



Gestational exposure to glyphosate-based herbicide and glucose homeostasis in rats: effects on dams during pregnancy and post-term periods, and on their progeny

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ABSTRACT

Exposure to glyphosate-based herbicides (GBH) may affect metabolism and induce endocrine alterations. This study evaluated whether environmentally relevant low doses of GBH affect glucose homeostasis during pregnancy and the metabolism of offspring later in life. Pregnant Wistar rats received daily oral doses of either 0.5 or 50 mg/kg of GBH or water (control) throughout the gestation period. No significant changes were observed in biometric and metabolic parameters during pregnancy. However, hepatic alterations were detected in GBH-treated rats, including reduced glycogen content and decreased global genome methylation. Offspring exhibited a transient delay in neurodevelopmental reflexes. Body mass and food intake remained unchanged up to postnatal day 70. After this period, animals were subjected to a metabolic challenge with dexamethasone. While dexamethasone-treated animals showed metabolic alterations typical of glucocorticoid exposure (glucose intolerance, insulin insensitivity, dyslipidemia), maternal GBH exposure during pregnancy did not exacerbate these effects in the offspring. Additionally, changes in DNA methylation-related gene expression were observed in female but not male offspring. *In vitro*, GBH exhibited greater cytotoxicity than glyphosate alone, significantly reducing the viability of 3T3-L1 adipocytes and HepG2 hepatocytes, whereas glyphosate alone had no significant effects, even at higher concentrations. Overall, gestational exposure to GBH under regulatory safe doses does not cause substantial metabolic disturbances in dams or their offspring. However, the existence of alterations in hepatic glucose metabolism and effects on hepatic epigenetic regulation at these low doses, along with the cytotoxic *in vitro* effects of GBH at high concentrations, warrants attention regarding human exposure and health impacts.

1. Introduction

Glyphosate (N-(phosphonomethyl)glycine) is a broad-spectrum

organophosphate compound and the active ingredient in many commercially available formulations known as glyphosate-based herbicides (GBH) (Benbrook, 2016). Globally, approximately 600-750

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thousand tonnes are used annually (Maggi et al., 2020). The mechanism of action of glyphosate consists of inhibiting the activity of the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase, which in turn impairs the biosynthesis of aromatic amino acids essential for plant development (Duke and Powles, 2008). Glyphosate is classified as a low-risk agent for animals because it targets enzymes absent in human cells. The scientific consensus about the genotoxic and probably carcinogenic effects of glyphosate on humans is still controversial (IARC, International Agency for Research on Cancer, 2016; Portier et al., 2016). Despite being classified as a low-risk agent for animals (Duke and Powles, 2008), there is increasing data suggesting that the massive use of glyphosate can affect vertebrate and invertebrate organisms (Benachour et al., 2007; Tan et al., 2022).

Human exposure to glyphosate may occur through ingestion of contaminated food and water, dermal contact, or inhalation of residue-containing particles, which can occur in both occupational and non-occupational contexts (Bai and Ogbourne, 2016; Lima et al., 2023). Glyphosate and its primary metabolite aminomethylphosphonic acid (AMPA) have been identified in human urine, plasma, placenta, and umbilical cord of pregnant individuals (Kongtip et al., 2017; Gillezeau et al., 2019). In this context, pregnancy represents a critical period for fetal development, during which environmental factors can significantly influence long-term health outcomes. Early-life exposure to xenobiotics, including glyphosate, can cross the placenta and affect offspring development (Benachour et al., 2007; Lorenz et al., 2020). Moreover, fetal exposure to environmental pollutants such as glyphosate may interfere with hormonal signaling, leading to epigenetic changes and predisposing the appearance of metabolic disorders later in life (Benachour et al., 2007; Kubsad et al., 2019; Muñoz et al., 2021; Rossetti et al., 2021; Miranda et al., 2023; La Merrill et al., 2025).

Previous preclinical and clinical studies have demonstrated that GBH exposure (e.g., RoundUp®) can disrupt glycemic and lipid metabolism in adult individuals, suggesting that GBH may act as an obesogenic and diabetogenic agent (Kubsad et al., 2019; Miranda et al., 2023; Rosolen et al., 2024; Feng et al., 2025). Metabolic impairments in adult rats have been associated with increased expression of proinflammatory cytokines and reduced antioxidant defenses, even at glyphosate doses considered by regulatory agencies to be no-observed-adverse-effect levels (NOAELs) (e.g., 50 mg/kg) (Prasad et al., 2022). Nevertheless, studies addressing these potential diabetogenic effects of GBH exposure during pregnancy remain poorly understood, and the available evidence indicates negative or absent impacts on metabolic outcomes in adult offspring (Gomes et al., 2022).

In addition to metabolic and endocrine disturbances, emerging evidence suggests that glyphosate and GBHs may also interfere with epigenetic regulatory mechanisms. Experimental studies have reported alterations in DNA methylation patterns and transcriptional responses following exposure to glyphosate or its formulations. For instance, transcriptomic and epigenetic disturbances in the liver of rats exposed to GBHs may indicate that even low-dose exposure may affect molecular pathways related to cellular stress responses and metabolic regulation (Mesnage et al., 2022a). Likewise, glyphosate exposure can reduce global DNA methylation and modify promoter methylation of genes involved in cell-cycle regulation and apoptosis in human peripheral blood mononuclear cells *in vitro* (Woźniak et al., 2020). Together, these findings suggest that epigenetic mechanisms may represent an important pathway linking glyphosate exposure to long-term biological effects.

Despite the widespread global use of GBH and ongoing debates regarding its potential adverse health effects, few studies have examined the impact of prenatal GBH exposure as a diabetogenic factor during gestation and in adult offspring. Therefore, we aimed to evaluate the effects of GBH exposure at environmentally relevant doses—within the range of the NOAEL and acceptable daily intake (ADI)—during pregnancy on glucose and lipid homeostasis throughout gestation and the post-term period, as well as during early and later stages of offspring life.

2. Methods

2.1. *In vivo study*

2.1.1. *Ethics statement*

The experimental protocol was approved by the Federal University of Santa Catarina Committee for Ethics in Animal Experimentation (ID n°. 3797220921) following the ARRIVE guidelines and the National Research Council of the National Academies guide for the care and use of laboratory animals (eighth edition, revised 2011).

2.1.2. *Animals*

Male and female Wistar rats (40 days old) were housed in a temperature and humidity-controlled environment and kept on a 12-h light-dark cycle (lights on 06:00–18:00). All rats had *ad libitum* access to food (Puro Lab 22 PB; Puro Trato Nutrição Animal, Santo Augusto, RS, Brazil) and filtered tap water. The Federal University of Santa Catarina's Animal Breeding Center supplied the animals. When female rats reached 90 days old, they were treated as described thereafter.

2.1.3. *Pilot studies*

We first conducted pilot studies to determine the optimal administration dose (Pilot 1) and the gestational exposure window (Pilot 2) that do not interfere with murinometric and gestational parameters. For Pilot Study 1, 15 females were paired with five males (3:1) during the night until the detection of a copulatory plug. Each pregnant rat was then housed individually and received GBH (Roundup Original® Mais, Brazil), containing di-ammonium salt N-(phosphonomethyl) glycine (Glyphosate) 577.0 g/L (57.7% w/v), acid equivalent di-ammonium salt N-(phosphonomethyl) glycine (Glyphosate) (480.0 g/L 48% w/v) and “other ingredients” listed as 678.0 g/L (67.8% w/v), in the drinking water at concentrations of 0.5% or 1% based on previous studies (Cattani et al., 2017; Gomes et al., 2022) for the 21 days of gestation. The control group received filtered water without GBH. Based on daily water intake, the amount of GBH ingested ranged from 168 to 308 mg/kg in the 0.5% group and 728 to 1075 mg/kg in the 1% group, exceeding the pre-established NOAEL for rats, which is around 79 mg/kg body mass (b.m.) (EFSA, 2023). Due to adverse effects observed, such as body mass loss and stillbirths, this dosage was discarded following veterinary advice. In Pilot Study 2, the same number of animals was used. We treated half of the pregnant rats on gestational day 1 (GD1) and the other half on GD6 with a GBH dose of 50 mg/kg b.m. by orogastric gavage, a dose around the NOAEL (EFSA, 2023), which has been evaluated in previous rodent studies (Romano et al., 2012). No adverse biometric or gestational outcomes were observed in either group, ruling out any toxic effect that could bias the study.

2.1.4. *Experimental design in the F0 animals*

Twelve female rats per group and 12 male rats (3:1) were paired as described before. Oral gavage administration of GBH (Roundup Original® Mais) began at GD1 with either 0.5 or 50 mg/kg (0.5 GBH and 50 GBH groups, respectively). The control group (CTL) received only filtered water via orogastric gavage (Fig. S1). We chose a dose of 0.5 mg/kg, which is the ADI approved by the European Food Safety Authority (EFSA, 2023). We ran the experiments in two separate groups of rats to assess reproducibility. The final number of pregnant rats used in this study was 8–12 per group for the Control, 0.5 GBH and 50 GBH groups, respectively.

An oral glucose tolerance test (oGTT) was carried out on GD14 in all pregnant rats. Pregnant rats received GBH until term (GD21 or GD22). At birth, the neonates were gently weighed, and litter sizes were standardized to eight pups per dam (four females and four males; the F1 generation) to minimize potential litter effects (Natividade da Silva et al., 2019). The dams (F0) and their offspring (F1) remained together until the end of the lactation period (21 days), after which the animals were sorted (2 males and 2 females from each dam) into their respective

groups (further described in the F1 section). The F0 rats were kept for one additional week after weaning and underwent a second oGTT on post-term day 28. Two days later, they were subjected to an intraperitoneal insulin tolerance test (ipITT), after which they were sacrificed on the same day. The F0 dams were daily monitored for biometric parameters (except during the post-term period, when the animals were monitored weekly), including body mass and food intake.

2.1.5. Developmental tests

Soon after birth, physical development was assessed through daily records of fur growth, eruption of upper and lower incisors, and eye opening, with the day of appearance of each event documented for every litter. Offspring developmental reflexes were also evaluated following the procedures described by Lorenzon et al. (2023). Sensory-motor function was assessed using negative geotaxis, performed every other day from postnatal day (PND) 7 to PND 13, and the righting reflex, evaluated daily from PND 5 to PND 8. In addition, the day on which each pup first exhibited the grasp and startle reflexes was recorded to characterize reflex maturation.

2.1.6. Experimental design F1

The F1 offspring (male or female) were distributed into four animals per cage per sex. After reaching 70 days of age, rats were treated daily (~8:00 a.m.) with either intraperitoneal saline (1 mL/kg, b.m.) or dexamethasone (1 mg/kg, b.m.; Decadron®, Aché) for 6 days (Fig. S1), according to prior studies (Giozzet et al., 2008; Natividade da Silva et al., 2023). This resulted in 12 groups of animals ($n = 8$ per group). This glucocorticoid treatment serves as a metabolic challenge to reveal potential vulnerabilities not observed in healthy animals (Natividade da Silva et al., 2023; Gomes et al., 2019). On the 5th day of treatment with dexamethasone, the animals underwent an oGTT and, the following day, an ipITT. Biometric parameters and food intake were taken weekly.

2.1.7. Glucose and insulin tolerance tests

Oral glucose tolerance tests (oGTT) were performed on GD14 and post-term day 28 in progenitors and on PND75 in the offspring (Fig. S1), following the procedures described by Bruxel et al. (2023). Intraperitoneal insulin tolerance tests (ipITT) were conducted on post-term day 30 in progenitors and on PND76 in the offspring (Fig. S1), as described by Bruxel et al. (2023), with the exception that animals were subjected to a 2-h fasting period prior to testing.

2.1.8. Euthanasia and tissue sampling

Euthanasia and tissue sampling were performed in 8-h-fasted rats at post-term day 31 (progenitors) and PND77 (approximately 12:00 a.m.) by administering an overdose of ketamine and xylazine, followed by decapitation. Immediately after, trunk blood was collected in tubes containing the anticoagulants EDTA-NaF (Hemstab/Glistab-Labtest®; Lagoa Santa, MG, Brazil). The tubes were then centrifuged at 3500 rpm for 10 min at 4 °C, and the plasma was aliquoted and stored at -80 °C for subsequent biochemical analyses. The masses of organs (visceral fat, liver, pancreas, and adrenal glands) were measured using a digital balance (TECNAL, Piracicaba, São Paulo, Brazil) and normalized to individual body mass.

2.1.9. Biochemical analysis

Plasma insulin was measured using the ELISA method (RAB0904; Sigma-Aldrich, St. Louis, MO, USA). Triacylglycerol, total cholesterol, HDL-cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and cholinesterase activity were determined by spectrophotometry according to the manufacturer's instructions (Analisa® and Labtest®, Lagoa Santa, Minas Gerais, Brazil). Circulating low-density lipoprotein (LDL)- and very low-density lipoprotein (VLDL)-cholesterol were calculated using the Friedewald equation.

2.1.10. Hepatic and adipose morphology, glycogen, and triacylglycerol contents

Liver and perigonadal adipose tissue (from the same anatomical region) were collected and fixed in 10% buffered formalin (pH 7.4) for 24 h at room temperature. Samples were then dehydrated, embedded in paraffin, and sectioned at 5 μ m for subsequent Hematoxylin and Eosin (HE) staining for morphological analysis. Hepatic histological evaluation focused on the identification of inflammatory foci and other structural alterations in hepatic tissue. Inflammatory foci were defined as localized aggregates of leukocytes within the hepatic parenchyma or periportal regions, according to morphological criteria commonly used in experimental toxicology and previous studies evaluating hepatic alterations following glyphosate exposure (Gomes et al., 2022; Saleh et al., 2018). Prior to the analysis, the histological criteria for identifying inflammatory infiltrates were discussed with guidance from a trained pathologist to ensure appropriate interpretation of the morphological findings. Histological evaluation was performed independently by two investigators, blinded to the experimental groups, to minimize observational bias. Adipocyte size and other morphometric parameters were measured as previously published (Santos et al., 2021). Images were acquired using an Olympus BX41 microscope (Tokyo, Japan) at final magnifications of 100 $\times$ and 400 $\times$. Hepatic triacylglycerol and glycogen content were measured as previously described (Santos et al., 2021; Giozzet et al., 2008).

2.1.11. DNA methylation and gene expression

We evaluated DNA methylations in liver tissue fragments from progenitors and adult offspring by first extracting the genomic DNA using the phenol/chloroform method. We subsequently treated the extracted DNA with the endonucleases T4- β -glucosyltransferase (T4-BGT, cat. #M0357S), *MspI* (Cat. #M0215S), and *HpaII* (Cat. #M0214S) (New England Biolabs, Beverly, MA, USA). The complete, fully detailed protocol and the primer sequences used for gene expression and DNA methylation were previously described in a study from our group (Bruxel et al., 2023). We opted to evaluate *Line1* (L1TD1 – LINE-1 type transposase domain-containing 1) gene methylation because it constitutes approximately 18% of the genome, and methylation levels in its promoter region are used as a surrogate marker for global genome methylation.

2.2. In vitro study

We conduct viability assays using adipocytes and hepatocytes to determine whether low doses of GBH or glyphosate exert cytotoxic effects.

2.2.1. Differentiation of 3T3-L1

The 3T3-L1 (8×10^4 cells/well) preadipocyte cell line was cultured in a 48-well plate and differentiated as described elsewhere (Jiménez-Sánchez et al., 2017). After differentiation, hypertrophic adipocytes were obtained using a high-glucose medium containing insulin (4.5 mg/mL).

2.2.2. Cell viability tests

The cytotoxic effect of HepG2 (8×10^4 cells/well) and differentiated 3T3-L1 was performed using resazurin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity assays. **Resazurin:** Cells were seeded at a density of 2×10^4 cells per well into 96-well plates and incubated for 24 h in the complete growth media. After reaching confluence, the cells were treated with either RoundUp Original Mais® (GBH) or glyphosate salt (glyphosate 98%, Pestanal, 45521, Sigma, St. Louis, CA, USA), supplemented to the complete growth media at concentrations ranging from 6 μ M to 4 mM, for 24 h. After treatment, the supplemented media was removed, and 200 μ L of 1 mg/mL resazurin dye-supplemented media was added to each well, followed by 2 h incubation at 37 °C in 5% CO₂. Subsequently, 100 μ L of the resazurin-dye-

supplemented media was removed from each well and transferred to a new 96-well plate. The absorbance was measured at dual wavelengths (570 and 600 nm) using a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany). Cell viability was calculated as the percentage reduction in the dye, as previously described. The data was collected on at least three independent triplicates. **MTT:** Cells were plated into 96-well plates and, after confluence, were exposed to glyphosate or GBH (10 μ M – 4 mM/mL) for 24 h. Cells were then washed with phosphate-buffered saline (PBS) and incubated for 2 h with MTT (0.5 mg/mL). Formazan crystals were solubilized by adding DMSO (100 μ L/well), and the colored solutions were read at 570 nm (Mosmann, 1983). The dataset was analyzed in at least three independent replicates.

2.3. Statistical analysis

All analyses were performed using GraphPad Prism Version 10.1.1 software (GraphPad Inc., La Jolla, CA, United States). The results were expressed as mean $\pm$ SD for parametric data and as median and inter-quartile range for nonparametric data. Because normality and variance homogeneity tests can yield discordant results, particularly with small sample sizes and when group distributions are only mildly non-Gaussian, we used a panel of complementary normality tests (Kolmogorov–Smirnov, Shapiro–Wilk, D'Agostino–Pearson, and Anderson–Darling). To improve transparency and avoid over-interpreting a single test, data were considered approximately symmetric only when normality was not rejected consistently across the panel for all groups in a given comparison (i.e., at least one test supported symmetry for all

groups). Conversely, to minimize inflation of type I error in multiple comparisons, heteroscedasticity was treated conservatively: variance heterogeneity was assumed if any of the variance tests indicated unequal variances (Bartlett, Brown–Forsythe, or Welch). In the presence of heteroscedasticity, we applied nonparametric alternatives, as described below. For line graphs, a 2-way RM ANOVA with Tukey's post hoc test was applied. For scatter plots with bar graphs, 1-way ANOVA with Tukey's post hoc test for symmetric/homoscedastic data or Kruskal–Wallis with Dunn's post hoc test for asymmetric/heteroscedastic data was used.

3. Results

3.1. GBH exposure during pregnancy did not affect pregnant and post-term outcomes in mothers

GBH exposure did not alter gestational length, which corresponded to 21 days for the GBH-treated and the Control pregnant groups. Litter size remained unaffected across all GBH groups compared with the Control, with 10 ± 2 , 9 ± 4 , and 11 ± 3 pups in the Control, 0.5 GBH, and 50 GBH groups, respectively.

Exposure to GBH did not affect body mass during pregnancy either at post-term period (Fig. 1A). Likely, food intake remained unchanged during pregnancy and post-term period, regardless of GBH exposure (Fig. 1B). Basal glycemia and glucose tolerance evaluated during gestation (GD14) and after the lactation period (post-term day 28) did not reveal any impact of GBH exposure on both treated groups in

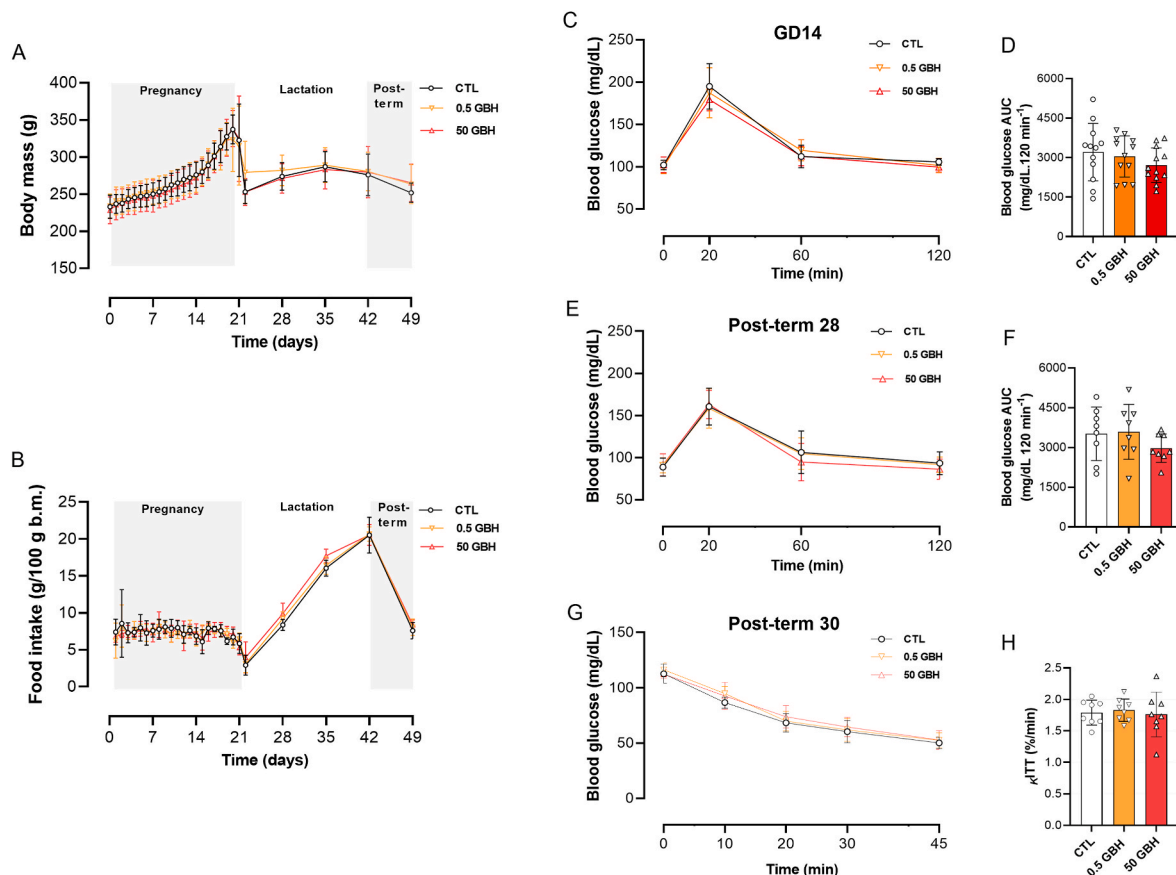


Fig. 1. GBH exposure during pregnancy did not affect pregnant and post-term outcomes in mothers. Body mass (A) and food intake (B) throughout gestation and the post-term period. Blood glucose levels during an oral glucose tolerance test (oGTT) (2 g/kg body mass) at gestational day (GD) 14 and postnatal day (PN) 28 (C,E, respectively). The respective incremental areas under the curve (AUCs) for blood glucose (D,F) were calculated from C and E, respectively. Blood glucose levels during an intraperitoneal insulin tolerance test (ipITT) at PN30 (G), and the corresponding blood glucose decay constant (k_{ITT}) (H). Results are expressed as mean $\pm$ SD. Line graphs were analyzed using ordinary 2-way with RM ANOVA followed by Tukey's *post hoc* test. Bar's graph were analyzed using ordinary 1-way NOVA with Tukey's *post hoc* test. $n = 12$ per group for (A, C and D), $n = 8$ per group for (B, E-H).

relation to the Control group (Fig. 1C–F). Furthermore, GBH exposure did not affect fed glycemia or insulin sensitivity on post-term day 30, regardless of the administered dose, as evidenced by constant blood glucose decay (k_{ITT}) values (Fig. 1G and H).

3.2. GBH exposure during pregnancy reduced plasma cholinesterase activity and exerted minor effects on several biochemical and morphological parameters

GBH exposure during pregnancy reduced plasma cholinesterase activity in both treated groups at GD14 (Fig. 2A), which was normalized after the lactation period (Fig. 2B; post-term day 31). Notably, none of the GBH treatments exerted a significant impact on circulating insulin, triacylglycerol, total cholesterol, or its fractions – HDL, LDL, and VLDL (Fig. 2C–H). Similarly, plasma creatinine values and the enzymatic activity of plasma AST and ALT remained unchanged in GBH-treated animals (Fig. 2I–K).

Hepatic histological analysis revealed inflammatory infiltrates in liver sections following GBH exposure (Fig. 3A and B). No change in hepatic triacylglycerol content was noted at the biochemical level in the treated groups (Fig. 3C). Of note, a dose-dependent reduction in hepatic glycogen content was observed, with significantly lower levels in the 50 GBH group (Fig. 3D). The *L1td1* gene methylation, a global molecular marker of hypo- or hypermethylation, was lower in both GBH-treated groups. Yet, no changes in *Line1* gene expression were observed in these groups (Fig. 3E and F, respectively).

Perigonadal adipose tissue histology revealed no impact of GBH exposure on adipocyte area, perimeter, or number (Fig. S2A–D), corresponding with unchanged adipose tissue depot masses (Table S1).

Furthermore, gestational exposure to GBH did not significantly affect the mass of metabolic tissues, including the liver, pancreas, and spleen. However, we observed a reduction in adrenal gland mass in the 50 GBH group compared to the Control group (Table S1).

3.3. Prenatal exposure to GBH affected the performance of offspring in some developmental tests

We explored neurodevelopmental parameters and found no alteration in the onset of the forearm grasp reflex, fur growth, incisor eruption, or eye opening, regardless of sex or prenatal GBH exposure (Fig. S3A–D). The startle reflex, a key indicator of sensory processing and motor responses, remained unaltered among the groups (Fig. S3E). Evaluation of sensory-motor function revealed higher latency of righting reflex at PND5 in female and male rats from 50 GBH- to 0.5 GBH-treated mothers, respectively, which was recovered in the subsequent days (Fig. S3F and G, respectively). A similar pattern was observed in females for negative geotaxis latency (Fig. S3H and I).

3.4. Intrauterine exposure to GBH did not affect glucose homeostasis later in life or exacerbate the diabetogenic effects of glucocorticoid treatment

Body mass values in offspring from mothers receiving GBH did not change in relation to controls until PND70, regardless of sex and dose of GBH exposure (Fig. 4A,C). Likely, no change in food intake was observed over the course of the evaluation in these animals (Fig. 4B,D). To reveal potential metabolic vulnerabilities not present under naïve conditions, animals were challenged with glucocorticoids, whose diabetogenic effects are well-defined. Prenatal GBH exposure did not exacerbate the body mass or food intake reduction caused by high-dose dexamethasone treatment (Fig. 4E–H).

The diabetogenic action of dexamethasone was evidenced by GTT experiments (Fig. 5A–D), in which the expected glucose intolerance was observed in all groups receiving glucocorticoid. Prenatal exposure to GBH rendered neither glucose intolerance later in life nor any exacerbation of glucose intolerance caused by dexamethasone administration in both sexes. Similarly, rats treated with dexamethasone had elevated blood glucose levels during the ipITT, reflecting the GC's effects on glucose metabolism and insulin sensitivity (Fig. 5E–H). Again, prenatal exposure to GBH did not exacerbate the insulin insensitivity induced by

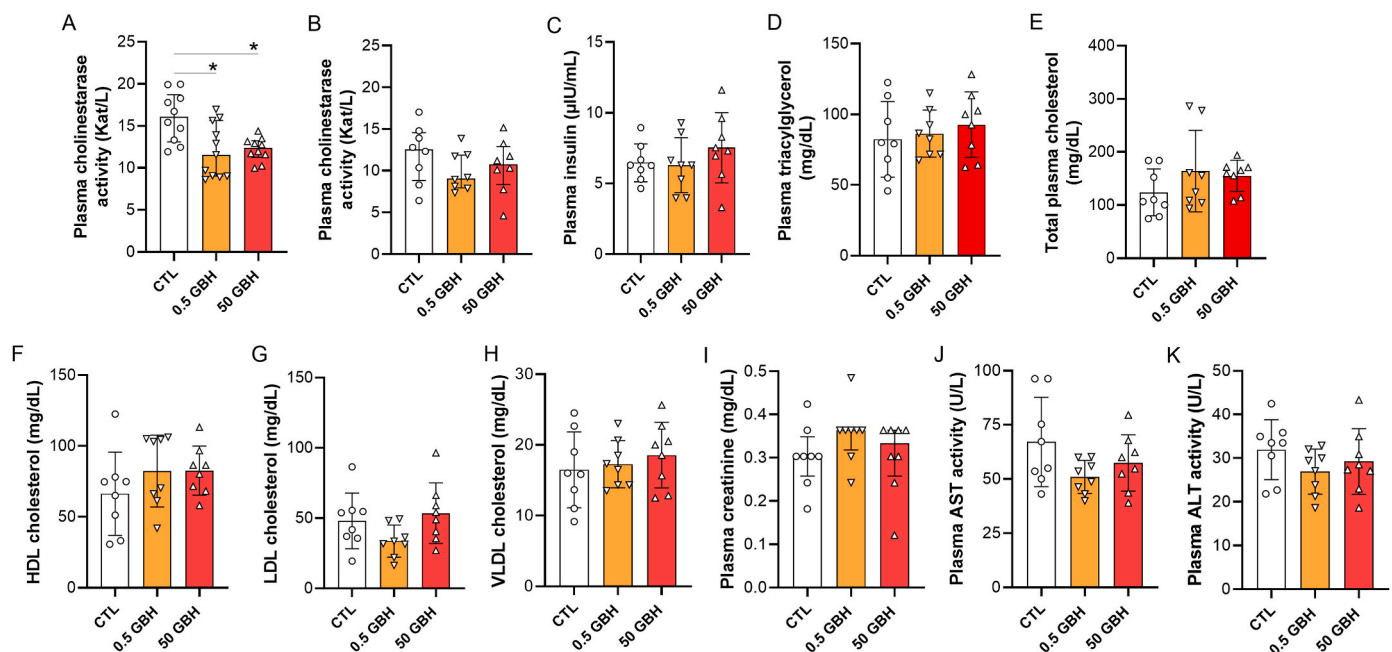


Fig. 2. GBH exposure during pregnancy reduced plasma cholinesterase activity and exerted minor effects on several biochemical and morphological parameters. Plasma cholinesterase activity on GD14 (A) and PND31 (B). Plasma insulin (C), triacylglycerol (D), total cholesterol (E), HDL cholesterol (F), LDL cholesterol (G), VLDL cholesterol (H), and creatinine (I) values on euthanasia day. Plasma aspartate transaminase (AST) (J) and alanine transaminase (ALT) activity (K) on euthanasia day. Values are expressed as mean $\pm$ SD (A,C,H,J,K) and as median $\pm$ interquartile range (B,I). Connecting lines indicate intergroup differences. * $p < 0.05$, using ordinary 1-way ANOVA with Tukey's *post hoc* test or the Kruskal-Wallis with Dunn's *post hoc* test. $n = 10$ –11 per group for A, $n = 8$ per group for the remaining graphs.

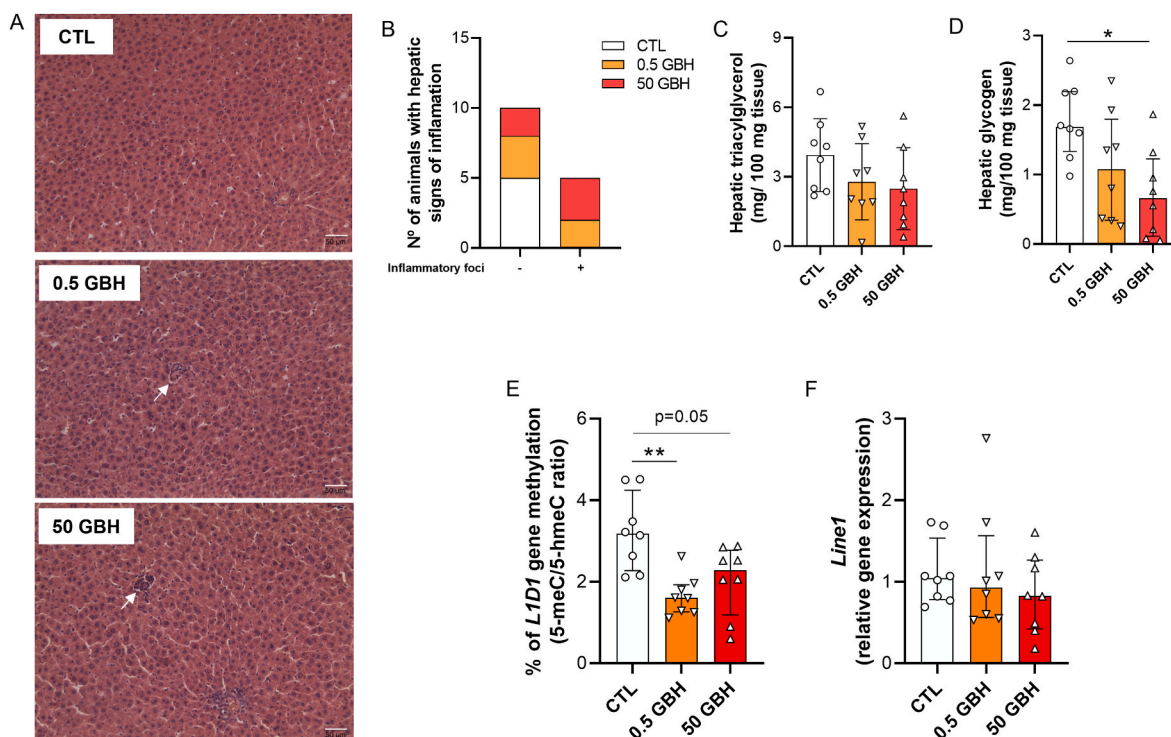


Fig. 3. Prenatal GBH exposure affected hepatic parameters in progenitors. Representative histological liver sections (HE stained), from the Control (CTL) group and the GBH-treated groups (0.5 or 50 GBH) (A). In (B), the number of animals with hepatic signs of inflammation. Hepatic triacylglycerol (C) and glycogen (D) contents. In (E) and (F), the hepatic *L1D1* gene methylation and *Line1* gene expression, respectively. Values are expressed as a contingency table (B), as mean $\pm$ SD (C,D) and as median $\pm$ interquartile range (E,F). Connecting lines indicate intergroup differences. * $p < 0.05$, using ordinary 1-way ANOVA with Tukey's *post hoc* test or the Kruskal-Wallis with Dunn's *post hoc* test. $n = 8$ per group. Scale bar means 50 μ m.

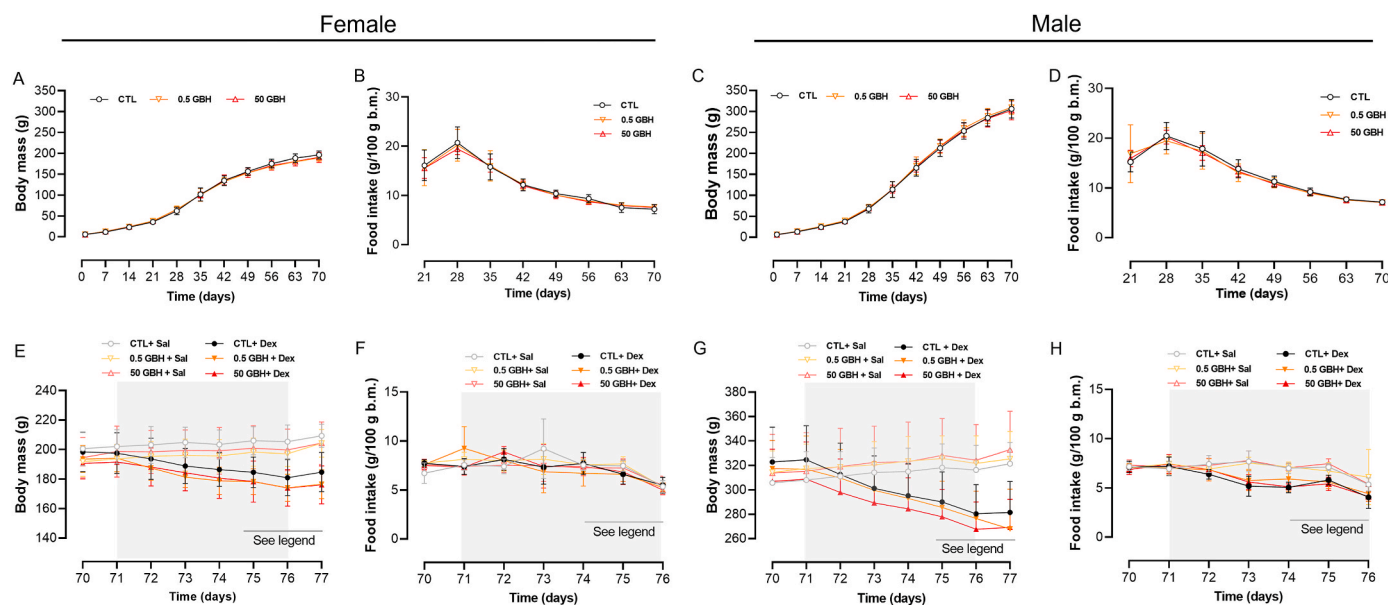


Fig. 4. Intrauterine exposure to GBH did not affect murinometric parameters or exacerbate the diabetogenic effects of glucocorticoid treatment. Body mass (A,C) and food intake (B,D) from PND1 to PND70 of naïve female and male offspring, respectively. Body mass (E,G) and food intake (F,H), following dexamethasone (Dex) or saline (Sal) treatment (shaded area indicates treatment period). Results are expressed as mean $\pm$ SD. Line graphs were analyzed using ordinary 2-way with RM ANOVA followed by Tukey's *post hoc* test. When indicated 'see legend', it means different from the respective control group. $n = 16$ per group for (A-D), $n = 8$ per group for (E-H).

glucocorticoids.

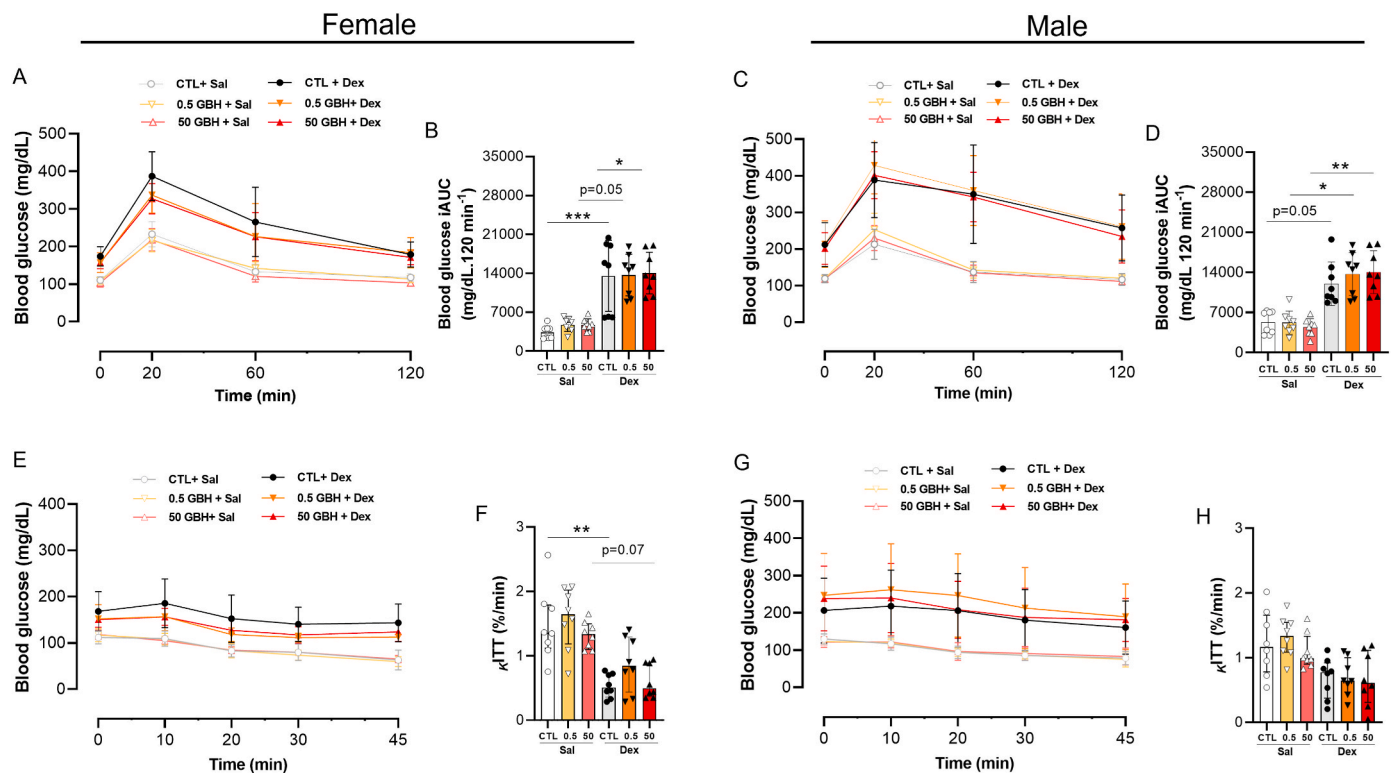


Fig. 5. Intrauterine exposure to GBH did not affect glucose homeostasis or exacerbate the diabetogenic effects of glucocorticoid treatment. Blood glucose levels during an oral glucose tolerance test (oGTT) (2 g/kg body mass) in female (A) and male (C) offspring on PND75. The respective incremental areas under the curve (IAUCs) for blood glucose (B,D) were calculated from A and C, respectively. Blood glucose levels during an intraperitoneal insulin tolerance test (ipITT) (1 IU/kg body mass) in female (E) and male (G) offspring. The respective blood glucose decay (κ ITT) for blood glucose (F,H) were calculated from E and G, respectively. Values are expressed as mean $\pm$ SD (A–D, E, and G) and as median $\pm$ interquartile range (F,H). Line graphs were analyzed using ordinary 2-way with RM ANOVA followed by Tukey's *post hoc* test. Bar's graph was analyzed using Kruskal-Wallis with Dunn's *post hoc* test (heteroscedastic data). Connecting lines indicate intergroup differences, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. $n = 8$ per group.

3.5. Intrauterine exposure to GBH had no major impact on biochemical plasmatic or hepatic parameters later in life, or exacerbated the dexamethasone-caused alterations

Plasma insulin remained unaltered in adult offspring from the 0.5 GBH and 50 GBH compared with the respective controls, and prenatal exposure to GBH did not worsen the hyperinsulinemia caused by glucocorticoid treatment, regardless of the sex and dose of GBH studied (Fig. 6A,D). Similarly, adult offspring prenatally exposed to GBH developed no alteration in the plasma lipid profile (Fig. 6B,C,E,F), and did not exacerbate the hypertriglyceridemia caused by dexamethasone treatment (Fig. 6B,E). Either renal or hepatic function estimated indirectly through plasma creatinine and transaminases remained unaffected in adult female and male offspring from mothers treated with GBH (Table S2).

The expected increase in hepatic lipid content under high glucocorticoid exposure was attenuated in adult rats from mothers exposed to GBH, regardless of sex or dose (Fig. 6G,K). A similar pattern was observed with the hepatic glycogen data (Fig. 6H,L). The *L1td1* gene methylation was lower in liver of female offspring from the 0.5 GBH group than in the control, although no changes were detected in *Line1* gene expression. Neither *L1td1* gene methylation nor *Line1* gene expression was observed in the male GBH-treated groups (Fig. 6I,J,M,N).

The mass of metabolic tissues such as liver, pancreas, and adipose tissue remained unaltered in adult offsprings from the 0.5 GBH and 50 GBH groups (Table S3). The lower adrenal and spleen and higher liver masses occurred as a result of dexamethasone treatment, with no additional effect of prenatal exposure to GBH in both sexes (Table S3).

3.6. GBH was more harmful to HepG2 and 3T3-L1 cells than glyphosate salt at high molar concentrations

Using two methods to determine cell viability, treatment of HepG2 cells with GBH or glyphosate for 24 h revealed a toxic effect of GBH at 400 μ M, with high cytotoxicity observed at 1 mM (Fig. 7A and B). Under 1 mM glyphosate treatment, HepG2 cells remained 100% viable, with loss of viability observed only at high concentrations (4 mM) (Fig. 7A and B). The adipocyte lineage 3T3-L1 showed reduced cell viability with GBH treatment, with a decrease in viability starting at 1 mM and a marked increase in cell death at 1.6 mM GBH (Fig. 7C and D). Conversely, 3T3-L1 cells remained unaffected by glyphosate treatment even at 4 mM (Fig. 7C and D).

4. Discussion

Our findings indicate that low-dose gestational exposure to GBH did not produce profound changes in biometric parameters, glucose tolerance, insulin sensitivity, or lipid metabolism in either the dams or their adult offspring. Furthermore, treatment of the progeny with the diabetogenic agent dexamethasone reveals no metabolic vulnerability that could be masked in unchallenged conditions. It is important to emphasize, however, that exposure to low doses of GBH was not totally exempt of effects, particularly in the liver.

Our *in vivo* findings align with those of Panzacchi et al. (2018), who exposed female rats to 1.75 mg/kg/day of either glyphosate or Roundup® (GBH) from gestational day 6 (GD6) to approximately postnatal day 73 or 125 and reported no detectable biometric alterations in dams during gestation or lactation. Similarly, pregnant rats exposed to GBH or pure glyphosate (2 mg/kg/day) from GD9 to PND21 showed

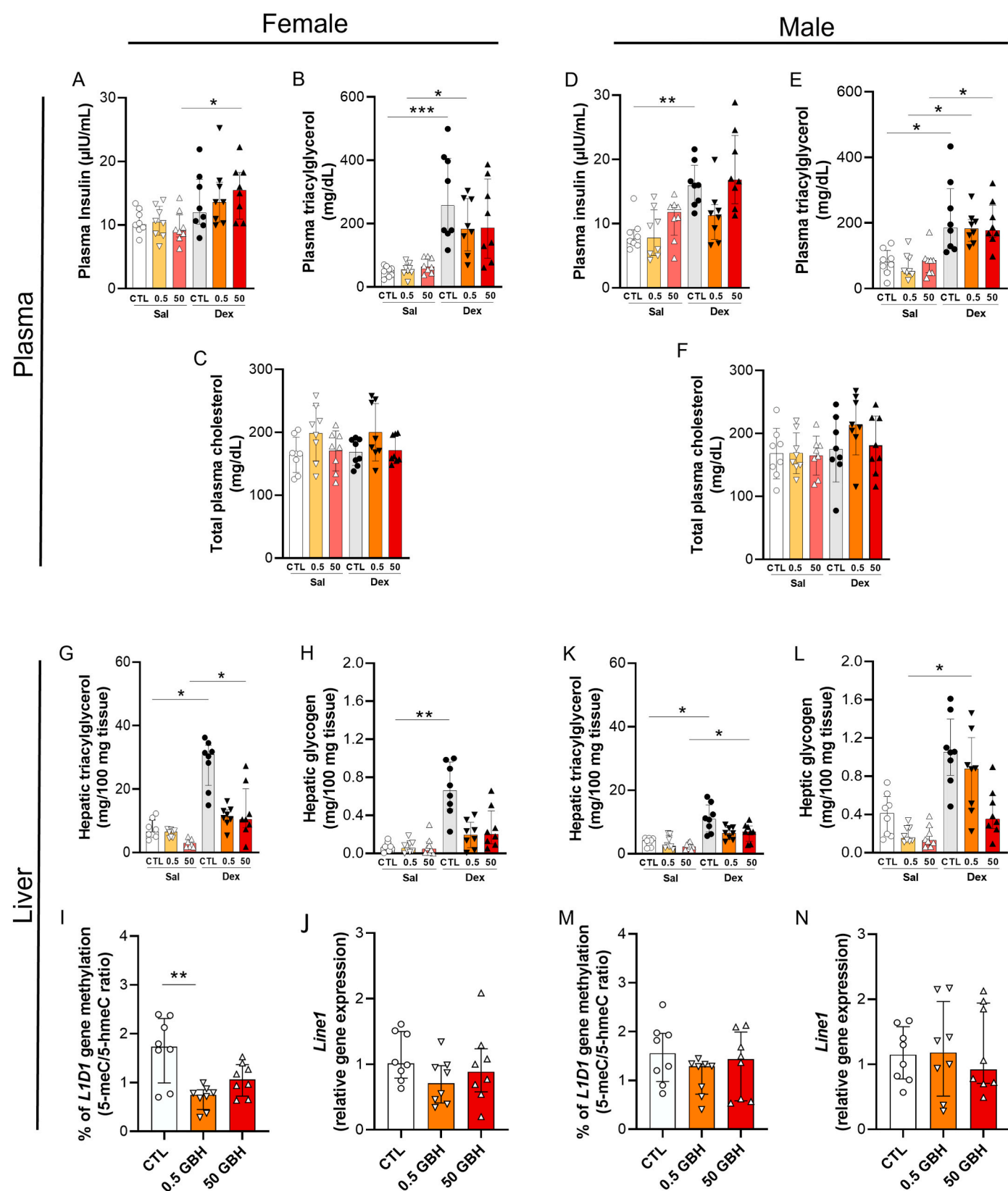


Fig. 6. Intrauterine exposure to GBH had no major impact on biochemical plasmatic or hepatic parameters later in life, or exacerbated the dexamethasone-caused alterations. Plasma insulin (A,D), triacylglycerol (B,E), and total cholesterol (C,F) in female and male offspring, respectively. Hepatic triacylglycerol (G,K) and glycogen (H,L) contents in female and male offspring, respectively. Hepatic *L1D1* gene methylation (I,M) and relative *Line1* gene expression (J,N) in female and male offspring, respectively. Values are expressed as median $\pm$ interquartile range. Connecting lines indicate intergroup differences. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ using Kruskal-Wallis with Dunn's *post hoc* test. $n = 8$ per group.

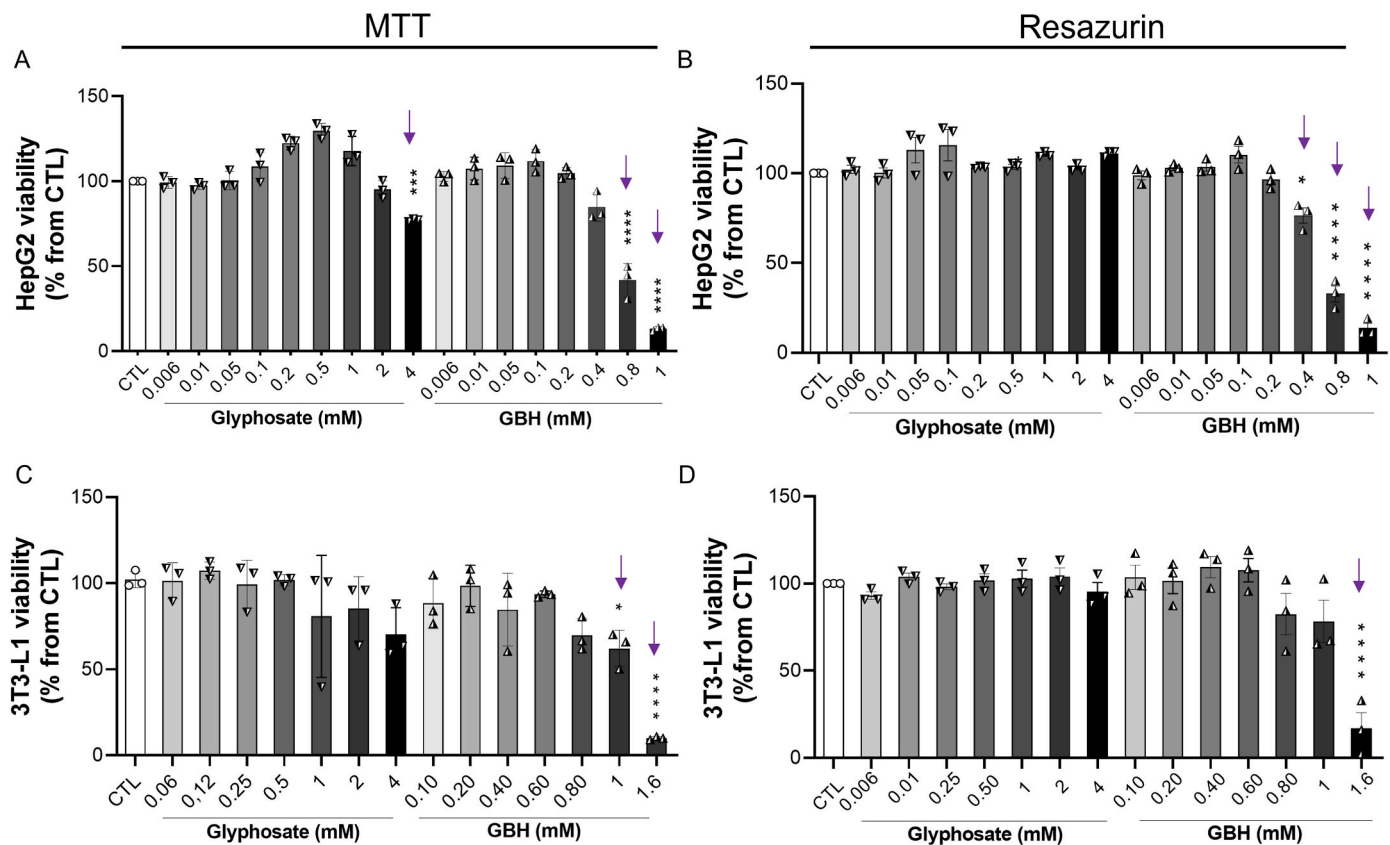


Fig. 7. GBH was more harmful to HepG2 and 3T3-L1 cells than glyphosate salt at high molar concentrations. Cell viability in HepG2 and 3T3-L1 cells was assessed by MTT (A,C) and resazurin (B,D) assays, respectively. Tested concentrations ranged from 6 μ M to 4 mM. Values are expressed as mean $\pm$ SD. Connecting lines indicate intergroup differences. * p < 0.05, *** p < 0.001, and **** p < 0.0001 using ordinary 1-way ANOVA with Tukey's *post hoc* test. Triplicates were performed in three independent experiments.

no changes in maternal body mass (Lorenz et al., 2020). Although studies directly evaluating the metabolic effects of glyphosate or its formulations during pregnancy remain scarce, available evidence indicates that GBH can influence glycemic control, typically at substantially higher doses. For instance, mice exposed to 0, 50, 250, or 500 mg/kg/day of GBH for 30 days exhibited significant increases in blood glucose only at the highest dose (500 mg/kg) (Qi et al., 2023), consistent with our findings, as no alterations were observed at 50 mg/kg/day. Additionally, prenatal and lactational exposure to 0.5% GBH in drinking water (approximately 420 mg/kg/day based on intake) does not necessarily induce overt metabolic disturbances in dams; instead, increased insulin sensitivity and enhanced glucose responsiveness were reported in offspring (Gomes et al., 2022).

We observed a transient reduction in plasma cholinesterase activity at GD14 in GBH-treated rats, likely reflecting an acute response to herbicide exposure during pregnancy. Organophosphate and organophosphate-like compounds—including certain adjuvants present in commercial GBHs—can interact with cholinergic signaling, although with lower potency than classical inhibitors. This temporary decrease may result from short-term inhibitory effects, whereas the absence of alterations at PND31 suggests that compensatory homeostatic mechanisms restored enzyme activity after exposure ceased (Milić et al., 2018). These findings indicate that subtle biochemical disruptions may occur during critical windows of fetal development, even in the absence of overt clinical signs. The doses used in this study (0.5 and 50 mg/kg) are particularly relevant, as they fall within the regulatory ranges for the acceptable daily intake (ADI) and the no-observed-adverse-effect level (NOAEL) for glyphosate, underscoring their importance for risk assessment (EFSA, 2023). Numerous studies suggest that glyphosate exhibits endocrine-disrupting properties, although regulatory agencies have not

yet formally classified it as an endocrine disruptor (Muñoz et al., 2021; Stone et al., 2025). In this context, evaluating low-dose exposures is essential, as many endocrine-disrupting chemicals exhibit non-monotonic dose-response relationships, with effects emerging at environmentally relevant concentrations below regulatory thresholds (Vandenberg, 2022).

Although dams showed no overt systemic alterations, exposure to the highest GBH dose (50 mg/kg) reduced hepatic glycogen content, with a similar trend at 0.5 mg/kg. Both exposure groups also displayed hepatic inflammatory infiltrates, which occurred more frequently at the higher dose. In addition, dams exposed to 0.5 mg/kg showed reduced hepatic relative methylation of the *L1td1* gene, with a comparable trend at 50 mg/kg. LINE-1 constitutes one of the most abundant classes of transposable elements in mammalian genomes, accounting for approximately 18% of genomic DNA. Epigenetic mechanisms tightly regulate their activity, particularly DNA methylation, which suppresses retrotransposition and helps maintain genomic stability (Slotkin and Martienssen, 2007). Because of this property, LINE-1 methylation levels are widely used as surrogate markers of global DNA methylation in studies investigating the epigenetic impact of environmental exposures (Bollati and Baccarelli, 2010). Despite the absence of detectable changes in *Line1* gene expression, the reduction in hepatic *L1td1* methylation observed here suggests that GBH exposure may alter hepatic epigenetic regulation. Hypomethylation states can compromise genomic stability and have been associated with inflammatory processes, ageing, and metabolic disorders such as diabetes (Urban et al., 2021). Experimental evidence also supports the potential for glyphosate exposure to induce epigenetic disturbances. Rats exposed to GBHs exhibit transcriptomic and epigenetic alterations affecting molecular pathways involved in cellular stress responses and metabolic regulation (Mesnage et al.,

2022a). Similarly, glyphosate exposure reduces global DNA methylation and alters promoter methylation of tumor suppressor genes involved in cell-cycle control and apoptosis in human peripheral blood mononuclear cells (Woźniak et al., 2020). While these findings support the potential of glyphosate to interfere with epigenetic mechanisms, our results extend this evidence by showing hepatic DNA methylation changes *in vivo* after gestational exposure to environmentally relevant GBH doses, with indications of sex-specific modulation of genes involved in DNA methylation pathways in the offspring. Together, these observations indicate that gestational GBH exposure can induce early epigenetic modifications in the liver, even in the absence of detectable alterations in basal glycemic control, highlighting the liver's sensitivity to GBH exposure and suggesting that such early epigenetic disruptions may increase susceptibility to metabolic dysregulation not captured by the assays used in this study. Genome-wide epigenetic analyses will be required to determine whether these alterations reflect broader reprogramming events.

The liver, a central organ in xenobiotic detoxification and energy metabolism, is particularly susceptible to subtle inflammatory or metabolic disturbances that may impair biotransformation capacity and increase vulnerability to reactive metabolites and metabolic dysfunction (Ford et al., 2017; Gomes et al., 2022). Experimental evidence supports the sensitivity of hepatic metabolic pathways to glyphosate-based exposures. Xiao et al. (2025) reported hepatic glycogen depletion in mice exposed to pure glyphosate, which may be associated with reduced expression of glucose transporter 2. Similarly, Daruich et al. (2001) demonstrated dose-dependent modulation of glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase activities in pregnant rats exposed to GBH, with inhibition at low doses and compensatory upregulation at higher doses, suggesting a shift in NADPH-generating pathways to counteract oxidative stress. Such enzymatic adjustments may contribute to the glycogen depletion observed in our study, either by reducing glucose oxidation efficiency at lower doses or by diverting glucose-6-phosphate toward the pentose phosphate pathway at higher doses, thereby limiting glycogen synthesis. Consistently, exposure to other organophosphate pesticides, such as chlorpyrifos, has been associated with hepatic glycogen reduction through alterations in cytochrome P450 systems or mitochondrial transporters, underscoring the vulnerability of hepatic glycolytic flux during xenobiotic detoxification (Elsharkawy et al., 2013).

Despite the absence of overt metabolic alterations in adult offspring, our findings are consistent with previous reports showing no major metabolic disturbances following gestational GBH exposure under standard dietary conditions (Gomes et al., 2022). In contrast, other studies have documented metabolic or endocrine alterations after prolonged or higher-dose exposures. For example, hepatic lipid and glycogen disturbances have been reported in rodents exposed to glyphosate at doses exceeding the NOAEL (Ren et al., 2019; Prakasam et al., 2021), and transgenerational metabolic and reproductive effects have been associated with early-life exposure (Kubsad et al., 2019). Clinically, observational studies have suggested that higher urinary glyphosate or AMPA levels correlate with impaired glucose metabolism and elevated HbA1c, particularly among metabolically vulnerable individuals, such as those who are overweight (Feng et al., 2025). Divergences among studies likely reflect differences in exposure duration, route, and developmental timing, as well as the presence or absence of metabolic challenges such as high-fat diets or gestational stress. Importantly, the lack of metabolic alterations in our study does not exclude potential effects in other physiological systems—such as reproductive or neurodevelopmental pathways—which may be more sensitive to low-dose herbicide exposure during critical developmental windows.

As previously noted, Feng et al. (2025) reported a dose-dependent association between urinary glyphosate concentrations and glycemic dysfunction, including elevated HbA1c levels, particularly among overweight individuals. Their findings suggest that an obesogenic

environment plays a decisive role in the manifestation of metabolic alterations. In our study, however, a key limitation is that, aside from pregnancy, which is itself a metabolic challenge due to physiologically induced insulin resistance, we did not introduce a high-fat diet or any additional metabolic stressor that could create a permissive context for metabolic disruption in the progenitors. The absence of such an obesogenic background may have limited the detection of subtle metabolic effects that could otherwise emerge under conditions of metabolic challenge.

With respect to offspring development, prenatal GBH exposure did not significantly affect body growth, food intake, or primary neurodevelopmental parameters. However, the early delays observed in the righting reflex and negative geotaxis indicate that gestational exposure may transiently disrupt neurodevelopmental processes. Although these effects were reversible, evidence from other environmental pollutants suggests that early-life developmental disturbances, even when temporary, can predispose individuals to later-life dysfunctions (Anarghou et al., 2024; Bartholomew et al., 2024). Thus, the potential for latent effects remains a concern, particularly given ongoing environmental contamination with glyphosate.

Following a glucocorticoid-induced metabolic challenge with dexamethasone, offspring prenatally exposed to GBH did not exhibit exacerbated metabolic impairments. All dexamethasone-treated groups showed impaired glucose tolerance, reduced insulin sensitivity, and elevated plasma triacylglycerol levels, consistent with previous reports (Natividade da Silva et al., 2023; Santos et al., 2014). However, prenatal GBH exposure did not intensify these alterations, indicating that the exposure conditions used in this study did not increase offspring susceptibility to metabolic disturbances. These findings align with those of Gomes et al. (2022), who also reported no significant effects of gestational glyphosate exposure on offspring metabolic parameters.

As with dams, no profound systemic alterations were detected in the offspring; however, those born to mothers exposed to the higher GBH dose (50 mg/kg) exhibited reduced hepatic glycogen content. Because hepatic glycogen is essential for maintaining glucose homeostasis during fasting, its depletion suggests early hepatic metabolic disturbances. This effect may stem from oxidative stress, mitochondrial dysfunction, or interference with insulin signaling pathways, mechanisms previously implicated in glyphosate-related toxicity (Prasad et al., 2022; Endirlik et al., 2022). Although these alterations were not reflected in basal glycemia or in responses to dexamethasone, they reinforce the liver's sensitivity as a target organ and indicate that subtle metabolic dysregulation may occur below the detection threshold of conventional assays.

The reduction in *L1td1* gene methylation observed in the liver of females born to mothers exposed to the lowest GBH dose (0.5 mg/kg) indicates that prenatal exposure may trigger epigenetic alterations even in the absence of immediate changes in *Line1* gene expression. Epigenetic modifications arising during critical windows of fetal development may persist into adulthood, reflecting the dynamic and potentially long-lasting nature of DNA methylation-mediated regulation (Benachour et al., 2007; Kubsad et al., 2019; Rossetti et al., 2021; Bruxel et al., 2023). The absence of comparable effects in males suggests a sex-specific response to GBH exposure, a pattern commonly reported in models of endocrine disruption (Kundakovic et al., 2013; Bruxel et al., 2023). Collectively, these findings indicate that even low-dose gestational exposure to GBH can modulate hepatic epigenetic marks in a sex-dependent manner, raising the possibility that early molecular alterations may precede overt metabolic dysfunction and contribute to long-term physiological outcomes. Although the observed effect was gene-specific, broader epigenomic disturbances cannot be excluded, and genome-wide analyses will be required to clarify potential reprogramming events.

The dexamethasone-induced increase in hepatic triacylglycerol and glycogen reflects the expected glucocorticoid-driven metabolic response, characterized by enhanced lipolysis, gluconeogenesis, and

glycogen storage that maintains the liver in a state of metabolic readiness (Motta et al., 2015; Liu et al., 2011; Sprangers et al., 2001). In the present study, however, offspring prenatally exposed to GBH (0.5 and 50 mg/kg) did not exhibit the anticipated increase in hepatic triacylglycerol and glycogen following glucocorticoid challenge, suggesting a potential programming effect on hepatic metabolic responses. Notably, reduced glycogen content was also observed in the dams. Although transgenerational alterations in these parameters have not been demonstrated, several studies have reported decreased intrahepatic glycogen in adult rodents. For example, rats exposed to glyphosate for 10 weeks at doses of 50, 100, and 250 mg/kg/day showed reduced hepatic glycogen associated with decreased glycogen synthase mRNA expression and increased glycogen phosphorylase mRNA expression (Prakasam et al., 2021). These findings suggest that glyphosate disrupts the balance between glycogen synthesis and degradation, ultimately leading to endogenous glycogen depletion.

Given the widespread human exposure to glyphosate residues, it is noteworthy that the concentrations used in our *in vitro* experiments fall within ranges reported in biological fluids of exposed populations. Under these conditions, GBH formulations showed greater cytotoxicity than glyphosate alone in hepatocyte and adipocyte models, supporting previous evidence that formulation adjuvants substantially contribute to the biological activity and toxicity of glyphosate-based herbicides (Defarge et al., 2016; Mesnage et al., 2022b; Teleken et al., 2026). This observation reinforces the view that the toxicological properties of GBH products cannot be attributed solely to the active ingredient, particularly since environmental and occupational exposures occur to complete formulations rather than isolated glyphosate (Martini et al., 2012; Mesnage et al., 2015). Although the concentration required to induce cytotoxicity *in vitro* exceeds typical environmental exposure levels, these findings highlight the intrinsic sensitivity of metabolically relevant cell types to GBH mixtures and suggest that formulation components may influence biological responses beyond those predicted from glyphosate alone. In the context of the present study, such effects may provide complementary mechanistic insight into the hepatic molecular alterations observed *in vivo*. The apparent divergence between *in vitro* cytotoxicity and the limited systemic effects observed *in vivo* likely reflects differences in effective exposure levels, physiological protective mechanisms in whole organisms, or the greater susceptibility of immortalized cell lines to chemical stress.

5. Conclusion

Overall, gestational exposure to GBHs at doses relevant to regulatory standards did not induce overt metabolic disturbances in dams or offspring, even under metabolic stress conditions. However, the hepatic metabolic alterations, transient neurodevelopmental effects, and pronounced *in vitro* cytotoxicity observed in this study underscore the need for caution. Given the widespread environmental contamination and human exposure to GBHs, further research addressing long-term, multigenerational, and molecular consequences of such exposures remains strongly warranted.

CRediT authorship contribution statement

Aline Barbosa Lima: Writing – review & editing, Writing – original draft, Investigation, Data curation. **Victoria Cristina Malinski:** Writing – review & editing, Investigation, Data curation. **Morgana Contini:** Writing – review & editing, Investigation, Data curation. **Natália Stinghen Tonet:** Writing – review & editing, Investigation, Data curation. **Bianca Franco Leonardi:** Writing – review & editing, Investigation, Data curation. **Rodrigo Augusto Foganhali da Silva:** Writing – review & editing, Resources, Methodology, Investigation, Data curation. **William T. Festuccia:** Writing – review & editing, Resources, Methodology. **Ivan Quesada:** Writing – review & editing, Resources, Methodology. **Alex Rafacho:** Writing – review & editing, Writing – original

draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the authors used AI platforms such as ChatGPT, Copilot, and Grammarly to improve grammar and text semantics. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2026.127942>.

Data availability

Data will be made available on request.

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