Objective: To evaluate the morphology and morphometry of the muscles extensor digitorium longus and soleus of C57BL/6 females, who were exposed to glyphosate during pregnancy and lactation. Methods: Twelve female mice from the C57BL/6 lineage were used. After detection of pregnancy, they were divided into a Control Group, which received only water, and a Glyphosate Group, which received water with 0.5% glyphosate during pregnancy and lactation. Both groups received ad libitum standard diet. After weaning, the females were euthanized and weighed; naso-anal length was measured, and fats were collected and weighed. The muscles extensor digitorium longus and soleus were collected, and their length and weight were measured. Then, the muscles were fixed in Methacarn to perform the histological study of muscle fibers. Results: Glyphosate Group presented lower weight gain during pregnancy and also lower final body weight and naso-anal length; however, the other body parameters evaluated did not present a significant difference in relation to the Control Group. Significant differences were also not observed in the analysis of muscle fibers and connective tissue. Conclusion: Exposure to 0.5% glyphosate during pregnancy and lactation resulted in lower weight gain during pregnancy, final weight, and naso-anal length. Despite not directly altering the morphology of muscle tissue, these results may indicate enough exposure to interfere with animal metabolism.

Keywords: Glyphosate; Pesticides; Toxicity; Maternal exposure; Muscle fibers, skeletal; Mice, inbred C57BL
INTRODUCTION

Glyphosate (N-(phosphonomethyl)glycine) is an organophosphorus compound that ranked first in the list of the ten best-selling active ingredients in Brazil, in 2018. It is present in the formulation of Roundup® Original DI (Monsanto do Brasil LTDA., São Paulo, SP, Brazil), one of the most widely used herbicides in the world, which accounted for almost 72% of global pesticide use in 2016. Its mechanism of action consists in inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase of the shikimate pathway, responsible for the production of the intermediate chorismate, a compound required in the synthesis of aromatic amino acids essential for plant development.

Although this pathway is not present in mammals, studies have shown that the herbicide is toxic in rats and mice, as well as in humans associated with the genesis of several diseases.

In Brazil, there are still no limits set on glyphosate or any other herbicide in water or soil by regulatory agencies. According to the Environmental Protection Agency (EPA), an agency from the United States, the glyphosate limit in drinking water is 700µg/L, with an acceptable daily dose of 0.05mg/kg per body weight.

However, it is common for the stipulated dose to be exceeded, which in turn, reflects in the increased concentration of this compound in the environment, promoting contamination of rivers and surface waters, and becoming a potential source of exposure in the work environment, during the mixing of chemical compounds, in the application of pesticides, in the cleaning of equipment, or even indirectly, during the handling of contaminated crops or food.

Although some experimental studies report that exposure to glyphosate promotes changes in some tissues, and in the metabolism of the offspring of rats and mice, the effects of exposure to this herbicide during pregnancy and lactation are not known yet. Thus, this study is of great importance for understanding of possible musculoskeletal changes promoted by exposure to glyphosate.

OBJECTIVE

To evaluate the morphology and morphometry of the muscles extensor digitorum longus and soleus of C57BL/6 females exposed to glyphosate during pregnancy and lactation.

METHODS

Obtaining the animals

Initially, 30 C57BL/6 mice of reproductive age were used, 20 females and 10 males, aged between 60 and 90 days, with a mean body weight of 20g to 25g. The animals were kept under controlled temperature (28±2°C) and light conditions (12 hours light/dark), and received standard rodent chow (Supralab, São Leopoldo, RS, Brazil) and filtered water ad libitum throughout the experiment.

All experiments reported in this study were conducted in accordance with national and international legislation, as per the guidelines of the National Council for the Control of Animal Experimentation and the Public Health Service Policy on
Humane Care and Use of Laboratory Animals (PHS Policy), under approval of the Ethics Committee on Animal Use of the Universidade Estadual do Oeste do Paraná (Unioeste), ordinance 3.730, of September 16, 2016, in the city of Cascavel (PR).

Crossbreeding
After 7 days of acclimatization, vaginal smears were taken to follow the estrous cycle of females, which were allocated for mating when they were in proestrus, with the proportion of two females to one male, during the night. In the morning of the following day, the vaginal smear was taken again to identify spermatozoa and the estrous cycle was determined to confirm pregnancy. Females considered pregnant showed the presence of spermatozoa or a 4-day stay in the diestrous phase after mating. The females that were not pregnant were again submitted to the mating process until pregnancy was confirmed.

Glyphosate administration
Once pregnancy was confirmed, the females were placed in individual boxes, separated into Control Group (CTL, n=6), which received filtered water during the entire period of pregnancy (21 days) and lactation (30 days), and Glyphosate Group (GF, n=6), which received the herbicide 0.5% glyphosate Roundup® Original DI in drinking water, from the fourth day of pregnancy until the end of lactation. This dosage had been used in a previous study, (20) and was chosen because it mimics direct groundwater contamination, because it is similar to the amount of pesticide found in water after agricultural practices. (21) The commercial formulation of Roundup® Original DI glyphosate used contained 445g/L of N-(phosphonomethyl)glycine diammonium salt, equivalent to 370g/L (37.0%m/v) of the active component glyphosate [N-(phosphomethyl)glycine].

Euthanasia of females
After 30 days of lactation, weaning occurred and the females were euthanized after completing two estrous cycles. The animals were anesthetized with xylazine hydrochloride (Anasedan®, Vetbrands, Axxon Group, Rio de Janeiro, RJ, Brazil) and ketamine hydrochloride (Dopalen®, Vetbrands, Axxon Group, Rio de Janeiro, RJ, Brazil) at concentrations of 9mg/kg and 90mg/kg, respectively, and were finally euthanized.

Collection of the muscles extensor digitorum longus and soleus
To collect the extensor digitorum longus (EDL) muscle, the skin was detached and the tibialis anterior muscle was removed for dissection and removal of the EDL muscle. The gastrocnemius muscle was removed for dissection and removal of the soleus (SOL) muscle. The EDL and SOL muscles were weighed (g) on analytical scales (Shimadzu UX620H, São Paulo, SP, Brazil) and their length (mm) was measured with the aid of a digital pachymeter (Digimess®, São Paulo, SP, Brazil).

Histological study
For the study of muscle fibers, the EDL and SOL muscles of the right antimer of the pelvic limbs were removed and stored in a glass container with Methacarn fixative. After 24 hours, they were transferred to 70% alcohol and embedded in paraffin, with an n-buty alcohol embedding protocol.

From the analysis of ten microscopic fields (40x lens) for each animal, the EDL and SOL muscles were transversely cut and submitted to hematoxylin-eosin (22) staining, for morphological analysis of the muscle fibers, quantification of the numbers of nuclei and fibers, nucleus-fiber ratio, area, and major and minor diameter of each muscle fiber. The same cutting procedure was performed for Masson’s trichrome staining, (23) which allows quantifying connective tissue, by analysis of ten microscopic fields for each animal (20x lens).

The images of the muscle fibers were obtained using an Olympus BX60® microscope, coupled to an Olympus DP71 camera (Tokyo, Japan), with the aid of the DP Controller 3.2.1 276 software. The Image-Pro Plus 6.0® software (Media Cybernetics, Maryland, USA) was used for morphological and morphometric analysis of the materials.

Statistical analysis
The data obtained were submitted to statistical analysis using the GraphPad Prism® (La Jolla, CA, USA) software, taking into consideration the results of the normality tests. For the data found to be normal, the statistical test used was the Student’s t test, whereas for the data that were not normal, the Mann-Whitney test was used. Values of p<0.05 were considered significant.
RESULTS

Pregnancy and lactation data
The GF Group had lower gestational weight gain (p=0.0327) when compared to the CTL Group (Table 1). However, the data for weight loss during lactation, gestation time, and litter size showed no statistical differences when compared to the CTL Group (Table 1).

Morphological and morphometric analysis of muscle fibers
The evaluation of the muscle fibers of the EDL and SOL muscles showed fibers with preserved morphology, maintaining the polygonal aspect, the presence of peripheral nuclei, and the eventual presence of central nuclei in the two groups studied (Figure 1).

As to the morphometric analysis of the muscle fibers of the EDL and SOL muscles, none of the parameters evaluated showed significant differences between Groups CTL and GF (Table 3).

Table 1. Pregnancy and lactation data from mice in the Control and Glyphosate Groups

| Groups  | Control (CTL) | Glyphosate (GF) |
|---------|--------------|----------------|
| Weight gain during pregnancy, g | 12±1.4 | 9.5±1.9* |
| Weight loss during lactation, g | 1.8±1.9 | 1.3±2.4 |
| Time of pregnancy, days | 19±1.4 | 20±0.82 |
| Litter size | 6.7±1.6 | 5.0±1.5 |

Values expressed as mean±standard deviation.
* p<0.05. Student’s t test.

Table 2. Body parameters of mice in Control and Glyphosate Groups

| Body parameters | Control (CTL) | Glyphosate (GF) |
|-----------------|--------------|----------------|
| Final body weight, g | 23±1.3 | 22±0.79* |
| NAL, cm | 9.7±0.5 | 9.2±0.30* |
| Weight of fats, mg | 469±42 | 602±173 |
| EDL muscle weight, mg | 7.2±1.8 | 10±4.9 |
| EDL muscle length, mm | 7.3±0.98 | 7.9±1.0 |
| Soleus muscle weight, mg | 6.1±0.81 | 6.2±2.2 |
| Soleus muscle length, mm | 6.1±1.3 | 6.3±1.2 |

Values expressed as mean±standard deviation.
* p<0.05. Student’s t test. Mann Whitney test.

Table 3. Morphometric analysis of the muscle fibers and connective tissue of the extensor digitorum longus muscle of mice in the Control and Glyphosate Groups

| Muscle | Control (CTL) | Glyphosate (GF) |
|--------|--------------|----------------|
| EDL muscle | | |
| Area, \( \mu m^2 \) | 115±34 | 152±96 |
| Major diameter, \( \mu m \) | 15±1.8 | 17±4.8 |
| Minor diameter, \( \mu m \) | 9.6±1.4 | 11±3.2 |
| Fiber density | 536±131 | 578±243 |
| Number of peripheral nuclei | 617±109 | 707±195 |
| Number of central nuclei | 1.0±0.71 | 0.80±1.3 |
| Nucleus/fiber ratio | 1.2±0.16 | 1.3±0.22 |

SOL muscle | | |
| Area, \( \mu m^2 \) | 157±24 | 187±69 |
| Major diameter, \( \mu m \) | 18±1.4 | 19±3.1 |
| Minor diameter, \( \mu m \) | 11±0.83 | 12±2.7 |
| Fiber density | 414±123 | 430±175 |
| Number of peripheral nuclei | 927±237 | 927±265 |
| Number of central nuclei | 2.2±2.4 | 1.8±2.5 |
| Nucleus/fiber ratio | 2.3±0.34 | 1.9±0.15 |

Values expressed as mean±standard deviation.
Student’s t test. Mann Whitney test.
EDL: extensor digitorum longus; SOL: soleus; CTL: Control Group; GF: Glyphosate Group.
Analysis of the amount of connective tissue

Masson trichrome staining showed the presence of connective tissue between muscle fibers, especially in the perimysium involving the fascicles (Figure 2). Regarding the estimated amount of connective tissue in the two muscles studied, no statistical differences were observed between the CTL and GF Groups (Figure 3).

DISCUSSION

The study showed the exposure of females to glyphosate during pregnancy and lactation promoted lower weight gain during gestation, which was also observed in other studies that exposed pregnant rats to 1% (14,24) and 0.5% (2) glyphosate concentrations. Furthermore, glyphosate exposure also resulted in lower final body weight and NAL of exposed animals, which corroborates the findings of Teleken et al., (20) who also exposed mice to 0.5% glyphosate during these phases of their life cycle.

Although the present study did not verify assess the animals’ water and food consumption, Beuret et al., (24) and McKenna et al., (25) showed animals exposed to glyphosate had lower water and food consumption compared to unexposed animals, which justifies the lower weight gain and the lower final body weight and NAL of the GF Group, since glyphosate administration may reflect in reduced palatability of ingested water, or promote changes in the thirst regulatory centers, due to the effects of the herbicide and its metabolites. (14)

As to muscle fibers, Bright et al., (17) demonstrated that rats exposed to sublethal doses of sarin presented with degeneration in muscle fibers and mononuclear infiltrates in the diaphragm muscle, when euthanized 24 hours and 3 days after exposure, respectively. De Bleecker et al., (18) noted that exposure to paraoxon compound promoted fiber necrosis in several muscle groups of rats, with predominance in the diaphragm muscle. However, mixed muscles, such as masseter and soleus, were also affected. In view of the results, the authors observed a correlation between the oxidative capacity of muscles and their susceptibility to necrosis, with mixed muscles showing a predominance of oxidative fibers being more prone to necrosis.

Although the literature findings demonstrated exposure to organophosphorus compounds promotes degeneration and necrosis of muscle fibers, as well as the relation between predominance of fiber type and susceptibility to necrosis, the same was not observed in the EDL and SOL muscle fibers upon exposure to glyphosate. This may be justified by recent findings showing the toxic potential of this herbicide during direct exposure is minimal, despite the current association of glyphosate exposure with occurrence of diseases. (26) Thus, the absence of changes in the morphological and morphometric parameters of the EDL and SOL muscles of the females, and of any necrotic processes pointing to possible fiber degeneration, may be associated with the low toxicity of the herbicide in this first exposure.
However, even if the low toxicity of glyphosate in direct exposure is demonstrated, a study showed that, despite not causing effects in the first generation, this herbicide promotes an increase in the occurrence of diseases in the offspring of exposed rats. Hence, its ability to promote epigenetic changes, which will be transmitted to subsequent generations.\(^{(27)}\) Due to the effects promoted by exposure, glyphosate has been investigated as a potential chemical endocrine disruptor,\(^{(26)}\) which consists of a substance capable of altering the maternal environment, and influencing the stages of intrauterine development, as well as increasing the risk of chronic diseases in adulthood.\(^{(29)}\)

Despite low toxicity during direct exposure, the potential action of glyphosate as an endocrine disruptor can promote changes in exposed offspring, and it is strictly necessary to take this fact into account in the etiology of diseases in future generations.

## CONCLUSION

Exposure to 0.5% glyphosate during pregnancy and lactation promoted lower weight gain during gestation and lower body weight and size of females. Although the morphological characteristics of muscle tissue were not altered, the change in body parameters indicates glyphosate may interfere in the metabolism of the animal, promoting changes in its cycle of obtaining and storing energy.

## ACKNOWLEDGMENTS

To the grant program of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financing this research, by the Graduate Support Program (PROAP), number 817693/2015.

## AUTHORS’ CONTRIBUTION

Ariadne Barbosa: data curation, research, editing and methodology. Mylena de Campos Oliveira: data curation and methodology. Camila Kuhn-Fraga: methodology. Lucinéia de Fátima Chasko Ribeiro: review and supervision. Sandra Lucinei Balbo: methodology. Márcia Miranda Torrejais: methodology, review of writing and supervision.

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