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Glyphosate-based Herbicide Exposure Causes Reduction in Endocrine Pancreatic Mass of Female Mice Submitted to Ovarian Hormone Deprivation

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HIGHLIGHTS

- Glyphosate-based herbicide (GBH) may act as endocrine disruptor.
- Here, at 50 mg/kg GBH exposure reduced islet pancreatic mass in ovariectomized mice.
- At 0.5 mg/kg, GBH exposure reduced islet and β-cell areas in SHAM female mice.
- GBH exposure at doses stated as safe can negatively impact endocrine pancreatic morphology.

Abstract: Glyphosate-based herbicides (GBHs) have been related to various organic impairments, however, its effects on endocrine pancreas and body glucose control are yet unclear. Here, we investigated the effects of GBH exposure on glucose homeostasis and endocrine pancreatic morphology in adult female mice. Furthermore, it was evaluated whether the effects of the herbicide are influenced by circulating concentration of ovarian hormones, particularly estrogen. Adult female *C57Bl/6* mice underwent either SHAM operation or bilateral ovariectomy to induce ovarian hormone deprivation. Subsequently, SHAM and ovariectomized (OVX) females received a daily oral gavage of 0.2 mL distilled water (SHAM0 and OVX0 groups), containing or not 0.5 mg/kg (SHAM0.5 and OVX0.5 groups), or 50 mg/kg of GBH (SHAM50 and OVX50 groups) for 60 days. The selection of these doses was based on the regulatory concentrations established for Acceptable Daily Intake (ADI) and No Observable Adverse Effect Level (NOAEL) of glyphosate, respectively. GBH exposure, at both concentrations, did not change body weight, pancreas weight, glucose tolerance or insulin sensitivity in SHAM and OVX mice. However, in OVX females exposed to 50 mg/kg/day, GBH reduced non-β cell area, as well as the islet, β-cell, and non-β cell masses in the pancreas. In SHAM females, a lower dose (0.5 mg/kg/day) of GBH reduced islet and β-cell areas/islet, without altering total endocrine pancreatic mass. These findings suggest that GBH exposure, even at regulatory threshold levels for ADI and NOAEL, may impair pancreatic islet structure. Importantly, the herbicide's detrimental effects appear to be more pronounced under conditions of low estrogen, such as in OVX females.

Keywords: Endocrine disruptors; Estrogen; Islets; Herbicides; Insulin; Ovariectomy.

INTRODUCTION

Glyphosate (N-phosphonomethyl-glycine) is the active ingredient of glyphosate-based herbicides (GBH)s, which are the most widely used pesticides in Brazilian agriculture [1]. It is an organophosphate derivative of glycine that acts by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which is involved in the biosynthesis of aromatic amino acids in plants and certain bacteria [2–4]. Since this pathway is absent in vertebrates [3], it was initially assumed that the herbicide would have no effect on these organisms, including humans. However, increasing evidence suggest that glyphosate and GBH exposure may act as potential endocrine disruptors [5–7], a term referring to exogenous compounds capable of affecting endocrine tissues and interfering with any aspect of hormonal action [8]. Such disruptions can compromise physiological homeostasis and increase susceptibility to various chronic diseases. This is particularly concerning given that glyphosate is recognized as a widespread environmental contaminant, frequently detected in soil, water, air, and food. A study elegantly demonstrated that glyphosate and its metabolite, aminomethylphosphonic acid (AMPA), are present in the urine of postmenopausal women, regardless of whether their diets consisted of organic or conventional foods [9]. Furthermore, both glyphosate and AMPA have been detected in household drinking water, with their concentrations increasing during periods of agricultural application [10]. These findings raise serious concerns about the potential health effects of chronic low-dose exposure to this herbicide over time.

Fine regulation of pancreatic islet hormone secretion and action is vital for maintaining glucose homeostasis. In mice, pancreatic islets consist of 60-80% β-cells, which are responsible for insulin synthesis and secretion, the primary hormone involved in blood glucose regulation. Other endocrine pancreatic cells include α and δ-cells, located at the islet periphery, which synthesize and secrete the counterregulatory glucose hormone, glucagon; and somatostatin, respectively [11]. Physiological and dietary conditions lead to modifications in the number of islet cells, mainly β-cells, ensuring glucose homeostasis [12, 13]. Failure to adapt to the increased hormonal demand can result in impaired body glucose control [13, 14]. Although GBH exposure has been shown to affect testes, ovaries, adrenal and thyroid glands [15–18], its impact on the endocrine pancreas remains largely unexplored. Existing data are mostly qualitative, and current findings on the effects of GBHs on glucose tolerance and insulin sensitivity are inconsistent [19–21].

Estrogen, the primary female sex hormone produced by the ovaries, plays a role not only in reproductive function but also in regulating the morphology and function of pancreatic islets. It exerts antidiabetic effects in women of reproductive age, a profile that shifts significantly following menopause [22–27]. Activation of the G-protein coupled estrogen receptor increases β-cell membrane potential, enhances intracellular Ca2+ concentrations, stimulating insulin secretion [22]. Moreover, classical estrogen receptors, ER-α and ER-β, are also expressed in endocrine pancreas, where they regulate insulin biosynthesis, glucose-induced insulin secretion, and β-cell survival [25]. Some studies suggest that glyphosate and GBHs may interfere with multiple aspects of estrogen signaling, including aromatase inhibition, altered ER-α activation and degradation, and modulation of gene transcription [5–7], Thus, it is plausible that GBH exposure would interfere in estrogen actions in pancreatic islets, leading to impairments in endocrine pancreas and in glucose homeostasis. Therefore, our study aimed to investigate the effects of GBH exposure at doses of 0.5 or 50 mg/kg, Acceptable Daily Intake (ADI) and No Observable Adverse Effect Level (NOAEL) for glyphosate, respectively, as defined by the Brazilian regulatory agency ANVISA [28, 29], on glucose homeostasis and the morphology of the endocrine pancreas in adult female mice. To further examine whether the herbicide's effects on such parameters are dependent on the circulating concentration of ovarian hormones, especially estrogen, our study included an experimental group of female mice that underwent bilateral ovariectomy, representing a state of ovarian hormone deprivation.

MATERIAL AND METHODS

Experimental model

Adult female C57Bl/6 mice (90-100 days-old) were obtained from the Central Animal Facility of UNIOESTE (Cascavel, PR, BRA) and maintained at the Núcleo Avançado para Estudos da Vida of the Biological Sciences and Health Sector of UEPG, under standardized conditions of temperature (23 ± 2ºC), humidity and luminosity (lights on: 7 am to 7 pm). All experimental procedures were approved by the Ethics Committee on the Use of Animals of UNIOESTE (Protocol no.: 18-21) and of UEPG (Process no.: 0533578/2021).

To verify whether the potential effects of GBH exposure are associated with the circulating concentration of ovarian hormones, a group of adult female C57Bl/6 mice underwent ovarian hormone deprivation by bilateral ovariectomy. In this way, the females were weighed and intraperitoneally (ip) anesthetized with 100 mg ketamine/kg body weight (BW) and 10 mg xylazine/kg BW [30]. A laparotomy was performed to remove the ovaries. Another group of females underwent a SHAM operation, in which the same anesthetic procedures and laparotomy were conducted, however the ovaries were only externalized, massaged, and returned to the abdominal cavity [31]. Fourteen days after bilateral ovariectomy, vaginal smear was collected and analyzed under an optical microscope to investigate anestrus in ovariectomized (OVX) females, a parameter indicating the induction of ovarian hormonal deprivation [32]. Subsequently, SHAM and OVX females received daily, via gavage, 0.2 mL distilled water (vehicle; SHAM0 and OVX0 groups, respectively), containing or not 0.5 mg GBH/kg BW (SHAM0.5 and OVX0.5 groups, respectively), or 50 mg GBH/kg BW (SHAM50 and OVX50 groups, respectively) for 60 days.

The doses of 0.5 and 50 mg GBH/kg were chosen because they are glyphosate regulated doses used as ADI and NOAEL by ANVISA [28, 29]. The herbicide used was Roundup Original® Mais (Monsanto do Brasil, São Paulo, SP, BRA), which contained 577 g/L N-(phosphonomethyl) glycine diammonium salt, corresponding to 480 g/L (48% m/v) of active component glyphosate.

Glucose and insulin tolerance tests

In the last week of the experimental period, females of all experimental groups, following a 12 h fast, were weighed and had their fasting blood glucose measured via a small cut at the tail tip, using of a glucometer (G-TECH Free, Accumed-Glicomed, Duque de Caxias, RJ, BRA). For the oral glucose tolerance test (oGTT), females received 2 g glucose/kg BW by gavage, and blood glucose levels were measured at 15, 30, 60 and 120 min during the oGTT.

Two days after the oral glucose tolerance test (oGTT), all female mice, following an overnight feeding period, underwent blood glucose measurements as described above. Then, the female mice were fasted for 2 h. Afterward, glycemia was measured again, and all female mice received an ip injection of 0.75 IU insulin/kg BW (Humulin R, Eli Lilly, Indianapolis, USA). Glycemia was recorded at 4, 8, 12, 16 and 20 min during the insulin tolerance test (ipITT). The glucose disappearance rate (K_{ITT}) during the ipITT was calculated using the formula: $0.693/(t_{1/2}) \times 100$ [33], with analysis conducted through GraphPad Prism Software© version 9.00 for Windows (San Diego, CA, USA).

Evaluation of general biometric parameters

After 60 days of exposure or not to GBH, all SHAM and OVX females were weighed, and the nasoanal length was registered. These parameters were used to calculate the rodent body mass index, Lee index, using the formula: $\frac{\sqrt[3]{BW}}{nasoanal\ length}\ x\ 1000$. Subsequently, all female mice were euthanized by decapitation and a laparotomy was performed to access the pancreases, which were dissected and weighed.

Histomorphometry of the endocrine pancreas

Pancreases of SHAM and OVX female mice exposed or not to GBH were fixed in 4% paraformaldehyde for 24 h. Then, dehydration was carried out with alcohol, clearing with xylene and the pancreases were included in paraplast (Surgipath, Leica Biosystems, São Paulo, SP, BRA). Semi-serial sections of 5 µm in thickness were taken, with a spacing of 100 µm between sections. Subsequently, pancreas sections were deparaffinized, hydrated, and incubated for 30 min at 95ºC with 10 mM citrate buffer. Afterwards, the sections were permeabilized and blocked for 1h with phosphate buffer solution (PBS) containing 5% albumin and 0.2% Triton X-100. Subsequently, the sections were incubated overnight at 4ºC with guinea pig-insulin polyclonal antibody (1:10000; Dako North America, Inc., CA, USA). Next, the endogenous peroxidase was blocked with 0.3% H2O2 in PBS, and the sections were incubated for 2h with peroxidase-conjugated rabbit anti-guinea pig IgG antibody (1:1500; Zymed Laboratories, Inc., San Francisco, CA, USA). Immunoreaction detection was performed using PBS containing 10% diaminobenzidine (Sigma-aldrich, St. Louis, MA, USA) and 0.02% H2O2. The sections were counterstained with Harris' hematoxylin.

For endocrine pancreas morphometry, all islets of 2 pancreatic sections of each mouse, were photographed with the Olympus DP72 camera (Olympus Optical, São Paulo, SP, BRA) coupled to the Olympus BX41TF light microscope (Olympus Optical, São Paulo, SP, BRA). The measurements of the islet, β-cell and non-β cell areas were carried out using the manual resource of FIJI Image J 2.14.0 free software (https://imagej.net/software/fiji/). The software was calibrated with a micrometric ruler (1 mm) enabling the

conversion of the values obtained in pixels to μm2. The ratio of β-cell/islet and non-β cell/islet were obtained by dividing the β-cell, or non-β cell area, by the islet area. For the distribution of islets by size, the number of small islets with an area <5000 μm2, medium islets with an area between 5000-10000 μm2, and large islets with an area >10000 μm2, were counted, and the number of small, medium and large islets of each section, was multiplied by 100 and divided by the total number of islets in the section [34, 35]. To obtain the islet, β-cell and non-β-cell masses, firstly each pancreatic section was registered with a camera (Axio Cam ERC5s Zeiss, Carl Zeiss Microimaging GmbH, Gottingen, Germany) coupled to a stereomicroscopy (Stemi 2000-C Zeiss, Carl Zeiss Microimaging GmbH, Gottingen, Germany). The pancreas section area was also manually measured using FIJI Image J software. The islet, β-cell and non-β cell masses were calculated by multiplying the pancreas weight by the total area of islets, or of the β-cell or of the non-β cell, in each pancreas section, and divided by the respective section area [36].

Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM). For statistical analysis, two factors were assumed: deprivation or not of ovarian hormones and exposure or not to GBH. Therefore, two-way ANOVA followed by the Bonferroni-Sidak post-test was used for statistical analysis with Prism 9.0 software (GraphPad Software, Boston, MA, USA). The level of significance was set at $p < 0.05$.

RESULTS

Figure 1 shows the BW and nasoanal length, recorded at the end of the experimental period, in female OVX or SHAM mice, exposed or not to GBH. Two-way ANOVA indicated a significant increase in BW due to ovarian hormone deprivation ($p < 0.006$; Figure 1A), with no observed impact of GBH exposure or interaction between both factors. However, the multiple comparison post-test did not reveal any differences in BW between the OVX and SHAM groups, regardless of GBH exposure (Figure 1A). Furthermore, no effect of ovarian hormone deprivation, GBH exposure, or interaction among of these factors, were observed on nasoanal length (Figure 1B) or Lee index (Figure 1C), between OVX and SHAM groups.

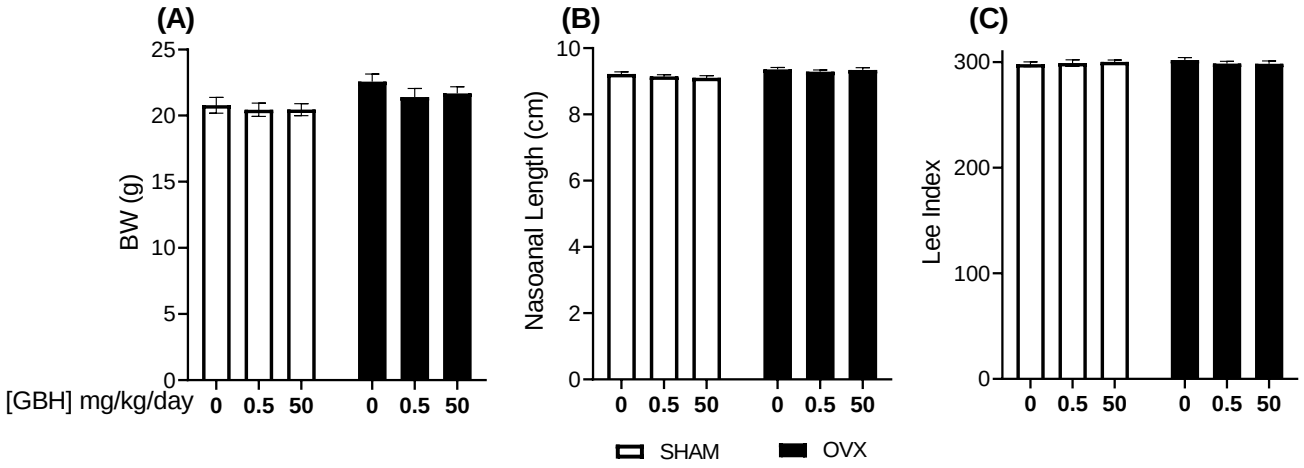

Figure 1. Mean ± SEM of body weight (BW; A), nasoanal length (B) and Lee index of SHAM0 (n = 6), SHAM0.5 (n = 6), SHAM50 (n = 7), OVX0 (n = 7), OVX0.5 (n = 7) and OVX50 (n = 8) female mice, registered at the end of the experimental period. Data were analyzed by two-way ANOVA followed by Bonferroni-Sidak post-test, $p < 0.05$.

Figure 2 depicts glucose homeostasis parameters evaluated in SHAM and OVX female mice, during the last week of the experimental period. Two-way ANOVA showed only an effect of ovarian hormone deprivation on fasting glycemia ($p < 0.003$; Figure 2A), with no observed impact of GBH exposure or interaction between these factors. However, the multiple comparison post-test did not indicate any significant differences between the OVX and SHAM groups (Figure 2A). Furthermore, fed glycemia was not affected by ovarian hormone deprivation, or GBH exposure, in OVX and SHAM groups (Figure 2B).

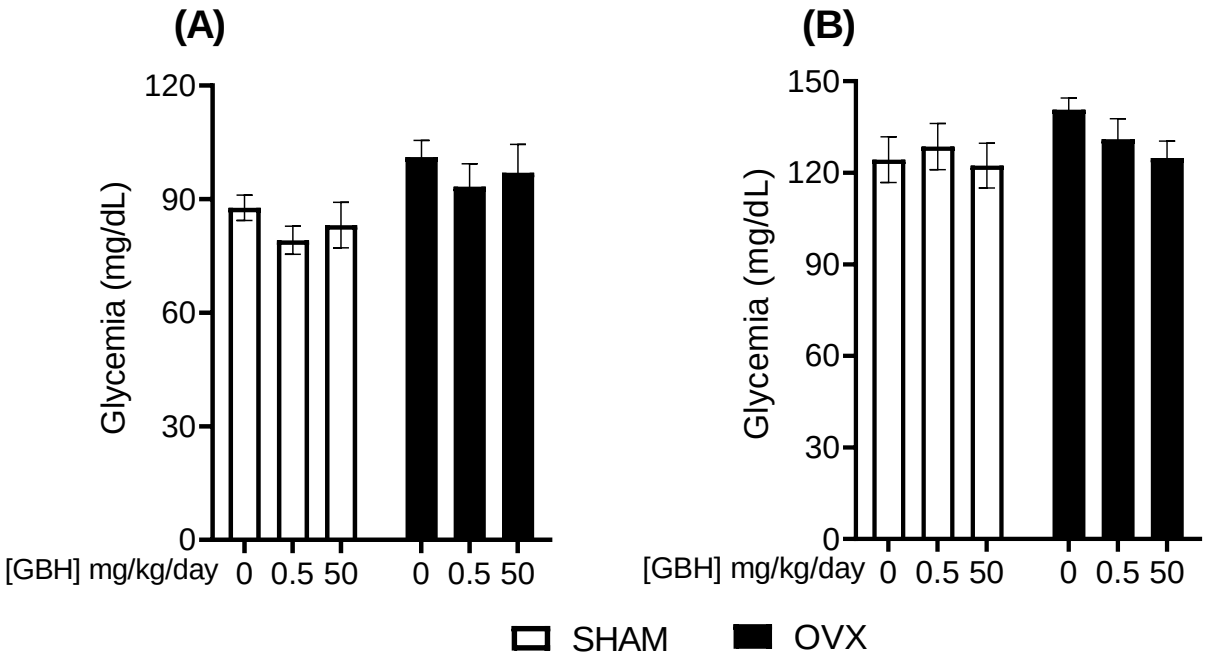

Figure 2. Mean ± SEM of fasting (A) and fed glycemia (B) of SHAM0 (n = 6), SHAM0.5 (n = 6), SHAM50 (n = 7), OVX0 (n = 7), OVX0.5 (n = 7) and OVX50 (n = 8). Data were analyzed by two-way ANOVA followed by Bonferroni-Sidak post-test.

In the oGTT, glycemia increased after glucose administration, reaching the highest values at 15 min, and returning near to basal levels after 120 min in all groups (Figure 3A and 3B). No differences in glucose tolerance were observed among OVX and SHAM females, exposed or not to GBH, during the oGTT (Figure 3A and 3B). Accordingly, the total glycemia during the test, expressed as the area under the glycemia curve (AUC), was similar between OVX and SHAM females exposed or not to GBH (Figure 3C).

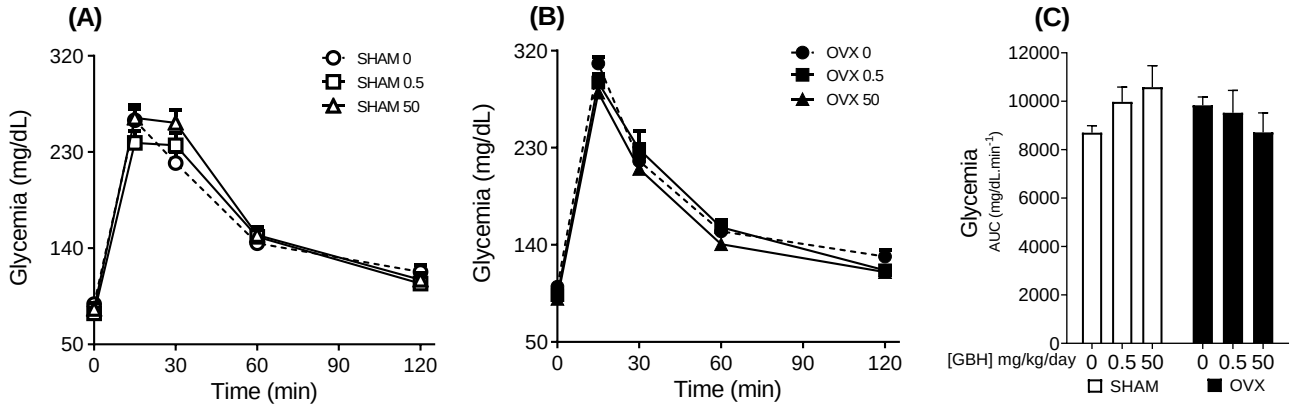

Figure 3. Mean ± SEM of glycemia profile during the oGTT in SHAM (A) and OVX (B) groups, and total glycemia during the oGTT (C), fed glycemia (E), glycemia profile during the ipITT in SHAM (F) and OVX (G) groups, and Kitt (H) of SHAM0 (n = 6), SHAM0.5 (n = 6), SHAM50 (n = 7), OVX0 (n = 7), OVX0.5 (n = 7) and OVX50 (n = 8). Data were analyzed by two-way ANOVA followed by Bonferroni-Sidak post-test.

In the ipITT, after insulin injection, a decrement in glycemia occurred after 4 min of the test in all groups. But, while in SHAM groups glycemia reached a steady state from 8 min to the end of the ipITT (Figure 4A), in OVX females, the decrease of glycemia was slower, continuing to reduce at 12 min of the test (Figure 4B). The glucose decay constant obtained from the ipITT (Figure 4C), was also similar among OVX and SHAM females, exposed or not to GBH.

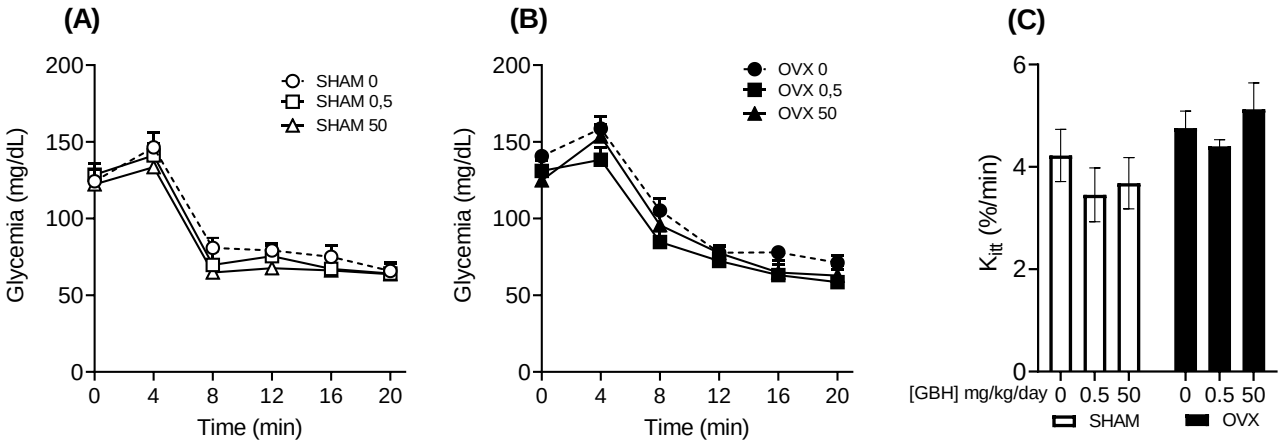

Figure 4. Mean ± SEM of glycemia profile during the ipITT in SHAM (A) and OVX (B) groups, and Kitt (C) of SHAM0 (n = 6), SHAM0.5 (n = 6), SHAM50 (n = 7), OVX0 (n = 7), OVX0.5 (n = 7) and OVX50 (n = 8). Data were analyzed by two-way ANOVA followed by Bonferroni-Sidak post-test.

Figures 5, 6 and 7, and Table 1 show histomorphometric analysis of the pancreases of SHAM and OVX female mice, exposed or not to GBH. Pancreas weight was similar among OVX and SHAM females (Table 1). Two-way ANOVA demonstrated only an effect of GBH exposure ($p < 0.01$) on pancreatic islets morphological features. Islets from OVX50 females exhibited reduced non-β cell area, when compared to those observed for OVX0 ($p < 0.05$; Figure 5 and 6C). While islets from SHAM0.5 females displayed lower islet and β-cell areas, in comparison with SHAM0 ($p < 0.05$; Figure 5, 6A and 6B). The β-cell area/islet area and non-β cell area/islet area ratios were also affected by the ovarian hormone deprivation ($p < 0.05$) and GBH exposure ($p < 0.01$). OVX0.5 and SHAM50 groups displayed an increase in β-cell area/islet area ratio, but a reduction in non-β cell area/islet area ratio, when compared to OVX0 and SHAM0, respectively ($p < 0.05$; Table 1).

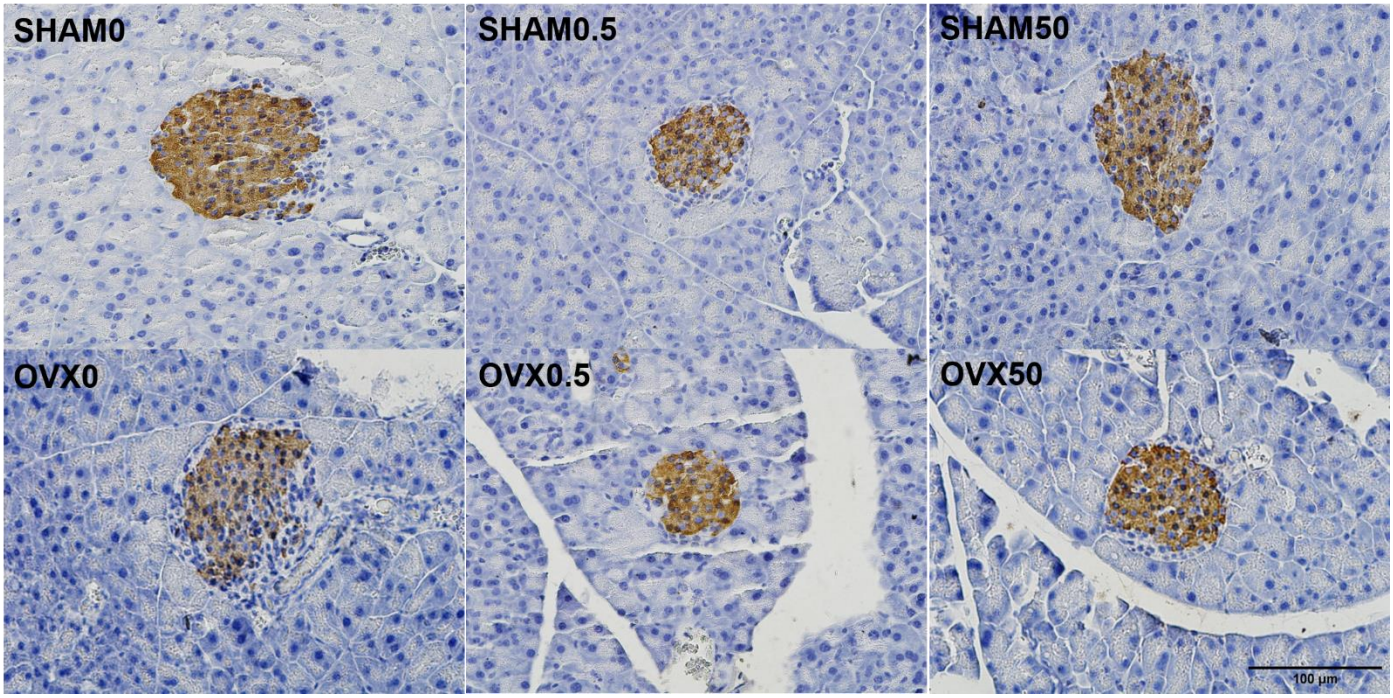

Figure 5. Representative histological images of 5-µm sections of the pancreases of SHAM0, SHAM0.5, SHAM50, OVX0, OVX0.5 and OVX50 female mice, which were subjected to immunohistochemistry for insulin (brown staining) and counterstained with Harris hematoxylin (blue). Scale bar = 100 µm.

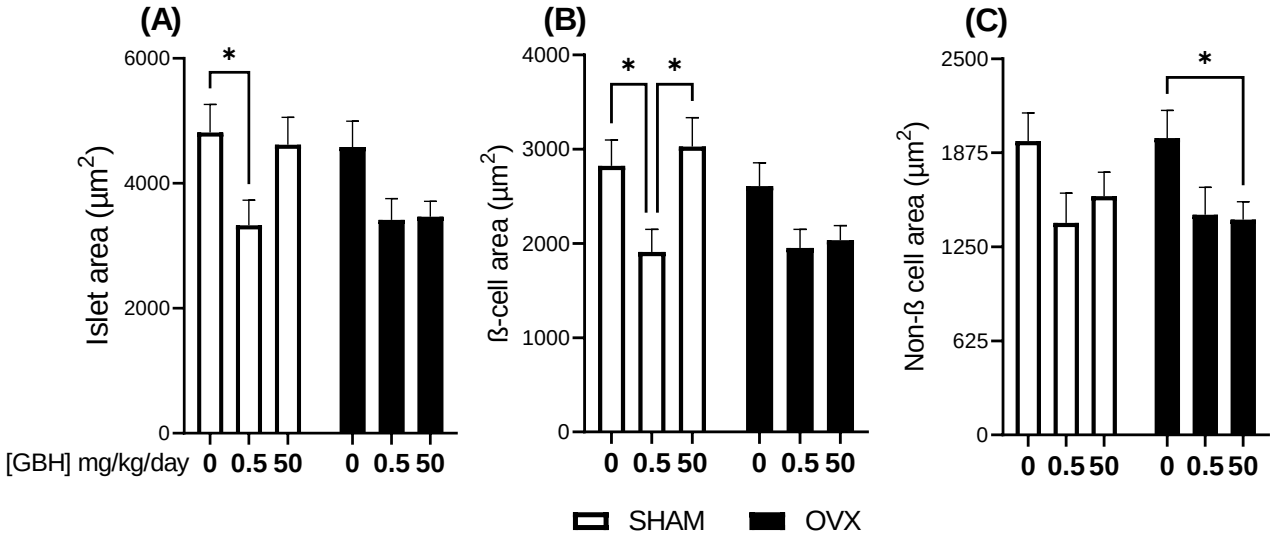

Figure 6. Mean ± SEM of islet (A), β-cell (B) and non-β cell areas (C) in the pancreases of SHAM0 (n = 6), SHAM0.5 (n = 6), SHAM50 (n = 7), OVX0 (n = 7), OVX0.5 (n = 7) and OVX50 (n = 8) female mice. The connecting lines under the bars indicate significant statistical differences. Data were analyzed by two-way ANOVA followed by Bonferroni-Sidak post-test, $p < 0.05$.

In addition, endocrine pancreatic mass was significantly affected by GBH exposure ($p < 0.05$), with OVX50 females exhibiting reductions of 46%, 44% and 47% in islet (Figure 7A), β-cell (Figure 7B) and non-β cell (Figure 7C) masses, respectively, when compared to those observed for OVX0 ($p < 0.02$, $p < 0.03$ and $p < 0.05$, respectively). No modifications among SHAM and OVX groups, due to ovarian hormone deprivation or GBH exposure, were observed in the percentage of islets distributed by size, or in the number of islets per section analyzed (Table 1).

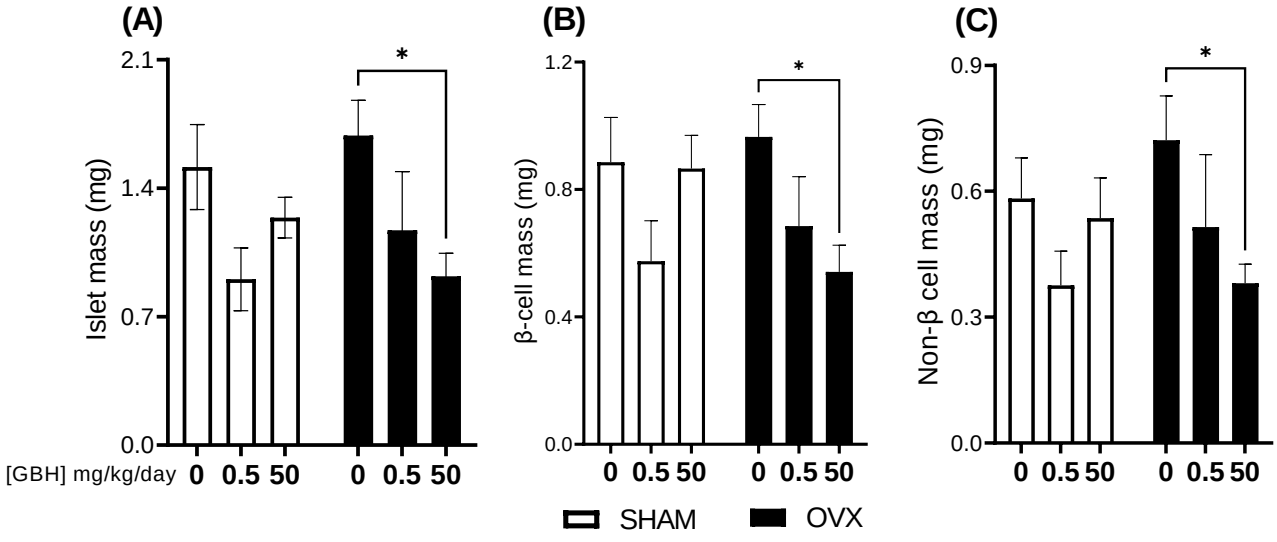

Figure 7. Mean ± SEM of islet (A), β-cell (B) and non-β cell masses (C) in the pancreases of SHAM0 (n = 6), SHAM0.5 (n = 6), SHAM50 (n = 7), OVX0 (n = 7), OVX0.5 (n = 7) and OVX50 (n = 8) female mice. The connecting lines under the bars indicate significant statistical differences. Data were analyzed by two-way ANOVA followed by Bonferroni-Sidak post-test, $p < 0.05$.

Table 1. Morphometric parameters of the endocrine pancreases from SHAM0 (n = 6), SHAM0.5 (n = 6), SHAM50 (n = 7), OVX0 (n = 7), OVX0.5 (n = 7) and OVX50 (n = 8) female mice.

	SHAM0	**SHAM0.5**	**SHAM50**	**OVX0**	**OVX0.5**	**OVX50**
Pancreas weight (mg)	202.2 ± 15.45	206.8 ± 11.91	200.7 ± 14.72	234.4 ± 18.25	245.2 ± 18.16	199.9 ± 13.9
β-Cell area/islet area ratio (%)	59.55 ± 1.62	64.94 ± 2.21	65.63 ± 1.60*	57.69 ± 1.46	63.20 ± 1.90#	60.73 ± 1.61
Non-β-cell area/islet area ratio (%)	40.45 ± 1.59	35.06 ± 2.21	34.37 ± 1.60*	42.31 ± 1.46	36.80 ± 1.90#	39.42 ± 1.61
% small islets/section (≤5000 μm2)	71.16 ± 4.12	72.02 ± 8.18	71.48 ± 4.10	75.92 ± 4.02	83.81 ± 4.24	71,55 ± 6.35
% medium islets/section (≥5000 and ≤10000 μm2)	13.85 ± 2.44	9.61 ± 2.11	13.76 ± 3.42	12.15 ± 2.37	11.14 ± 3.38	14.67 ± 2.52
% large islets/section (≥10001 μm2)	14.99 ± 3.29	6.15 ± 2.19	15.22 ± 2.82	11.94 ± 2.63	8.65 ± 1.54	6.97 ± 2.51
Total number of islets/section	22.45 ± 2.56	19.44 ± 3.89	19.50 ± 2.82	26.15 ± 1.84	17.67 ± 3.07	19.47 ± 2.93
Total islets analyzed	245	175	219	340	215	258

Data are means ± SEM. *indicate significant difference from SHAM0; # indicate significant difference from OVX0 (Two-way ANOVA followed by Bonferroni-Sidak post-test, $p < 0.05$).

DISCUSSION

Our study revealed that 60 days of GBH exposure to female mice, at concentrations referenced by ANVISA as ADI and NOAEL [28, 29], led to modifications in endocrine pancreas morphology. Notably, the dose of 50 mg GBH/kg/day, when combined with a condition of ovarian hormone deprivation, yielded the most alarming result. OVX50 females exhibited reductions in islet, β-cell and non-β cell masses. Other islet morphology modifications, which only lead a trend, but not a significant reduction in endocrine pancreatic mass, were the decrease in islet and β-cell areas per islet in SHAM females exposed to 0.5 mg GBH/kg/day. Interesting, such effects were not accompanied by modifications on glucose tolerance and insulin sensitivity in OVX50 and SHAM0.5 females, indicating that the herbicide can negatively impact islet morphology before of any changes can be observed in general circulating parameters related to glucose homeostasis.

It is important to emphasize that currently, in literature, it has only data about GBH effects on general pancreatic morphology, and that used high concentrations of the herbicide of those were used in our study. For example, in rats exposed to 375 mg GBH Bushfire®/kg, by gavage, for 8 weeks, only the exocrine pancreatic morphology was analyzed, reporting that the herbicide caused degeneration of acinar cells [20]. In a subsequent study, rats exposed to daily gavage of 375 or 750 mg GBH Bushfire®/kg/day, for 36 weeks, exhibited moderate and severe degeneration of the islets and acinar cells, respectively, despite no significant statistical differences in glycemia and insulinemia [21]. However, rats that were exposed to 187.17 mg/m3, 313.31 mg/m3 or 467.93 mg/m3 Roundup Original DI® by inhalation or through food nebulized with the herbicide, for 75 days, did not show any modification in pancreatic morphology [37]. Therefore, here we provided the first evidence that GBH regulated doses can pose a risk to endocrine pancreatic health.

Reduction in β-cell mass is a crucial factor in the development of diabetes. In type 2 diabetic subjects were observed decreases of 41% to 63% in β-cell mass, mainly due to increased β-cell apoptosis [14]. Maybe GBH exposure in OVX50 female mice can lead to changes in endocrine pancreatic cells turnover, reducing proliferation and enhancing apoptosis. However, despite the reduction in islet and β-cell masses, OVX50 females did not exhibit changes in glycemia or glucose tolerance. Possibly, GBH exposure in OVX50 can have changed insulin signaling pathway in target tissues, which might not be evident through the general insulin sensitivity evaluated by ipITT in our study. Previously, was reported that the male F1 offspring from female C57Bl/6 mice, that during gestation and lactation ingested water containing 0.5% Roundup Original DI®, displayed in adulthood enhanced insulin sensitivity, which contributed to maintain glucose tolerance, insulinemia and glycemia to values of those were observed in F1 offspring that were not exposed to the herbicide [19]. Thus, further investigations are necessary to verify the insulin signaling pathway in insulin-target tissues in SHAM and OVX females exposed to 0.5 and 50 mg GBH/kg, to evidence whether any change occurs in insulin signaling.

Another important point to consider is that GBH exposure, in our study, seems to impact endocrine pancreatic morphology, depending on the concentration of circulating ovarian hormones, especially estrogen. Estrogens have been demonstrated to regulate insulin and glucagon secretion, as well as β-cells survival. Such effects occur through estrogen interaction with estrogen receptor (ER)-α, ER-β and the G-coupled estrogen receptor [22, 25–27]. Remarkably, in different experimental models of endocrine pancreatic injury, pre-diabetes, and diabetes, a sexual dimorphism has been reported. There is evidence of a higher susceptibility of mal yuchies, compared to females, to display β-cell failure and disruptions in glucose homeostasis. This protective effect in females appears to be promoted by estrogens, as ovariectomy or menopause increases the susceptibility of females to endocrine pancreatic impairments and diabetes, like those observed in males [24, 25]. In this regard, studies have indicated that glyphosate alone and GBH formulations may act as endocrine disruptors of the hormonal action of estrogen [5, 7]. Therefore, it is possible that in our study, such endocrine-disrupting action of GBH on the estrogenic signaling pathway may have contributed to the reduction in islet area and β-cells in the SHAM0.5 group, as well as the decreased islet, β-cell and non-β-cell masses in OVX50 females. However, more studies are needed to explain why the negative impacts of GBH exposure on the morphology of the endocrine pancreas differ depending on the dose and the circulating concentration of ovarian hormones.

STUDY LIMITATIONS

While the findings presented here support the notion that exposure to a GBH can impair the structural integrity of the endocrine pancreas, particularly under conditions of ovarian hormone deprivation, several limitations should be acknowledged:

1. **GBH exposure and Relevance of Tested Doses**: The doses used in this study (0.5 and 50 mg/kg) were selected based on the ADI and NOAEL, as defined by the Brazilian regulatory agency (ANVISA). These doses were chosen to evaluate whether current regulatory thresholds are indeed safe for the endocrine pancreas. While our results raise important concerns, new studies are necessary to determine the actual levels of glyphosate present in food, drinking water, and air, and to assess the cumulative health impacts of chronic exposure in real-life scenarios. In addition, considering the pleiotropic actions often associated with endocrine-disrupting chemicals, it is plausible that longer exposure periods beyond the 60 days assessed here could reveal other alterations in glucose tolerance and insulin sensitivity in both SHAM and OVX mice which were not fully evident within the current timeframe.
2. **Mechanisms of Action Involved in GBH Effects on Endocrine Pancreas:** Although previous studies suggest that GBHs may interfere with estrogen signaling, the specific mechanism by which 50 mg/kg GBH exposure leads to the observed reduction in endocrine pancreatic mass in OVX females remains unclear. Further investigations are needed to elucidate how GBH modulates estrogen pathway in the endocrine pancreas of both ovarian hormonally intact and hormone-deprived females. Moreover, whether estrogen replacement therapy could prevent or mitigate these effects remains an important issue for future research.
3. **Animal Model Limitations**: The use of *C57Bl/6* mice allowed for detailed biochemical and histological analyses. However, rodent models may not fully replicate the complexities of human endocrine and metabolic physiology. Therefore, caution is warranted when extrapolating these findings to humans. Further research using clinical and epidemiological approaches are essential to evaluate the potential endocrine-disrupting effects of glyphosate exposure in women, especially under varying hormonal conditions such as pre- and postmenopause.

CONCLUSION

In summary, GBH exposure at the dose NOAEL of 50 mg/kg/day, reduced islet, β-cell and non-β cell masses, in female mice submitted to ovarian hormone deprivation. Additionally, at the ADI (0.5 mg/kg/day), GBH exposure reduced islet and β-cell areas in SHAM females. These findings are alerts that the concentrations of GBH exposure considered without risk to health can negatively impact endocrine pancreatic morphology, even before any signs of modifications in glycemia are evident.

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