly in the GBH group (**figure 1G,H**). It was also possible to observe an increase in type I

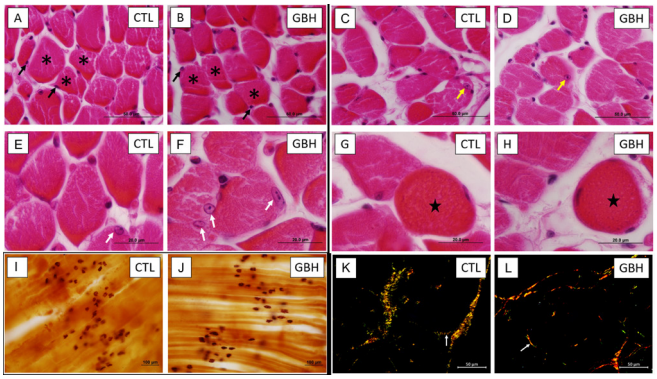

Figure 1. Photomicrographs of the diaphragm muscle of 150-day-old mice.

Cross section, HE (**A-H**). (**A,B**) Muscle fibers (asterisk) and peripheral nuclei (black arrows). (**C,D**) Centralized nuclei (yellow arrows). (**E,F**) Enlarged basophilic nuclei (white arrows). (**G,H**) Rounded muscle fibers with hypereosinophilic sarcoplasm (star). Longitudinal section, Nonspecific Esterase reaction (**I,J**). Morphological characteristics of JNMs. Picrosirius Red (**K,L**). Observe the perimysium (arrow). Collagen type I (in red), collagen type III (in green) and areas of collagen type I and III overlaps (in yellow). CTL: Control group. GBH: Glyphosate-based herbicide group..

Table I. Body characteristics.

Variable	CTL	GBH	P-value
Body Weight (g)	30.81 ± 1.04	27.75 ± 2.33[A]	0.0225
Nasoanal Length (cm)	9.11 ± 0.43	8.95 ± 0.18	0.4185
Lee Index (g/cm³)	342.13 ± 12.61	333.08 ± 7.49	0.1682
Retroperitoneal (g/100 g)	0.24 ± 0.02	0.24 ± 0.02	0.6752
Perigonadal (g/100 g)	1.01 ± 0.18	1.25 ± 0.49	0.4848
Adiposity (%)	1.24 ± 0.20	1.51 ± 0.51	0.5887
Diaphragm (g/100 g)	0.15 ± 0.02	0.15 ± 0.02	0.8721

CTL: Control group (n = 6); GBH: Glyphosate group (n = 6); A: statistical difference *versus* control group ($p < 0.05$).

collagen deposition in GBH animals, with a greater predominance of reddish colouration (**figure 1L**). The NMJs found in the diaphragm muscle of the animals were polymorphic, with an oval, round or elliptical shape, however, in the GBH animals it was possible to notice an increase in junctions in elliptical and oval shapes (**figure 1I,J**).

Diaphragm muscle fibers' structure

Regarding the structure of the muscle fibers (**table II**), it was possible to observe that the GBH animals presented a reduction of approximately 9% in fiber density (t = 5.8472, df = 9.4036, P-value = 0.0002), 25% in the ratio of nuclei per fiber (t = 5.9281, df = 5.8347, P-value = 0.0011), 37% in the percentage of central nuclei (t = 5.0659, df = 5.4176, P-value = 0.0031) and 32% in the ratio of capillaries to fiber (t = 5.1561, df = 7.4976, P-value = 0.0011).

However, the GBH animals showed an increase of 15% in the cross-sectional area (t = -5.0021, df = 9.9462, P-value = 0.0005), 10% in the largest diameter (t = -6.0698, df = 8.5282, P-value = 0.0002) and 7% in the smallest diameter (t = -3.9581, df = 9.3541, P-value = 0.0031) of the muscle fibers when compared to the CTL, however, there was no difference between the groups in the ratio of these diameters (t = -2.1834, df = 5.7764, P-value = 0.0734). Finally, the GBH animals showed an increase of approximately 60% in the myonuclear domain (W = 0, P-value = 0.0021) when compared to the CTL animals.

Extracellular matrix composition and neuromuscular junction

Regarding the characteristics of the extracellular matrix (**table III**), it was possible to observe that the GBH animals presented a 43% increase in the proportion of type I collagen (W = 0, P-value = 0.0021) when compared to the CTL animals. Conversely, the GBH animals showed a 23% reduction in the overlapping of collagens I and III (W = 36, P-value = 0.0049), when compared to the CTL. However, there was no difference between the groups in the quantification of type III collagen (W = 17, P-value = 0.9372), nor the quantification of total connective tissue (W = 11, P-value = 0.2971).

Table II. Diaphragm muscle fibers' structure.

Variable	CTL	GBH	P-value
Fiber Density (mm²)	1338.81 ± 27.94	1229.74 ± 36.14[A]	0.0002
Fiber Cross-Sectional Area (µm²)	699.85 ± 44.78	824.67 ± 41.60[A]	0.0005
Fiber Largest Diameter (µm)	35.43 ± 1.41	39.61 ± 0.91[A]	0.0002
Fiber Smallest Diameter (µm)	22.94 ± 0.69	24.62 ± 0.75[A]	0.0031
Diameters Ratio	1.54 ± 0.01	1.61 ± 0.04	0.0734
Nuclei per Fiber Ratio	1.38 ± 0.14	1.01 ± 0.04[A]	0.0011
Central Nuclei (%)	3.08 ± 0.11	1.94 ± 0.54[A]	0.0031
Mionuclear Domain (µm²) *	507.93 ± 32.79	817.63 ± 54.85[A]	0.0021
Capilar per Fiber Ratio	0.57 ± 0.08	0.38 ± 0.04[A]	0.0011

CTL: Control group (n = 6); GBH: Glyphosate group (n = 6); A: statistical difference *versus* control group (p < 0.05); *Mann-Whitney Test.

Table III. Extracellular matrix composition and neuromuscular junction.

Variable	CTL	GBH	P-value
Connective Tissue (% pixels)*	2.98 ± 0.97	3.43 ± 0.66	0.2971
Collagen Type III (% pixels)*	16.53 ± 2.24	16.74 ± 1.25	0.9375
Collagen Type I (% pixels)*	28.66 ± 3.84	40.54 ± 4.88[A]	0.0021
Collagen Merge (% pixels)*	54.82 ± 2.49	42.72 ± 6.05[A]	0.0049
NMJ Cross-Sectional Area (µm²)	259.93 ± 13.86	346.25 ± 21.07[A]	0.0000
NMJ Largest Diameter (µm)	24.05 ± 1.38	28.30 ± 0.57[A]	0.0002
NMJ Smallest Diameter (µm)	12.35 ± 0.32	14.21 ± 0.68[A]	0.0005
NMJ Diameters Ratio	1.95 ± 0.15	1.99 ± 0.07	0.5489
CSA Fiber / CSA NMJ Ratio	2.70 ± 0.25	2.39 ± 0.17[A]	0.0345

CTL: Control group (n = 6); GBH: Glyphosate group (n = 6); A: statistical difference *versus* control group (p < 0.05); *Mann-Whitney Test.

Meanwhile, the neuromuscular junctions (**table III**) showed an increase of approximately 32% in the cross-sectional area (t = -8.3817, df = 8.6472, P-value = 0.0000), 20% in the largest diameter (t = -6.943, df = 6.7036, P-value = 0.0002) and 16% in the smallest diameter (t = -6.0155, df = 7.1036, P-value = 0.0005) in the GBH animals when compared to the CTL. However, there was no difference between groups in the ratio of diameters (t = -0.62626, df = 7.486, P-value = 0.5498), an important predictor of structural alteration of the joints. Finally, the ratio between the cross-sectional area of muscle fibers and neuromuscular junctions (t = 2.4861, df = 9.0216, P-value = 0.0345) showed a reduction of approximately 13% in GBH animals when compared to CTL.

Histopathological index

Regarding the histopathological characteristics (**table IV**), it was possible to notice that the GBH animals presented scores approximately 300% higher than the CTL animals in the total index of alterations (W = 0, P-value = 0.0048). Similarly, across subdivisions of the index, GBH animals showed a 360% increase in the circulatory and inflammatory disorders (CID) score (W = 0, P-value = 0.0045), a 300% increase in the muscle fiber alterations (MFA) score (W = 0, P-value = 0.0042), a 550% increase in the associated tissue alterations (ATA) score (W = 0.5, P-value = 0.0055), and 110% increase in progressive changes (PC) score (W = 5.5, P-value = 0.0455) when compared to CTL animals.

Principal components analysis

In the case of Principal Components Analysis (**figure 2**), the multivariate arrangement of the data in significance showed that in only 3 principal components, there was a retention of 93.06% of the variance of the model data. Regarding the First Component (data = Total Index, Muscle Fiber Alterations, Mionuclear Domain, NMJ Cross-Sectional Area and Largest Diameter, Circulatory and Inflammatory Disorders, Nuclei per Fiber Ratio, Fiber Density, Associated Tissues Alterations, Collagen Type I and Collagen Merge; Eigenvalue = 15.05; Retention Variance = 75.27%), the GBH animals showed a positive displacement concerning the CTL, showing that these animals presented greater alterations in the muscle fiber, nuclear alterations, alterations histopathological changes in the characteristics of the neuromuscular junctions and the composition of the extracellular matrix (t = -25.58, df = 9.8254, P-value = 0.0000). As for the Second (data = Cross-Sectional Areas Ratio, Progressive Changes; Eigenvalue = 2.09; Retention Variance = 10.90%) and Third (data = NMJ Smallest Diameter, Capillary per Fiber Ratio,

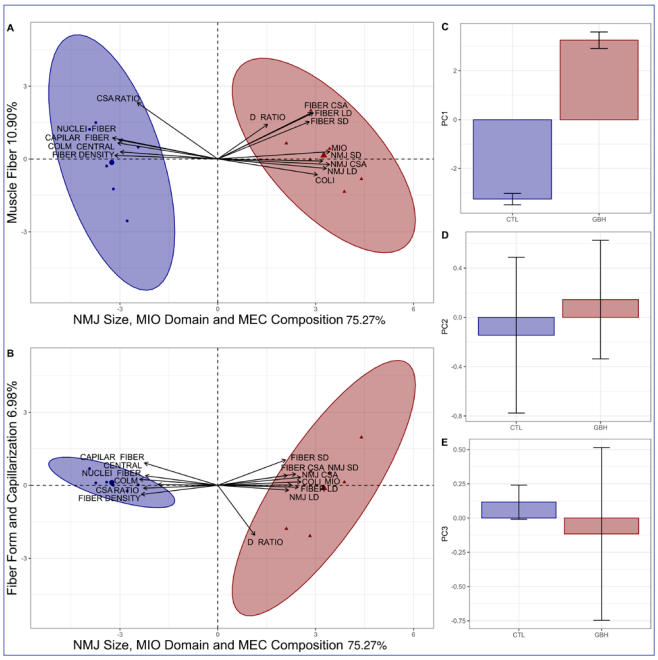

Figure 2. Principal Component analysis **(A, B)** and variation of data in each component **(C-E)**.

CTL: control group; GBH: glyphosate group. First Component (data = Total Index, Muscle Fiber Alterations, Mionuclear Domain, NMJ Cross-Sectional Area and Largest Diameter, Circulatory and Inflammatory Disorders, Nuclei per Fiber Ratio, Fiber Density, Associated Tissues Alterations, Collagen Type I and Collagen Merge; Eigenvalue = 15.05; Retention Variance = 75.27%); Second (data = Cross-Sectional Areas Ratio, Progressive Changes; Eigenvalue = 2.09; Retention Variance = 10.90%) and Third (data = NMJ Smallest Diameter, Capillary per Fiber Ratio, Central Nuclei, Fiber Cross-Sectional Area, Fiber Largest Diameter, Diameters Ratio; Eigenvalue = 1.01; Retention Variance = 6.89%) components.

Table IV. Histopathological index.

Variable	CTL	GBH	P-value
Total Index (max 320)*	18.00 ± 2.82	71.00 ± 3.03[A]	0.0048
CID (max 36)*	3.33 ± 1.63	15.33 ± 2.42[A]	0.0045
MFA (max 182)*	10.00 ± 2.19	40.00 ± 1.78[A]	0.0042
ATA (max 36)*	1.33 ± 1.63	8.67 ± 3.01[A]	0.0055
PC (max 36)*	3.33 ± 2.06	7.00 ± 2.75[A]	0.0455

CTL: Control group (n = 6); GBH: Glyphosate group (n = 6); circulatory and inflammatory disorders (CID); muscle fiber alterations (MFA); associated tissue alterations (ATA); progressive changes (PC); A: statistical difference *versus* the control group ($p < 0.05$). *Mann-Whitney Test.

Central Nuclei, Fiber Cross-Sectional Area, Fiber Largest Diameter, Diameters Ratio; Eigenvalue = 1.01; Retention Variance = 6.89%) components, there was no difference between the CTL and GBH groups (t = -0.1642, df = 9.9892, P-value = 0.8728; t = 0.12797, df = 6.7539, P-value = 0.9019, respectively).

DISCUSSION

The present study sought to evaluate the histomorphometric and histopathological changes in the diaphragm muscle of adult animals chronically exposed during the peripubertal period to a dose of 50 mg/Kg/day of glyphosate-based herbicide (GBH). Thus, we observed that the animals exposed to GBH showed a reduction in body weight, without alteration in the other evaluated body characteristics (NAL, LEE, Adiposity, Diaphragm weight). However, in addition to the morphological changes found, the GBH animals showed an increase in the size of muscle fibers (Cross-Sectional Area, Largest and Smallest Diameters), in addition to an increase in the myonuclear domain. On the contrary, they presented a reduction in the density of muscle fibers, in the ratios of nucleus and capillaries per fiber and a reduction in the percentage of central nuclei. There was also an alteration in the components of the extracellular matrix, with a reduction in the overlapping of collagen I and III, due to the increase in the deposition of type I collagen), in addition to a change in its predominant format, contributing to the decrease in the ratio between the areas of muscle fibers and NMJ. Finally, the histopathological analysis showed an increase in total muscle damage in the animals exposed to GBH, due to the increased presence of circulatory and inflammatory disorders, muscle fiber alterations, associated tissue alterations and progressive changes.

Exposure to GBH is associated with changes in the development of animals, especially during critical periods of life, such as during pregnancy (24), perinatal (25) and peripubertal periods (26). Chronic and subchronic exposures to doses of 250 mg/kg/day and 500 mg/kg/day, regardless of the sex of the animals, have been reported to reduce weight gain in animals in the peripubertal period, such as those described in the present study (27, 28). Alterations in the weight gain of the animals may be related to several factors, in particular the reduction in the body's accumulation of lipids, the reduction in the glycogen reserve and the reduction in the synthesis of complex proteins, in this sense an effect on the organism's ability to reserve energy (26). This reduction in reserve energy supply may be related to the large consumption of ATP in the reactions involved in phase I and phase II of xenobiotic detoxification, pathways classically altered in chronic exposure to organophosphates (28, 29). Another factor that may justify the change in weight gain of the animals is the possible endocrine-disrupting effect of this herbicide (1), leading to lower synthesis of steroidal hormones, responsible for the positive modulation effect on the body growth rate during puberty (4, 30).

As one of the main routes of human contamination with GBH is the nasal-oral route, intoxication by this and other organophosphates may be related to respiratory problems, such as cases of respiratory failure (8, 31, 32). One of the main consequences of chronic respiratory failure is the progressive increase in tone and strength of muscle contraction, due to the workload exerted on the muscle (33, 34). In cases of alteration in the diaphragmatic workload, there is a significant variation in the recruitment of motor units, causing muscle hyperactivation (33). In this sense, it is common to find hypertrophy in muscle fibers (35) and in the junctional postsynaptic compartments (36), as found in the present study.

Also, exposure to GBH may be related to functional changes in the cardiovascular system (25, 37). In experimental models of heart failure, after biochemical evaluation of the diaphragm muscle, there was evidence of induction of oxidative distress and morphological and functional changes (38). In this sense, it is possible to associate the diaphragmatic alterations found in the present study as a result of pulmonary congestion, resulting from cardiac mechanical alteration and, consequently, reduction in cardiac output, caused by the use of glyphosate (37).

In addition to changes in fiber size, any change in functional demands can make muscle tissue respond differently, altering its structural, metabolic and functional aspects (39). The increase in myonuclear volume and domain found in animals exposed to GBH may be related to the toxic effect of organophosphates on the tissue, since, as a consequence of the cellular toxicity promoted by GBH, there may be a greater expression of proteins of the antioxidant defense system and repair (40). That way. nuclei with increased volume may indicate an increase in the protein synthesis rate (41), as well as related to the muscle fiber regeneration process (42). However, the reduction in nuclear density in animals exposed to glyphosate impairs this feature. In this sense, the reduction of central nuclei may signal a failure or reduction in the regenerative capacity of the muscle tissue of these animals, since this position of the nuclei is the determining factor for maintaining the health of the tissues since their presence in the muscle fibers indicates correct and coordinated muscle regeneration process (43, 44).

Finally, the connective envelopes are responsible for the structural maintenance of muscle fibers and for allowing the individually generated force of contraction to act on the

entire muscle. It is important to note that several pathological alterations in the muscle are also associated with some degree of thickening of the extracellular matrix, or the collagen composition of this matrix (45). Therefore, due to muscle damage caused by exposure to GBH, there may be an imbalance in the proliferation of growth factors involved in muscle regeneration and mesenchymal growth, including fibroblast growth factor (FGF), which acts by stimulating tissue synthesis. connective tissue, especially in the synthesis of collagen I, which determines the tensile strength and rigidity of the tissue (45). In this sense, a structural alteration, such as an increase in fibrotic tissue, can negatively impact the arrival of muscle growth signalling factors. Although the increase in connective tissue is an indicator of muscle regeneration, collagen overproduction often results in the formation of scar tissue that impairs muscle function (46).

According to the literature, the toxicity of glyphosate is low in subchronic or chronic exposure in adult individuals. However, this herbicide can induce metabolic and structural alterations in critical periods, such as the peripubertal period, a fact that can lead to the emergence of several associated comorbidities (47, 48). In the present study, we could observe that chronic exposure to lower doses than those studied in the literature in the peripubertal period was sufficient to promote changes in muscle fibers that may indicate a change in the mechanism of the functionality of the diaphragm muscle. We also found results that suggest an important histopathological alteration, which together with the nuclear alterations, can translate into a reduction in the muscle repair capacity, which, associated with the alteration of the neuromuscular junctions, can contribute to accentuate these damages throughout the life of these animals.

CONCLUSIONS

Because of the results, it is concluded that peripubertal exposure to GBH generates muscle adaptations that are dictated throughout development, with changes in weight and diaphragmatic muscle tissue being found in adduct mice. GBH led to histomorphological adaptations with hypertrophy of muscle fibers, an increase in the nuclear domain and type I collagen and NMJs, and changes involved in the process of muscle remodelling.

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DATA AVAILABILITY

Data from this study can be accessed at: Zazula M, Torrejais MM. GBH diaphragm DOHaD. Mendeley Data. 2023. doi: 10.17632/z77jdscnyc.1.

CONTRIBUTIONS

MFZ, LFCR, MMT: data collection, analysis and interpretation of results, draft manuscript preparation, review of results, final version of the manuscript. APM: data collection, analysis and interpretation of results, draft manuscript preparation, review of results. MCO, AB: data collection, analysis of results. MLB: data collection, analysis and interpretation of results, draft manuscript preparation, review of results.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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